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Intranasolacrimal Immunization of Mice with Pneumococcal Surface Protein A Plus Poly(I:C) Protective Against Nasopharyngeal Carriage of *Streptococcus pneumonia*

Hiromi Nagano[1,2]*, Issei Nagano[1,2], Masaki Kawabata[1,2], Junichiro Ohori[1,2] and Yuichi Kurono[1,2]

[1]Department of Pediatric Dentistry, Immunobiology Vaccine Center, Institute of Oral Health Research University of Alabama at Birmingham, USA

[2]Department of Otolaryngology, Head and Neck Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, Japan

Abstract

Purpose: The purpose of the study was to investigate the efficacy of intranasolacrimal immunization for induction of Pneumococcal surface protein A (PspA)-specific Secretory IgA (S-IgA) Antibody (Ab) responses in the upper respiratory tract.

Methods: Mice intranasolacrimally immunized with PspA and Polyinosinic-polycytidylic acid (Poly(I:C)) four times at weekly intervals were compared with intranasolacrimal controls and intranasal immunization on the same schedule. One week after final immunization, Nasal Washes (NWs), saliva and plasma were evaluated with PspA-specific ELISA. Mice were challenged with *S. pneumoniae* strain and CFUs in NWs was determined.

Results: Higher PspA-specific IgG (10.71 ± 1.60, P<0.01) and IgA (5.71 ± 1.25, P<0.01) Abs were present in plasma of vaccinated mice compared with controls (0.0 ± 0.0, 0.0 ± 0.0). Significantly increased PspA-specific S-IgA Ab responses were seen in NW (3.0 ± 1.0, P<0.01) and saliva (5.87 ± 1.46, P<0.01) of mice given intranasolacrimal PspA+Poly(I:C) compared with controls (0.0 ± 0.0, 0.0 ± 0.0). Importantly, mice given intranasolacrimal PspA+Poly(I:C) had significantly lower numbers of bacteria CFUs in NWs (2.43 ± 0.41, P<0.05) and NPs (3.57 ± 0.73, P<0.05) compared with controls (3.21 ± 0.47, 4.29 ± 0.58).

Conclusion: These results show that intranasolacrimal immunization induces antigen-specific mucosal and systemic immune responses same as intranasal immunization.

Keywords: Intranasolacrimal immunization; *Streptococcus pneumoniae*; PspA; Poly(I:C)

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Correspondence:
Hiromi Nagano, Department of Otolaryngology, Head and Neck Surgery, Kagoshima University Graduate School of Medical and Dental Sciences, 8-35-1 Sakuragaoka, Kagoshima, 890-8520, Japan, Tele: 81992755410; Fax: 81992648296; E-mail: nagano@m.kufm.kagoshima-u.ac.jp

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Introduction

Streptococcus pneumoniae (pneumococcus) is a major human bacterial pathogen and a significant cause of morbidity, resulting in 40,000 deaths in the United States each year [1]. *S. pneumoniae* causes invasive diseases such as otitis media, sepsis, meningitis and pneumonia in children and elderly people. Every year, pneumococcal infections result in the deaths of an estimated 800,000 children <5 years of age worldwide [2]. Pneumococci are also responsible for 30% to 50% of all otitis media infections and a significant portion of cases of sinusitis [3,4].

Pneumococcal capsular polysaccharide and pneumococcal protein-capsular conjugate vaccines provide protective immunity against pneumoniae and invasive diseases in adults and infants, but do not protect against strains with different serotypes. Therefore, it is important to develop a new generation of vaccines for prevention of all potential *S. pneumoniae* infections.

Pneumococcal surface protein A (PspA) is an exposed virulence factor found in almost all pneumococcal strains. PspA is also a highly immunogenic antigen that affects host-pathogen interactions by inhibiting complement activation by the classical and alternative pathways. Furthermore, PspA elicits specific Antibody (Ab) responses that protect against nasal carriage, pneumonia and bacteremia in animal models. Taken together, PspA is an attractive candidate for protein-based pneumococcal vaccines. Indeed, we have shown that intranasal immunization with

PspA plus a plasmid encoding Flt3 ligand and CpG ODN induced PspA-specific S-IgA Ab responses that provided significant protection against *S. pneumonia* infection in mice [5].

In this study, we have examined the efficacy of intranasolacrimal immunization for induction of protective PspA-specific S-IgA Ab responses in the upper respiratory tract in order to develop a new and effective mucosal vaccine.

