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Intranasal immunization with a non-adjuvanted adhesive protein descended from *Pasteurella pneumotropica* and its preventive efficacy against opportunistic infection in mice

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ABSTRACT

Intranasal vaccination is one of the most effective means of protecting against invading and colonizing pathogens because the vaccine elicits a mucosal immune response. The exploitation of vaccine adjuvants and delivery systems for intranasal vaccines is an important way to evoke antigen immunogenicity and elicit a better immune response at the mucosal sites. In the present study, we assessed the potential of intranasal immunization using a non-adjuvanted bacterial adhesive protein toward the host organs. We evaluated intranasal immunization with modified recombinant PnxIIIA (MP3) from *Pasteurella pneumotropica* and its preventive efficacy against opportunistic infection caused by *P. pneumotropica*, without using any adjuvants or delivery systems. The 100-kDa MP3 was confirmed to retain its immunogenicity and binding activity to collagen type I similar to the parent PnxIIIA. When MP3 was fused to green-fluorescent protein and inoculated into C57BL/6J mice intranasally, fluorescence intensity in the intranasal airway could be observed until 3 h after inoculation. Mice were intranasally immunized with MP3 at a maximum of 4 doses, with 7-day intervals. The antibody titer of serum IgG and IgA specific for MP3, as well as that of bronchoalveolar lavage fluid IgA, showed more than 9 ($\log_2$) after 3 or 4 rounds of immunization. Experimentally infecting immunized mice with *P. pneumotropica* resulted in the inability to isolate the bacterium from the nasal cavity, trachea, conjunctiva, or cecum with more than 3 doses in the immunized mice. Although the detection in each organ seldom changed with less than 2 rounds of immunization, unlike that observed in the non-immunized mice, the detection remarkably decreased with 3 or more rounds of immunization. These results suggest that intranasal immunization with a non-adjuvanted adhesive protein could have preventive effects against opportunistic infection by *P. pneumotropica*.

1. Introduction

Intranasal vaccination is one of the most effective administrations on account of the easy accessibility and elicitation of both mucosal and systemic immune responses [1–3]. The antigens are processed by endocytic vesicles of lymphoid cells in nasal-associated lymphoid tissue (NALT) [2], and B-cells develop into IgA plasma cells followed by the activation of T-cells. Secretory IgA (sIgA) defends the mucosal surface against entry by pathogens, and it is known to be the most abundantly synthesized isotype in the system [2]. Because of its role in protecting against invading pathogens into the nasal epithelium, the production of pathogen-specific sIgAs is one of the most important factors in phylaxis.

To induce an effective immune response, the antigen is generally co-applied with an aluminum salt as an adjuvant to enhance the immunogenicity of the antigen. In addition, various adjuvants and functional proteins are being developed that focus on inducing receptor binding or targeting cells to enhance the immune response [4–9]. Furthermore, antigen delivery systems that include live viruses, DNA particles, and biopolymers have been developed and investigated as a means to efficiently transport antigens to the inductive sites [10–13]. Although each method has different

Abbreviations: MP3, modified recombinant PnxIIIA protein; rPnxIIIA, recombinant PnxIIIA protein; AG, green-fluorescent protein Azami-Green.

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advantages, improvements to those systems with respect to safety and enhancement of antigen immunogenicity are needed.

Pasteurella pneumotropica is the causative agent of rodent pasteurellosis [14]. In current studies, several RTX (repeats in toxin) toxins were identified in this species, and of these, 250-kDa PnxIIA contains bacterial Ig-like domains and hemagglutinin repeats that are thought to be indispensable for the protein's attachment and invasion of bacterial cells into host organs [15,16]. In fact, the recombinant PnxIIIA that lacks these domains failed to bind to the extracellular matrix (ECM) [16]. PnxIIIA is reportedly less cytotoxic toward leukocytes as compared to other RTX family proteins, and therefore, PnxIIIA is one of the candidate antigens for immunization against *P. pneumotropica* infection in rodents [15,16]. In this regard, ECM adhesive proteins help long-term retention in the nasal cavity, which results in enhanced opportunities for NALT cell recognition when PnxIIIA is used in a vaccine for intranasal immunization. Simultaneously, for immunization, it may be required that the antigen alone is recognized by the lymphoid cells without an adjuvant or antigen delivery systems. To address these hypotheses, PnxIIIA from *P. pneumotropica* was modified as a vaccine antigen, while retaining its immunogenicity and binding activity. Here, preventive efficacy from intranasal immunization with modified recombinant PnxIIIA (MP3) was demonstrated in the setting of opportunistic infection caused by *P. pneumotropica*. Concurrently, we assessed the potential of performing intranasal immunization with a non-adjuvanted adhesive protein to eliminate infection by opportunistic pathogens.

2. Materials and methods

2.1. Bacterial strains and culture media

The *P. pneumotropica* used in this study was ATCC 35149, and for the experimental infection, a spontaneous rifampicin-resistant derivative of ATCC 35149 was used. The rifampicin-resistant *P. pneumotropica* was grown and maintained in brain heart infusion (BHI) medium supplemented with a final concentration of 50 μg/mL rifampicin. *Escherichia coli* strains DH5α and BL21-AI were used in Luria-Bertani (LB) medium supplemented with 100 μg/mL ampicillin for expression vector modification experiments and production of recombinant protein, respectively.

