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Comparative Effects of Toll-Like Receptor Agonists on a Low Dose PspA Intranasal Vaccine against Fatal Pneumococcal Pneumonia in Mice

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Abstract

To develop a cost-effective pneumococcal vaccine, we compared the effects of a panel of Toll-like receptor (TLR) agonists on a low dose pneumococcal surface protein A (PspA) nasal vaccine in a fatal pneumococcal pneumonia model using a serotype 3 strain. The mice were nasally immunized with 10 μg of the TLR agonist (TLR 2, 3, 4 and 9) and 0.1μg of PspA once per week for three weeks. A high level of PspA-specific immunoglobulin G (IgG) was detected in sera of mice that were nasally administered a low dose of PspA plus each TLR agonist, while no PspA-specific IgG were detected in sera of mice that had been nasally administered a low dose of PspA alone. A relatively low level of PspA-specific IgG was also detected in the airway of mice that had been nasally administered a low dose of PspA plus each TLR agonist. The binding of PspA-specific IgG increased the deposition of C3 on the bacterial surface. Bacterial density in the lung and blood was significantly decreased in mice that had been administered a low dose of PspA plus each TLR agonist, compared with mice that received a low dose of PspA alone 24 h after a bacterial challenge. Furthermore, significant increases in survival rate were found in a murine model of fatal pneumonia that had been nasally administered a low dose of PspA plus each TLR agonist, compared with mice that received a low dose of PspA alone. The rank order of TLR agonists on the effect of increasing survival rate was LPS > Pam3CSK4 > Poly(I:C) and CpG 1826. These data suggest a potentially new strategy for the development of a cost-effective intranasal vaccine with a low dose PspA plus TLR agonist that would be effective against life-threatening bacteremic pneumococcal pneumonia.

Keywords: PspA; TLR agonist; Intranasal vaccine; *Streptococcus pneumoniae*; Pneumonia

Abbreviations: PspA: Pneumococcal surface protein A; TLR: Toll-Like Receptor; LPS: Lipopolysaccharide; Poly(I:C): Polyinosine-polycytidylic acid; CpG ODN 1826: CpG-Containing Oligodeoxynucleotide 1826; BALF: Bronchoalveolar lavage fluid; NW: Nasal wash

Introduction

S. pneumoniae is a leading human pathogen that causes a wide variety of diseases, ranging from otitis media to pneumonia, bacteremia, and meningitis in both children and adults. Pneumococcal infections can occur at any age but are more frequent in infants, the elderly and immunocompromised patients. Despite the development of effective treatments, the pneumococcus has remained a significant cause of morbidity and mortality worldwide [1,2]. Because of this, a clear need for an effective vaccine for the prevention of disease exists. Currently licensed polysaccharide-based pneumococcal vaccines only elicit protective antibodies against the infection of serotypes that are included in the vaccine. In addition, invasive diseases attributable to non-vaccine serotypes of *S. pneumoniae* have increased greatly [3,4]. Therefore, the search for new vaccine candidates that elicit protection against a broader range of pneumococcal strains is an important goal. To broaden the protection, the use of pneumococcal proteins represents a feasible and preferable alternative. Several pneumococcal proteins are currently under investigation as potential candidates for such a vaccine [5,6]. One of these proteins, PspA has recently undergone phase one clinical trials in humans and has been found to be safe and highly immunogenic [7,8]. PspA is a surface protein of *S. pneumoniae* that is found on all pneumococci and is broadly expressed among different serotypes of pneumococci [8]. Antibodies to PspA generated in mice [9,10] or humans [7,8] are capable of passively protecting mice against infections with different serotypes. PspA is, therefore, an attractive candidate for use as future protein-based pneumococcal vaccines.