Materials and Methods

Animals

Six- to 8-week-old female C57BL/6 mice were purchased from the Frederick Cancer Research Facility (National Cancer Institute, National Institutes of Health, Frederick, USA) and maintained in the animal facility of the University of Alabama at Birmingham (UAB) Immunobiology Vaccine Center (Birmingham, USA) under specific pathogen-free conditions. All mice used in the study were free of bacterial and viral pathogens. All animal experiments were performed in accordance with institutional guidelines at the UAB animal facility.

PspA and adjuvant

PspA was purified by nickel-affinity chromatography from *Escherichia coli* BL21(DE3) carrying pUAB055 [6], which comprised the first 302 of the 558 aa of PspA/Rx1, including all of the α-helical region and some of the proline-rich region [7,8]. We used Polyinosinic-polycytidylic acid (Poly(I:C)) (InvivoGen, USA), a synthetic analog of dsRNA, as was employed the adjuvant for PspA. Mice were anesthetized by intra-peritoneal administration of ketamine (2.46 mg/mouse) and xylazine (0.216 mg/mouse) in order to perform intranasolacrimal immunization.

Immunization and sample collection

Mice were immunized intranasal-acrimimally or intranasally four times at weekly intervals with 1 μg of PspA and 10 μg of Poly(I:C) as mucosal adjuvant. Control groups were immunized on the same timetable with 11 μL PBS alone, 1 μg PspA alone, or 10 μg Poly(I:C) alone. Plasma, Nasal Washes (NWs) and saliva were collected on day 28. Saliva was obtained from mice following injection of 100 mg sterile pilocarpine hydrochloride (Sigma-Aldrich, USA).

PspA-specific ELISA

PspA-specific Ab titers in plasma, saliva, and NWs were determined by ELISA [9]. Briefly, 96-well Falcon microtest assay plates (BD Biosciences, USA) were coated with 1 μg/ml PspA in PBS. After blocking with 1% BSA (Sigma-Aldrich, USA) in PBS, 2-fold serial dilutions of samples were added and incubated overnight at 4°C. HRP-labeled goat anti-mouse μ-, ϒ-, or α-H chain-specific Abs (Southern Biotechnology Associates, USA) were added to individual wells. For IgG subclass Ab analysis, biotinylated mAbs specific for IgG1, IgG2a, IgG2b, and IgG3 (BD Biosciences, USA) and peroxidase conjugated goat anti-biotin Ab (Vector Laboratories, USA) were used for detection. The color reaction was developed for 15 min at room temperature with 100 ml 1.1 mM ABTS (EMD Biosciences, USA). Endpoint titers were expressed as the reciprocal log2 of the last dilution that gave an OD at 415 nm of 0.1 greater than background.

PspA-specific ELISPOT

Mononuclear cells from spleen were isolated aseptically by gentle teasing through stainless-steel screens [10-12]. Nasal Passages (NPs) and Submandibular Glands (SMGs) were excised, teased apart and dissociated using collagenase type IV (Sigma-Aldrich, USA). Mononuclear cells were purified by discontinuous Percoll gradient centrifugation (Amersham Biosciences, USA). An ELISPOT assay was used to determine the number of PspA-specific Ab-Forming Cells (AFCs). Briefly, 96-well nitrocellulose plates (Millititer HA; Millipore, USA) were coated with 100 ng/ml PspA for analysis of anti-PspA-specific AFCs.

PspA-specific CD4+ T cell responses and detection of cytokines by ELISA

CD4+ T cells from spleen were purified using an Auto-MACS system (Miltenyi Biotec, USA) [10-12]. The purified CD4+T cell fraction was resuspended in RPMI 1640 (Cellgro Mediatech, USA) and 10% FCS (complete RPMI1640) (4×10^6 cells/mL) before culturing in the presence of 5 mg/mL PspA and T cell-depleted, irradiated (3,000 rad), splenic APCs for 5 days. Supernatants of T cell cultures were subjected to a cytokine-specific ELISA. Interferon gamma (IFN-ϒ) and Interleukin-4 (IL-4) in culture supernatants of CD4+ T cells from spleen were measured by cytokine-specific ELISA [10-12].

