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Nasal immunization with plasmid DNA encoding P6 protein and immunostimulatory complexes elicits nontypeable *Haemophilus influenzae*-specific long-term mucosal immune responses in the nasopharynx

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ABSTRACT

Nasal vaccination is an effective therapeutic regimen for preventing upper respiratory infection, while DNA vaccines represent a new approach for controlling infectious diseases. Here, we examined the efficacy of nasally administered DNA vaccine on upper respiratory infections.

A DNA plasmid encoding the P6 outer membrane protein of nontypeable *Haemophilus influenzae* (NTHi) was constructed. Mice were immunized 3 times intranasally with the DNA plasmid and Matrix-M, an immunostimulatory complex adjuvant. P6-specific immune responses were examined using purified P6 protein. Nasal-associated lymphoid tissue (NALT) $CD4^+$ T cells were purified and incubated with feeder cells in the presence of P6, and the expression of cytokine mRNA was examined. In addition, NTHi challenges were performed and the level of NTHi was quantified in nasal washes.

P6-specific nasal wash IgA and serum IgG were elevated following immunization with the DNA plasmid and Matrix-M. The number of specific IgA-producing cells increased in the nasal passages of the immunized mice. In addition to Th1 and Th2 cytokine expression, IL-17 was detected in P6-specific NALT $CD4^+$ T cells. Moreover, DNA vaccination enhanced bacterial clearance.

These findings suggest that a successful DNA vaccination protocol has been developed for inducing *in vivo* immune responses against NTHi. Nasal vaccination with P6 DNA vaccine and Matrix-M might be a new effective regimen for the induction of specific protective immunity in the upper respiratory tract.

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is a major pathogen of otitis media (OM) and upper respiratory tract diseases [1]. In patients with OM, NTHi is frequently isolated from the nasopharynx as well as middle ear effusions. Therefore, inhibition of NTHi colonization in the nasopharynx is considered effective in preventing OM. As the number of antibiotic-resistant strains of NTHi has increased in recent years, the development of vaccine against NTHi is considered an important public health goal. P6 is one of the outer membrane proteins of NTHi and is an antigen (Ag) common in all NTHi strains [2,3]. Systemic and mucosal immunization with P6 induces strong serum antibody responses in experimental animals [4]. Several lines of evidence indicate that immune responses to P6 are protective. Specifically, immunization with P6 protects against OM and pneumonia in animal models [5]. P6 is also a target of human bactericidal antibodies that are associated with protection against OM [6]. This makes P6 a suitable candidate for a mucosal vaccine [2,7].

Nasal immunization is the most effective therapeutic regimen for inducing mucosal secretory immunoglobulin A (sIgA) and systemic IgG responses [8,9]. To elicit maximal levels of Ag-specific immune responses in both mucosal and systemic compartments, it is necessary to use an appropriate mucosal adjuvant [8,10]. Cholera toxin (CT) is one of the most potent mucosal adjuvants for enhancing Ag-specific immune responses when co-administered with protein Ag both orally and nasally [8,10]. We previously demonstrated that P6-specific IgA and Th2 immune responses could be induced in the middle ear and nasopharynx by nasal vaccination with P6 and CT [11], which affords protection against NTHi-induced experimental OM [12]. CT has been shown to be an effective mucosal adjuvant in animal models but is toxic in humans [10]. Consequently, an alternative adjuvant is necessary for the development of an efficacious mucosal vaccine for humans.

DNA vaccines represent a new approach to the control of infectious disease [13,14]. DNA vaccine technology is a simple concept

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based on a relatively simple design and production technology. In particular, both cellular and humoral immune responses are induced without the attendant concerns associated with live, attenuated vaccines [15,16]. Another advantage of DNA vaccines over conventional protein vaccines is the low cost of producing a highly purified product [14]. Furthermore, DNA vaccine distribution is not dependent upon maintaining a cold chain, thus the vaccines can be easily distributed especially in developing countries. DNA vaccination works by using host cells as protein factories to produce the plasmid-encoded antigen. The translated protein is then processed and presented to the immune systems in a manner similar to process that occurs following a natural infection. The vast majority of DNA vaccines are delivered by parenteral routes, which rarely elicit immune responses at the mucosal site [14,17,18]. In the present study, we have investigated the efficacy of nasal DNA vaccination to induce protective immunity against NTHi in the nasopharynx, with the ultimate goal of developing a mucosal vaccine for preventing OM.

2. Materials and methods

2.1. Animals

Six-week-old specific pathogen-free BALB/c mice (Charles River Japan, Atsugi, Japan) were used in this study. All experiments were approved by the Committee on Animal Experiments of Oita University, Japan, and were performed according to the local guidelines.