Since *S. pneumoniae* enters the host primarily through the respiratory mucosa, vaccination strategies designed to target the airways are of great interest. An appropriate mucosal adjuvant is required to elicit an antigen-specific immune response in both the mucosal and systemic compartments [11]. We previously reported that each of the TLR agonists used in this study is an effective nasal adjuvant for the PspA antigen at a high dose (2.5 μg per mouse), and that it elicited the production of PspA-specific IgA in the airways and PspA-specific IgG in plasma. Because of this, it was capable of reducing the bacterial load in both the nasopharynx and lungs after a challenge with pneumococci with different serotypes [12]. Nasal immunization with a high dose of PspA alone could induce a certain level of PspA-specific IgG in the plasma and increased mouse survival, compared with mice that were nasally immunized with PBS alone, in a fatal pneumonia model in this study. These data suggest that nasal immunization with the reduced

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dose of PspA in combination with a TLR agonist was able to prevent the development of fatal pneumonia in this model. In the present study, we therefore examined the issue of whether nasal immunization using different TLR agonists in conjunction with a low dose (0.1μg per mouse) of PspA could confer protection against fatal pneumococcal pneumonia in healthy mice.

Materials and Methods

Mice and bacterial strains

Female C57BL/6 mice (6- to 8-wk-old) were purchased from Charles River Japan, Kanagawa, Japan. Mice were transferred to microisolators and maintained in horizontal laminar flow cabinets. They were provided sterile food and water in a specific pathogen-free facility. All mice used in these experiments were free of bacterial and viral pathogens. All animal experiments described in this study (protocol number; 08008) were performed in accordance with institutional guidelines for the Osaka University animal facility. *S. pneumoniae* WU2 strain with serotype 3, expressing PspA belonging to family 1, clade 2, was grown in Todd-Hewitt Broth (BD, Franklin Lakes, NJ) supplemented with 0.1% yeast extract (THY) to the mid-log phase and washed twice with phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$. Bacteria were suspended in THY, and aliquots were snap frozen in liquid nitrogen and stored at -80°C until used.

Recombinant PspA and adjuvants

Recombinant PspA/Rx1 (amino acids 1 to 302) was prepared as previously described [8]. To extensively remove LPS from the PspA preparations, we used an LPS removal column, ProteoSpin^R, (Norgen, Thorold, Canada). Four TLR agonists, including *Escherichia coli* K12 LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), Poly(I:C) (TLR3 agonist) or CpG ODN 1826 (TLR9 agonist) were selected to use as adjuvants. The LPS, Pam3CSK4 and Poly(I:C) were purchased from InvivoGen (san Diego, CA). CpG ODN 1826 was purchased from Hokkaido System Science (Sapporo, Japan). Each of these adjuvants was used in a dose of 10 μg for nasal immunization.

Nasal immunization

Mice were immunized intranasally three times at weekly intervals with 12μl of PBS containing 10μg of each TLR agonist and 0.1μg of PspA, 0.1μg of PspA alone or 12 μl of PBS on days 0, days 7 and days 14. The mice were euthanized on day 21 to obtain serum, bronchoalveolar lavage fluid (BALF) and a nasal wash (NW). The time points of nasal immunization and sampling for the determination of antibody levels were determined according to our previous study [13]. After removing the mandible, the nasal cavity was gently flushed with 1 ml of PBS from the posterior opening of the nose. The NW obtained from the anterior openings of the nose was collected. BALF was obtained by irrigation with 1 ml of PBS using a blunted needle inserted into the trachea after a tracheotomy [13].

PspA-specific antibody assays

PspA-specific antibody titers of IgG or IgA in Serum, BALF and NW were determined by ELISA as previously described [12]. PspA was used as the coating antigen (1 μg/ml). A 100μl sample was added to each well, followed by incubation at 37°C for 30 min. The plate was washed, and then reacted with 100μl of alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, IgG1 or IgG2a (Zymed, San Francisco, CA) and the OD at 405 nm was then measured. End-point titers were expressed as the reciprocal log_2 of the last dilution giving an OD_{405} of 0.1 OD unit above the OD_{405} of negative control samples obtained from non-immunized mice.