Bacterial infection

S. pneumoniae capsular group 19 strain EF3030 was obtained from Dr. Alan Parkinson at the Arctic Investigations Laboratory of the Centers for Disease Control (Anchorage, USA) [13]. *S. pneumoniae* strain EF3030 was among the human isolates of capsular group 19 examined previously and found to be essentially noninvasive in mice [14]. Three weeks after the fourth immunization, mice were challenged with $1\text{-}2 \times 10^6$ CFU EF3030 *via* the nasal route. Five days post challenge; NWs were collected as described above. NP tissues were removed from the nasal cavity and washed in 1 ml of PBS. The numbers of bacterial colonies were determined by plating NWs and NPs on blood agar (BD Biosciences, USA) and incubating at 37°C overnight [15].

Statistical analysis

Data are presented as mean ± SEM and compared by Mann-Whitney U test with $p<0.05$ considered significant. Analyses were performed using SAS v..9.3 (SAS Institute, USA).

Results

PspA-based mucosal vaccines induced specific IgM, IgG and IgA Ab responses in plasma

PspA-specific IgM, IgG and IgA were lower than the detection limit in all groups before immunization, and were lower than the detection limit in all control groups after immunization. There were significantly increased PspA-specific IgM, IgG and IgA responses in plasma of mice given intranasolacrimal (7.86 ± 0.90, $P=2.57 \times 10^{-11}$; 10.71 ± 1.60, $P=5.86 \times 10^{-10}$; 5.71 ± 1.25, $P=4.57 \times 10^{-8}$) and intranasal (7.0 ± 0.87, $P=4.82 \times 10^{-12}$; 15.78 ± 0.44, $P=5.26 \times 10^{-21}$; 6.78 ± 2.44, $P=1.11 \times 10^{-6}$) immunization of PspA+Poly(I:C), compared with all control groups (0.0 ± 0.0; 0.0 ± 0.0; 0.0 ± 0.0). There was no significant difference in PspA-specific IgM and IgA responses between intranasolacrimal and intranasal immunization, but PspA-specific IgG was significantly higher after intranasal immunization (15.78 ± 0.44 *vs.* 10.71 ± 1.60, $P=2.88 \times 10^{-7}$) (Figure 1A).

Comparison of the numbers of PspA-specific AFCs in spleen

PspA-specific IgM, IgG and IgA AFCs were lower than the detection limit in all control groups after immunization. There were significantly increased levels of PspA-specific IgM, IgG and IgA AFCs in plasma of mice after intranasolacrimal (19.57 ± 14.38, $P=3.65 \times 10^{-}$

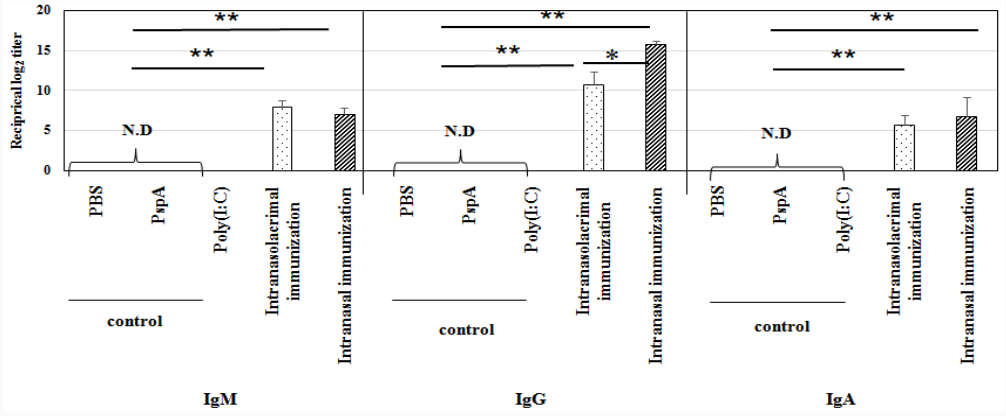

Figure 1A: PspA-Specific immunoglobulin (in plasma).
Significantly increased levels of PspA-specific IgM, IgG, and IgA responses were seen in plasma of mice given intranasolacrimal immunization PspA plus Poly (I:C) when compared with all control groups.