2.2. Plasmid DNA construction

A DNA plasmid encoding B-cell and T-cell epitopes of P6 was constructed. The sequence of immunogenic P6 epitope for mice has been reported [19]. cDNA was subcloned from plasmid pUC57 into the Not I site of the mammalian expression vector pCMVβ (CLONTECH, Sunnyvale, CA). The pCMVβ vector consists of a pUC backbone with the cytomegalovirus immediate-early gene promoter, an intron, a polyadenylation signal from SV40, and an ampicillin resistance gene. The plasmid was transformed into *Escherichia coli* DH5α and purified by alkaline lysis and double cesium chloride gradient ultracentrifugation followed by ethanol precipitation. The plasmid DNA was then resuspended in endotoxin-free water. The spectrophotometric A260/A280 ratios were between 1.8 and 2.0, and the endotoxin content was determined as <5 ng/mg of plasmid DNA by the Limulus amebocyte lysate assay (Sigma, St. Louis, MO).

2.3. Immunization

Immunization was conducted using the P6-encoding plasmid (pCMVβ/P6) and a non-coding control plasmid (pCMVβ/c). Matrix-M (AbISCO; ISCONOVA, Uppsala, Sweden) was used as an adjuvant. Mice were immunized 3 times intranasally with 10 or 100 μg of plasmid and 10 μg of Matrix-M on days 0, 7, and 14. Control animals were immunized 3 times intranasally with 10 μl of phosphate-buffered saline (PBS) containing 10 μg of P6 protein, which was purified from NTHi (strain 76) in our laboratory, as described previously [11]. PBS administration without an antigen was used as a negative control. The animals were sacrificed 1 week after the final immunization and samples were collected. For elucidation of the long-term effects of nasal vaccination, P6-specific immune responses were also examined 4 months after the final immunization.

2.4. Western blot analysis for P6 expression

Western blot analysis was performed to determine the expression of P6 protein in the nasal mucosa. For detection of P6 protein, we used serum of systemically P6-immunized mice, which were intraperitonealy administered with 10 μg of P6 protein weekly, total 3 times [11]. Nasal-associated lymphoid tissue (NALT), a mucosal inductive site, was taken from the palatine, and cells were prepared by a gentle teasing through sterile stainless steel screens [20]. Cell lysates were mixed in loading buffer with 5% 2-mercaptoethanol (ME). Nasal wash samples were also examined. Samples were subjected to 14% sodium dodecyl sulfate-poly-acrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The blots were incubated with serum of P6-immunized mouse for 1 h at room temperature. Membranes were washed with 0.5% Tween TBS and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse IgG secondary antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 30 min at room temperature. Protein bands were visualized with ECL substrate (GE Healthcare, Amersham, Bucks, England).

2.5. Flow cytometry

Mononuclear cells (MNCs) were isolated from NALT, and the number of $CD11c^+$ dendritic cells (DCs) in NALT as well as the expression of functional markers on DCs were analyzed by flow cytometry, as described previously [20,21].

The following monoclonal antibodies (mAbs) were used in this study: fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11c (HL3), phycoerythrin (PE)-conjugated anti-mouse CD11b (M1/70), Cy-Chrome-conjugated anti-mouse CD8α (53-6.7), Cy-Chrome-conjugated anti-mouse CD11c (HL3), FITC-conjugated anti-mouse MHC class I ($H\text{-}2K^b$, AF6-88.5), FITC-conjugated anti-mouse MHC class II (I-A/I-E, 2G9), FITC-conjugated anti-mouse CD80 (B7-1; 16-10A1), and FITC-conjugated anti-mouse CD86 (B7-2; GL1). These mAbs were purchased from BD Pharmingen (San Diego, CA). MNCs were incubated with various combinations of mAbs for 30 min at room temperature [21]. Samples were analyzed using FACS Calibur (Becton Dickinson, Sunnyvale, CA). Absolute number of $CD11c^+$ DCs in NALT per mouse were calculated according to the absolute number of isolated lymphocytes and the mean frequency of $CD11c^+$ DCs [21].

2.6. P6-specific antibody assays

P6-specific antibody titers in nasal wash and serum were determined by enzyme-linked immunosorbent assay (ELISA) with purified P6 protein, as described previously [21]. Briefly, 96-well plates (Nunc, Roshilde, Demmark) were coated with P6 (0.5 μg/well) and incubated overnight at 4 °C. Plates were washed and blocked with 1% bovine serum albumin (BSA)/PBS for 1 h at 37 °C. After washing, serial dilutions of the samples were added and incubated for 4 h at room temperature. After washing, biotinylated goat anti-mouse IgM, IgG, or IgA was added (1:1000 dilution; Southern Biotechnology Associates, Birmingham, AL). Following incubation and washing, HRP-conjugated streptavidin was added (1:2000 dilution; Gibco BRL, Gaithersburg, MD). The plates were incubated overnight at 4 °C. After washing, color reaction was developed at room temperature with 100 μl of ABTS (2,2′-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer at pH 4.2 containing 0.01% H_2O_2; Moss Inc., Pasadena, CA) after incubation for 15 min. Endpoint titers were expressed as the reciprocal log 2 of the last dilution, which gave an optical density at 405 nm (OD405) of >0.1 OD units above that of the