IgG binding and C3 deposition assays

Antibody binding was analyzed by whole cell ELISA. Frozen stock of *S. pneumoniae* WU2 (family 1 and clade 2) were plated onto blood agar, incubated overnight and then grown in THY to an OD_{600} of 0.4~0.5 and harvested by centrifugation. The bacterial cells were washed, resuspended in PBS, and fixed with 80% ethanol at room temperature for 1 h. The ethanol-killed bacteria were washed twice with PBS, and the pellet resuspended in PBS to an OD_{600} of about 0.2. 50μl of the ethanol-killed bacteria were coated in ELISA overnight at 4°C. The following day, the wells were washed 3 times with 0.05% of Tween-20 in PBS (PBST). The plates were then blocked with 3 % of skim milk in PBST at room temperature for 1 h. After 3 washes with PBST, 50μl of diluted serum in 1 % of skim milk in PBST were added to the plates, which were then incubated at 37°C for 2 h. The plates were washed 3 times with PBST, and then reacted with 100μl of alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, San Francisco, CA). The OD at 405 nm was then measured. The end-point titers were expressed as the reciprocal log_2 of the last dilution giving an OD_{405} of 0.1 OD unit above the OD_{405} of negative control samples obtained from non-immunized mice.

C3 deposition was analyzed by flow cytometry. 10μl or 20μl of heat-inactivated serum was incubated with washed *S. pneumoniae* WU2 cells in 100μl of a reaction mixture achieving at a final concentration of 10^8 cfu/ml at 37°C for 30 min. The live bacteria were washed once with PBS and then incubated with 10% fresh normal mouse serum as the source of complement in gelatin veronal buffer with Ca^{2+} and Mg^{2+} (Sigma, St. Louis, MO) at 37°C for 30 min. After washing, live bacteria were incubated with 100μl of fluorescein isothiocyanate-conjugated anti-mouse C3 IgG (MP Biomedicals, Solon, OH) in PBS for 30 min on ice. The samples were fixed with 2% formaldehyde after two washing steps and stored at 4°C. Flow cytometry analysis was conducted using a FACSCalibur apparatus (Becton Dickinson), and 10,000 gated events were recorded.

Fatal pneumococcal pneumonia model

To determine the protective effects of nasal immunization with PspA plus each TLR agonist, *S. pneumoniae* WU2 strain at a dose of 2.0 × 10^7 cfu (3 x LD_{50}) suspended in 30μl of sterile saline was intranasally administered to both the immunized and untreated mice 2 weeks after the last immunization. The 2-week interval between the last immunization and the bacterial challenge was maintained to avoid the influence of each TLR agonist on pulmonary defense, since some TLRs are involved in the innate immune response *to S. pneumoniae* [14-16]. The blood or lungs were aseptically removed from mice that had been anesthetized or euthanized with pentobarbital at 24 h post-bacterial challenge. The lung tissue was homogenized in 2 ml of sterile saline per whole lung tissue prior to culturing. To prevent coagulation, the blood was collected in tubes containing heparin. Quantitative bacterial cultures of blood or lung tissues were performed on horse blood agar. Mortality was monitored for 16 days following the pneumococcal challenge.

Statistics

Statistical analyses were performed using one-way ANOVA and Tukey's multiple comparison method for antibody titers and bacterial clearance [17], and a log rank (Mantel-Cox) test for analysis of the survival curve with Graphpad Prism (GraphPad Software, Sandiego,

CA). Data were considered to be statistically significant if the p-values were less than 0.05.

Results

Immune responses to PspA in mice after nasal immunization with a low dose of PspA plus TLR agonists

While no PspA-specific IgG was detected in sera of mice that had been immunized nasally with PspA alone, increased high levels of PspA-specific IgG were detected in sera of mice that had been immunized nasally with PspA plus and either LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 1A). The levels of PspA-specific IgG in the sera of mice immunized PspA plus LPS were significantly higher than those of mice that had been immunized with PspA plus and either Poly(I:C) ($p < 0.01$) or CpG 1826 ($p < 0.01$). No significant difference was found among the levels of PspA-specific IgG among mice that had been immunized nasally PspA plus Pam3CSK4, Poly(I:C) or CpG 1826.