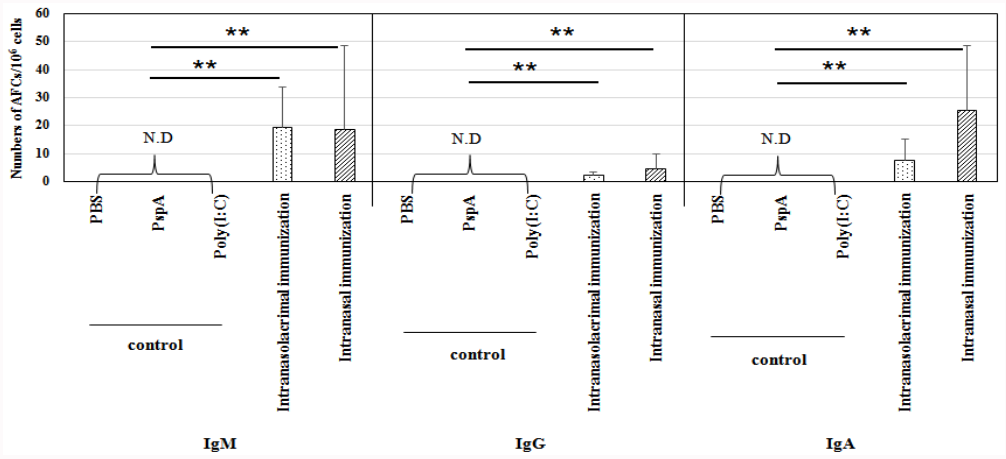

Figure 1B: PspA-Specific ELISPOT Assay.
Both intranasolacrimal and intranasal immunization elicited elevated numbers of PspA-specific IgM, IgG and IgA AFCs in spleen of mice when compared with those responses in control groups.
Results are expressed as mean ± SEM. *p<0.05; **p<0.01.

^{3}; 2.42 ± 0.98, P=2.60 × 10^{-5}; 7.86 ± 7.42, P=1.60 × 10^{-3}) and intranasal (18.71 ± 29.93, P=2.90 × 10^{-3}; 4.78 ± 5.4, P=3.58 × 10^{-5}; 25.56 ± 23.22, P=1.19 × 10^{-2}) immunization of PspA+Poly(I:C), compared with all control groups (0.0 ± 0.0; 0.0 ± 0.0; 0.0 ± 0.0). There was no significant difference in PspA-specific IgM, IgG, and IgA AFCs between intranasolacrimal and intranasal immunization (Figure 1B).

PC-specific IgA in NWs and saliva

PspA-specific IgA in NWs and saliva was lower than the detection limit in all control groups after immunization. There were significantly increased PspA-specific IgA responses in NWs and saliva of mice given intranasolacrimal (3.0 ± 1.0, P=9.32 × 10^{-7}; 5.87 ± 1.46, P=1.8 × 10^{-8}) and intranasal (8.22 ± 1.09, P=1.25 × 10^{-10}; 5.67 ± 2.0, P=2.5 × 10^{-7}) immunization of PspA+Poly(I:C), compared with all control groups (0.0 ± 0.0; 0.0 ± 0.0). PspA-specific IgA was significantly higher after intranasal immunization than after intranasolacrimal immunization (8.22 ± 1.09 *vs.* 3.0 ± 1.0, P=2.55 × 10^{-7}) in NWs, but there was no significant difference in saliva (Figure 2A).

Induction of PspA-specific IgA Ab responses in NPs and SMGs

PspA-specific IgA AFCs were lower than the detection limit in all control groups after immunization in NPs and SMGs. There were significantly increased levels of PspA AFCs in NPs and SMGs of mice after intranasolacrimal (1.66 ± 0.58, P=7.49 × 10^{-3}; 10 ± 0.0, P=6.52 × 10^{-5}) and intranasal (32.80 ± 11.25, P=2.74 × 10^{-3}; 31.71 ± 17.81, P=3.76 × 10^{-4}) immunization of PspA+Poly(I:C), compared with all control groups (0.0 ± 0.0, 0.0 ± 0.0). There was no significant difference in PspA-specific IgA AFCs between intranasolacrimal and intranasal immunization in SMGs, but PspA-specific IgA AFCs were significantly higher after intranasal immunization in NPs (32.80 ± 11.25 *vs.* 1.66 ± 0.58, P=3.56 × 10^{-3}) (Figure 2B).