2.2. Construction and expression of MP3

Prior to modifying the immunogenic recombinant PnxIIIA (rPnxIIIA), the major histocompatibility complex (MHC) class II-binding peptides and their affinities for PnxIIIA were predicted using the Immune Epitope Database (IEDB; http://www.iedb.org/). To determine the MHC class II-binding predictions, the IC_{50} (median inhibitory concentration) of H2-A and E was used as an indicator of rPnxIIIA modification. The MP3 expression vector was constructed using the entire PnxIIIA coding gene containing the entry vector pENTR/SD/D-TOPO (Invitrogen, Carlsbad, CA, USA) as a PCR template in 3 steps with the PrimeSTAR Mutagenesis Basal Kit (Takara Bio, Shiga, Japan). The N-terminus, corresponding to amino acid residues 1–285 (aa; Fig. 1A), coding region was deleted using the primer pair M3A-NF (5′-AGCCCTTCACCGCAGATGCGAATGACAAT-3′) and M3A-NR (5′-GCATCTGCGGTGAAGGGCTCCTTCTTAAAGTT-3′). The C-terminus, at aa positions 1668–2453, coding region was deleted using the primer pair M3A-CF (5′-AGGTAATAAGGGTGGGCGCGCCGACCCAGCT-3′) and M3A-CR (5′-CCACCCTTATTACCTTCAACACCCCC-3′). Finally, the coding region aa at position 737–1095 was deleted using the primer pair M3A-MF (5′-TACTGCAGCCGGAGTTGATAATACA-3′) and M3A-MR (5′-CTCCGGCTGCAGTATTACCTGCCGG-3′), yielding pENTR-M3A. Subsequently, pENTR-M3A was recombined with pBAD-DEST49 (Invitrogen) to yield pBAD-PnxM3A. For the *in vivo*-binding assays in the mouse nasal cavity, MP3 was expressed together with the green-fluorescent protein AzamiGreen (AG), which originated from pAG-S1 (Medical and Biological Laboratories, Nagoya, Japan). The AG and MP3 coding genes were fused together, yielding pENTR-M3A-AG. The pENTR-M3A-AG vector was recombined with pDEST17 (Invitrogen) to yield pDEST-M3A-AG. The expression and purification of recombinant proteins were performed in the same manner as that used for the parent PnxIIIA [16]. The supernatant of recombinant *E. coli* culture was filtered using a 0.45-μm filter unit (Millipore, Billerica, MA) after the cells were sonicated, and was loaded onto a 1-mL His-trap HP affinity column (GE Healthcare, Amersham, UK) mounted on an ÁKTAprime plus fast protein liquid chromatography device (FPLC device; GE Healthcare). FPLC was performed by running a program for histidine-tagged protein purification according to the manufacturer's instructions.

2.3. Enzyme-linked immunosorbent assay (ELISA)

To determine the *in vitro* binding activity toward collagen and the antibody titers, ELISA was performed with rPnxIIIA derivatives and animal samples, respectively. The method for determining the binding affinities was the same as in a previous study [16]. For verification of the MP3 *in vitro* binding activity, we used non-collagen coated plate as the control.

To determine the IgG and IgA antibody titers in sera and the IgA antibody titer in bronchoalveolar lavage fluids (BALs), 96-well microtitre plates were coated with 1.0 μg/mL of MP3 and incubated overnight at 4 °C. Subsequently, the plates were blocked with 10% BSA for 30 min, and then the serially diluted samples were incubated in each well for 1 h. The plates were washed 3 times with PBS-T, and the AP-labeled anti-mouse IgG (KPL) or AP-labeled anti-mouse IgA diluted in 10% BSA at 1:1000 was then added into each well, followed by a 1 h incubation. Subsequently, the plates were washed 3 times with PBS-T and developed using the BluePhos Microwell Phosphatase Substrate System (KPL, Gaithersburg, MD, USA). AP-labeled anti-mouse IgA was prepared using non-labeled anti-mouse IgA (Invitrogen) and an AP Labeling Kit (Dojindo Laboratories, Kumamoto, Japan), according to the manufacturer's instructions. All the sample sera were confirmed to be shown higher antibody titers than the untreated control sera.

2.4. Intranasal immunization and P. pneumotropica infection

All the animal experimental procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Medical University. Specific-pathogen-free (SPF) 6–8-week-old female C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan) and were maintained under SPF conditions. For the experimental infection, intranasal immunization was performed at 7-day intervals with a maximum of 4 doses. Mice were divided into 5 groups (10 mice per group): each group received 0 (untreated control group), 1, 2, 3, or 4 doses of immunization for comparative studies. Mice were intranasally administrated with 2 μg of non-adjuvanted MP3 following anesthetization by a 1 mg intranasal pentobarbital sodium injection (Nembutal; Abbott Laboratories, Abbott Park, IL, USA). For 7 days after each final immunization, 5 mice in each group were euthanized by carbon dioxide asphyxiation, and then the heart blood and BALs were collected. To collect BALs, a catheter was placed in the trachea, and 0.8 mL PBS was instilled into the lung and then collected. The other mice were intranasally infected with 1.0×10^6–3.0×10^6 CFU/mL of rifampicin-resistant *P. pneumotropica* at 7 days after each final immunization. *P. pneumotropica* solution (20 μL) was intranasally administrated to each group of