The levels of PspA-specific IgG in the BAL fluids and NWs of mice that had been nasally immunized with PspA plus the LPS were 5.17 ± 0.98 and 1.67 ± 1.03, respectively. In contrast, negligible levels of PspA-specific IgG were induced in the BAL fluids and NWs from mice that had been nasally immunized PspA plus Pam3CSK4, Poly(I:C) and CpG 1826. PspA-specific IgA was not detected in the BAL fluid or NWs from these mice that had been immunized nasally with PspA plus the TLR agonist.

No PspA-specific IgG1 or IgG2a was detected in sera of mice that had been immunized nasally with PspA alone (Figure 1B). In contrast, increased levels of PspA-specific IgG1 or IgG2a were found in mice that had been immunized nasally with PspA plus the TLR agonist. The levels of PspA-specific IgG1 in sera of mice that had been immunized nasally with PspA plus LPS were significantly higher than those of mice that were immunized nasally with PspA plus Pam3CSK4 ($p < 0.05$), PspA plus Poly(I:C) ($p < 0.01$) or PspA plus CpG 1826 ($p < 0.01$). No

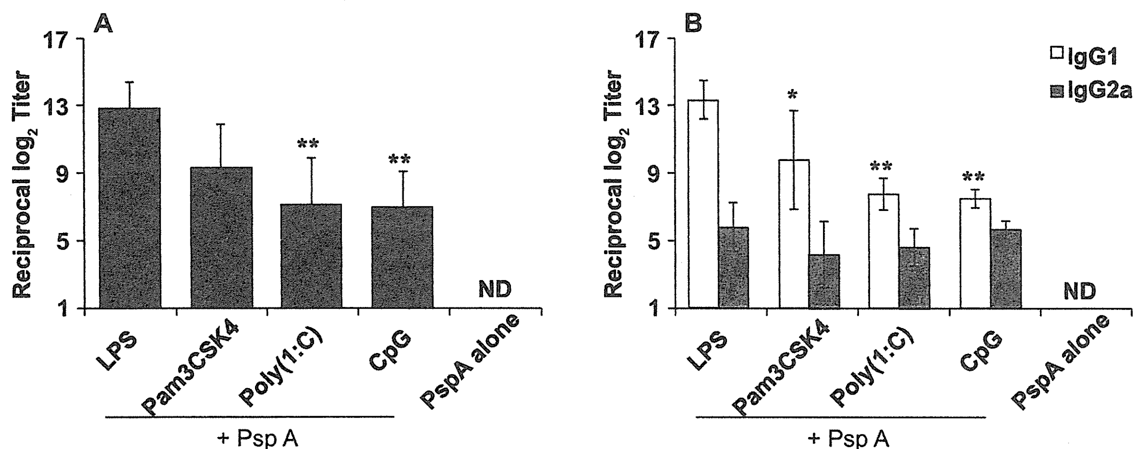

Figure 1: Induction of PspA-specific IgG (closed bars)(A), PspA-specific IgG1 (open bars) and IgG2a (gray bars) (B) in serum by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times at weekly intervals with 10 µg of TLR agonist and 0.1 µg of PspA. One week after the final immunization, the mice were euthanized to obtain serum, and PspA-specific antibody titers were determined using ELISA. The results are expressed as the mean ± S.D. for six mice per group. *$p < 0.05$, **$p < 0.01$, when compared with mice that were nasally immunized PspA plus LPS. ND, not detected.