Anti-PspA IgG subclass Ab responses in plasma of mice given intranasolacrimal PspA plus poly(I:C)

PspA-specific IgG1, IgG2a and IgG2b were lower than the detection limit in all groups before immunization and in all control groups after immunization. There were significantly increased PspA-specific IgG1, IgG2a and IgG2b responses in plasma of mice given intranasolacrimal (10.14 ± 1.68, P=1.84 × 10^{-9}; 9.17 ± 0.75, P=4.19 × 10^{-11}; 8.5 ± 1.73, P=6.44 × 10^{-5}) and intranasal (13.33 ± 2.0, P=9.57 × 10^{-13}; 6.11 ± 1.62, P=4.62 × 10^{-9}; 10.89 ± 1.54, P=3.73 × 10^{-13}) immunization of PspA+Poly(I:C) compared with all control groups (0.0 ± 0.0; 0.0 ± 0.0; 0.0 ± 0.0). PspA-specific IgG1 and IgG2b were significantly

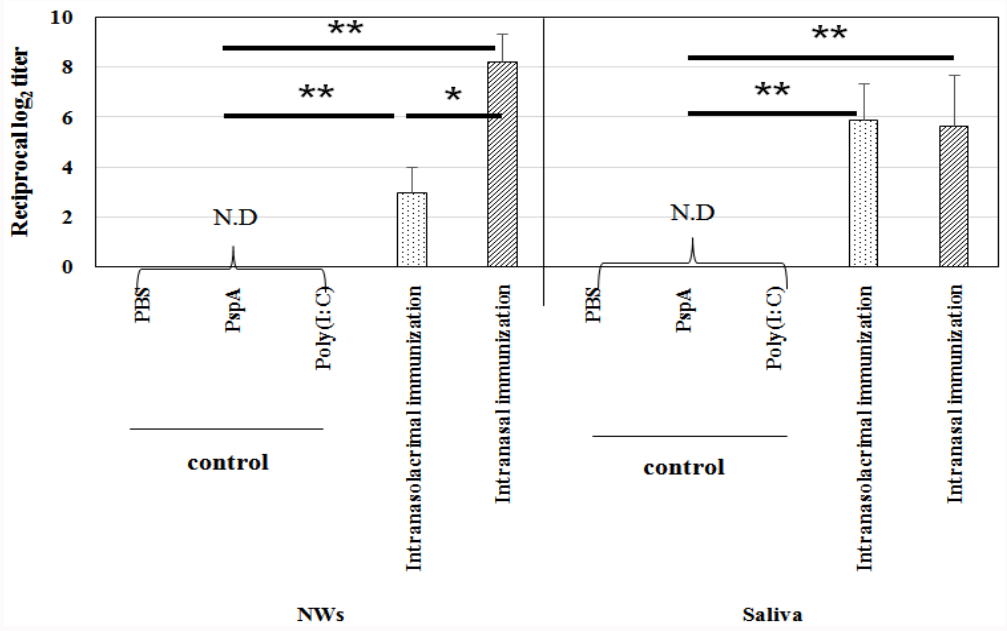

Figure 2A: PspA-specific IgA.
There were significantly increased PspA-specific IgA responses in NWs and saliva of mice given intranasolacrimal and intranasal immunization of PspA plus Poly(I:C), compared with all control groups. PspA-specific IgA was significantly higher after intranasal immunization than after intranasolacrimal immunization in NWs, but there was no significant difference in saliva.

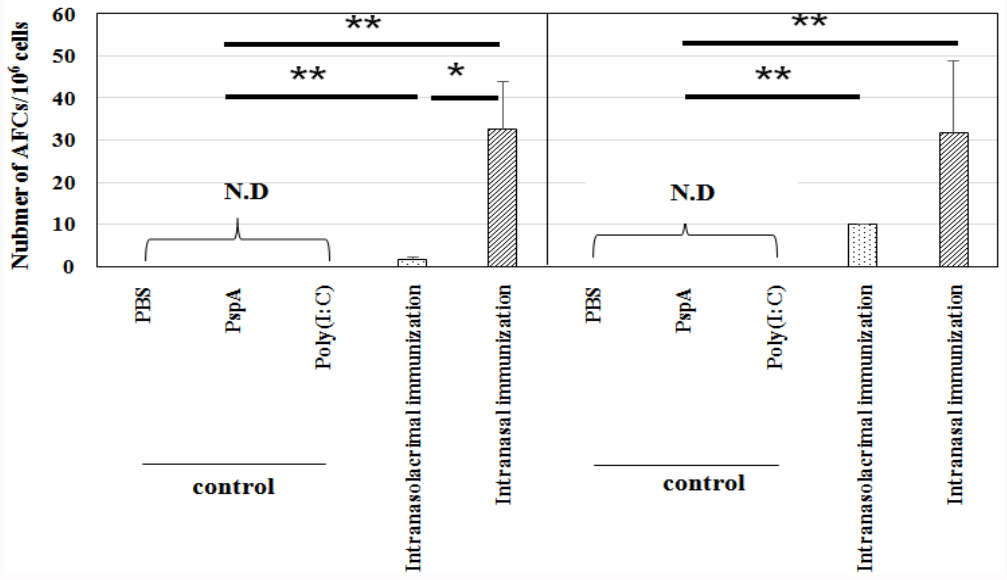

Figure 2B: PspA-Specific ELISPOT Assay.
Intranasolacrimal immunization revealed PspA-specific IgA AFCs when compared with control groups, these numbers were lower than those seen in NPs of mice given vaccine via the nasal route.
Results are expressed as mean ± SEM. *p<0.05; **p<0.01.

higher after intranasal immunization (13.33 ± 2.0 *vs.* 10.14 ± 1.68, P=4.4 × 10^{-3}), whereas PspA-specific IgG2a was significantly higher after intranasolacrimal immunization (9.17 ± 0.75 *vs.* 6.11 ± 1.62, P=8.7 × 10^{-4}) (Figure 3A).

Cytokine production from splenic CD4+ T cells

There was no significant difference in IFN-ϒ levels intranasolacrimal immunization and intranasal immunization (0.91 ± 0.59 *vs.* 0.89 ± 0.54, P=0.95). IL-4 was lower in intranasolacrimal immunization than intranasal immunization (0.21 ± 0.08 *vs.* 1.46 ± 0.74, P=0.02).

Intranasolacrimal immunization induced functional anti-PspA Abs to prevent nasal carriage of *S. pneumoniae*

Mice had significantly lower bacteria counts in NWs and NPs after intranasolacrimal (2.43 ± 0.41, P =3.80×10-2; 3.57 ± 0.73, P=3.35×10-2) and intranasal (1.75 ± 0.82, P=1.83×10-2; 2.93 ± 0.38, P=1.62×10-2) immunization of PspA+Poly(I:C), compared with controls (3.21 ± 0.47; 4.29 ± 0.58). There was no significant difference in bacteria counts in NWs and NPs between intranasolacrimal and intranasal

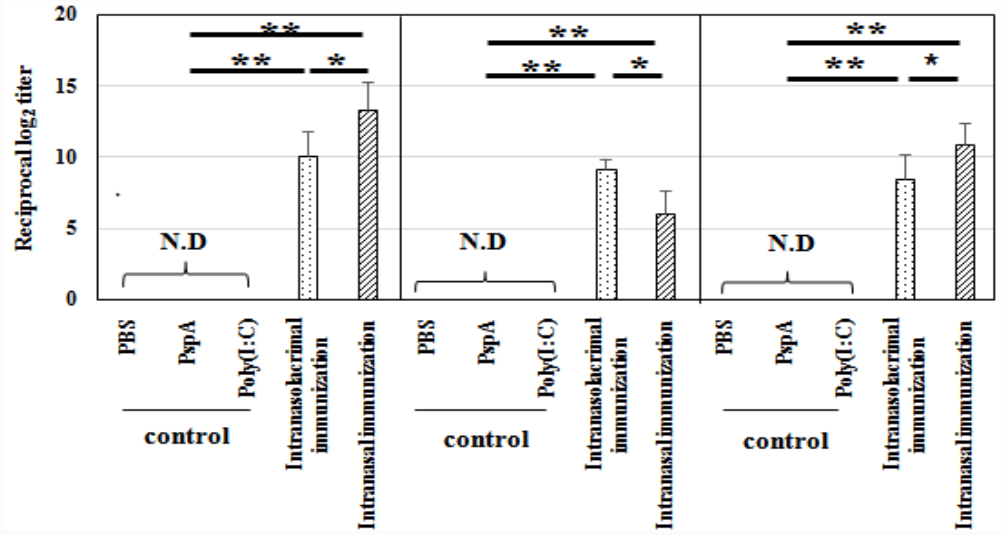

Figure 3A: PspA-specific IgG subclass.
Significantly increased levels of PspA-specific plasma IgG1, IgG2a and IgG2b Ab responses were induced by intranasolacrimal and intranasal immunization with PspA plus Poly(I:C) when compared with control groups.

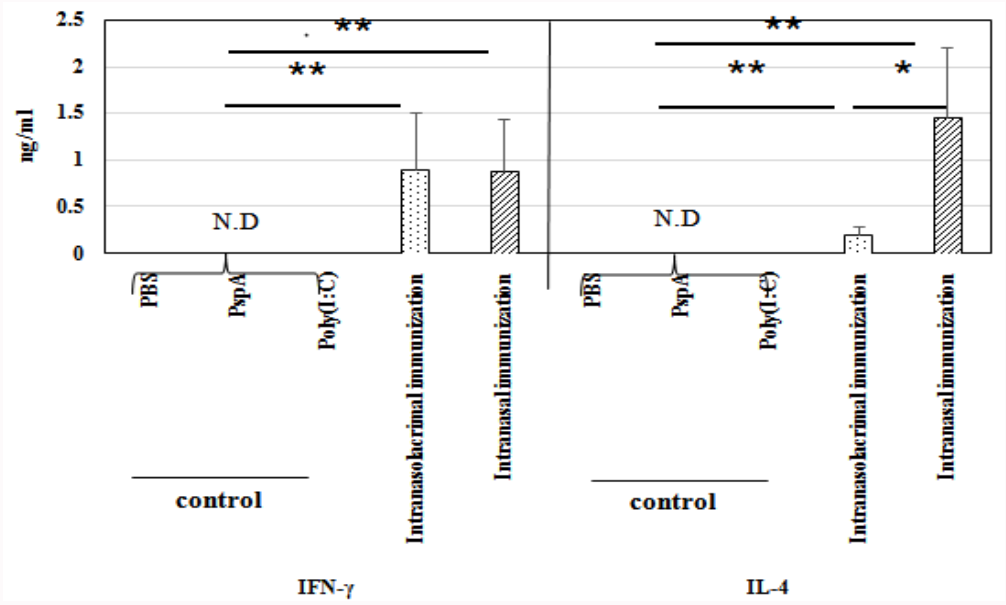

Figure 3B: Cytokine production from splenic CD4+ T cells.
There was no significant difference in IFN-ɤ levels intranasolacrimal immunization and intranasal immunization. IL-4 was lower in intranasolacrimal immunization than intranasal immunization.
Results are expressed as mean ± SEM. *p<0.05; **p<0.01.

immunization (Figure 3B, 4A, 4B).

Discussion

Systemic administration of vaccines can be used, including subcutaneous injection and transmucosal administration of intranasal and sublingual vaccines, but facial nerve paralysis may occur as an adverse event after transmucosal delivery of intranasal vaccine [16]. Therefore, an administration route with fewer adverse events has been sought and this has led to interest in intranasolacrimal immunization. The nasolacrimal ducts are at the border of the disciplines of ophthalmology and Otorhinolaryngology. However, little is known about the immunology of the nasolacrimal system. Our purpose was to investigate the influence of immunity in the nasolacrimal duct on nasal cavity infection, compared with intranasal immunization.

Higher levels of PspA-specific IgM, IgG and IgA Abs were noted in plasma of vaccinated mice compared with controls, and significantly increased PspA-specific S-IgA Ab responses were seen in NWs and saliva of mice given intranasolacrimal immunization of PspA plus Poly(I:C), compared with control groups. These results show that intranasolacrimal immunization induces antigen-specific mucosal and systemic immune responses.

The advantages of ocular instillation include avoidance of rapid increases in antigen and adjuvant concentrations in the nasal cavity because antigens flow into the nasolacrimal duct after storage in the lacrimal sac; Tear Duct-Associated and Nasopharynx-Associated

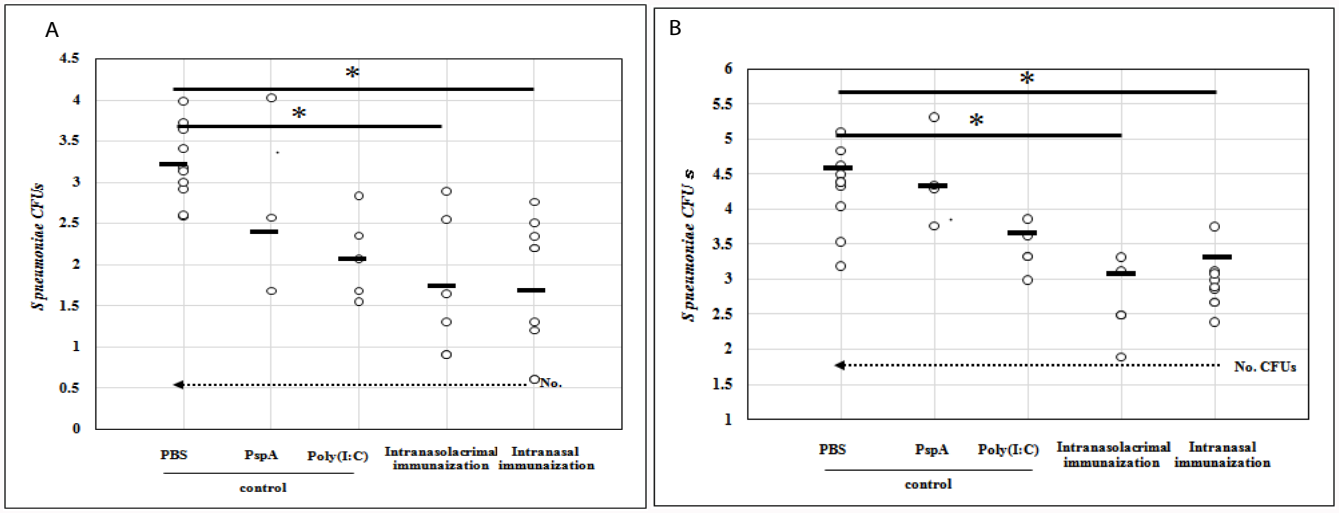

Figure 4A and 4B: Intranasolacrimal immunization induced functional anti-PspA Abs to prevent nasal carriage of *S. pneumoniae*.
Both NWs and NPs of mice given intranasolacrimal vaccine contained significantly lower bacteria when compared with those of mice given PBS alone via the same route.
Results are expressed as mean ± SEM. *p<0.05; **p<0.01.

Lymphoid Tissue (TALT and NALT) are activated; medical waste can be reduced because no needles are used; and the procedures causes no pain. In addition, since antigens and adjuvants flow from the nasolacrimal duct to the inferior nasal meatus, there is only a small possibility of direct flow into the olfactory cleft, where olfactory nerves are distributed, and the effects on the central nervous system should be low, compared with administration of nasal drops. TALT displays cytomorphological and immunophenotypic features of Mucosa-Associated Lymphoid Tissue (MALT) and is similar in humans and mouse. Expression of immunoglobulins and secretory components indicates that the conjunctiva belongs to the secretory immune system [17,18]. NALT and TALT lymphocytes are independent of these tissue-specific migration molecules [19].

In our studies, PspA-specific IgG in plasma, PspA-specific IgA in NWs, and the number of IgA AFCs were significantly higher in intranasal compared to intranasolacrimal immunization. This may be because some antigen is absorbed in the lacrimal duct and reaches the nasal cavity mucous membrane, which may also explain the absence of a significant difference in bacteria counts in NWs and NPs between intranasolacrimal and intranasal immunization. The balance of IgG1 and IgG2a may be particularly important, since it was recently reported that induction of a balanced IgG1 and IgG2a anti-PspA Ab response correlated with increased protection against systemic pneumococcal infections [20-22]. According to these IgG subclass Ab responses, induction of a balanced Th1- and Th2-type cytokine response may be an ideal host response for protection. In a cytokine analysis, there was no significant difference in IFN-ϒ levels intranasolacrimal immunization and intranasal immunization. But, IL-4 was lower in intranasolacrimal immunization than intranasal immunization. This suggests that intranasal immunization of PspA plus Poly(I:C) as adjuvant causes predominant induction of Th2 type antibody-mediated immunity, and also Th1 type. But intranasolacrimal immunization of PspA plus Poly(I:C) as adjuvant causes both of Th1 and Th2 responses.

Intranasolacrimal immunization with PspA plus Poly(I:C) resulted in significantly lower numbers of *S. pneumoniae* in NWs and NPs, compared with controls. These results show that intranasolacrimal immunization is an effective mucosal immunization strategy for induction of protective pneumococcal-specific Ab responses in blockade of *S. pneumoniae* colonization of the nasal cavity.

Conclusion

This approach may be a new and effective mucosal immunization strategy for induction of protective pneumococcal-specific Ab responses in blockade of *S. pneumoniae* colonization of the nasal cavity. There was no significant difference in the numbers of bacteria in NWs and NPs in intranasolacrimal and intranasal immunization.

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