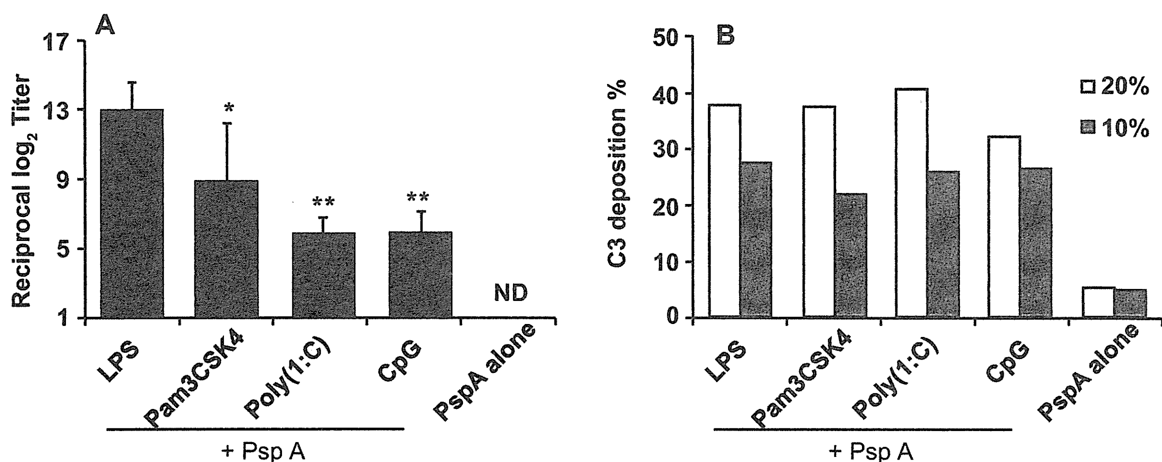

Figure 2: Binding of IgG antibodies (A) and C3 deposition (B) on the *S. pneumoniae* WU2 cell surface in the presence of sera from immunized and control mice. Binding The levels of IgG were analyzed by whole cell ELISA, and the C3 deposition were analyzed by flow cytometry using *S. pneumoniae* WU2 cells which were incubated with 20% (open bars) or 10% (gray bars) of pooled sera from mice that were immunized by intranasal immunization with either PspA plus each TLR agonist or PspA alone. The percentage of fluorescent bacteria (greater than 10 fluorescence intensity units) is shown as C3 deposition for each sample. *$p < 0.05$, **$p < 0.01$, when compared with mice that were nasally immunized PspA plus LPS. The results are expressed as the mean ± S.D. for six mice per group. ND, not detected.

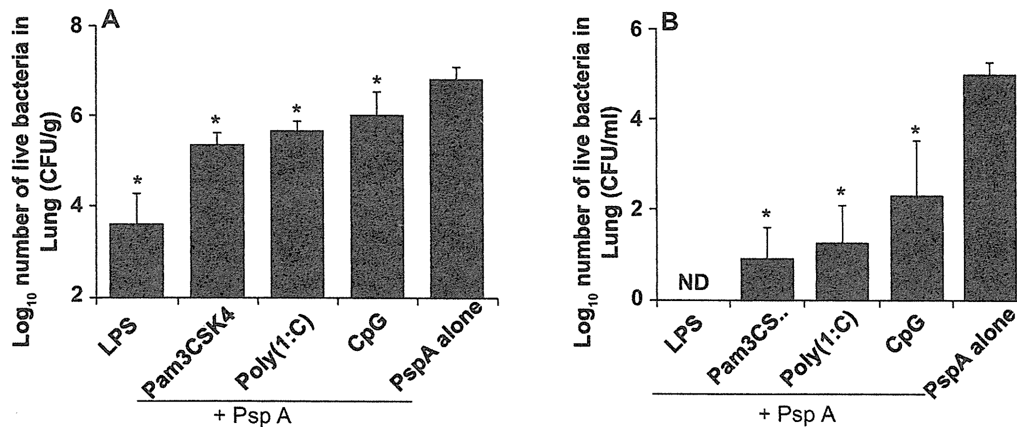

Figure 3: The effect of intranasal immunization with PspA plus each TLR against on the bacterial densities in Lung tissue (A) and blood (B) at 24 h post-challenge with the *S. pneumoniae* WU2 strain. An invasive dose of 2 × 10^7 cfu / mouse was nasally administered to mice that had been previously immunized with either PspA plus each TLR agaonist or PspA alone. Mice were euthanized to obtain lung tissues or blood from infected mice at the indicated time-point after bacterial challenge, and quantitative bacterial cultures of lung tissue or blood were performed. Values represent the Log_{10} cfu / g or Log_{10} cfu / ml (mean ± S.D.) for six mice per group. *$p < 0.0001$, when compared with mice that were nasally immunized with PspA alone.

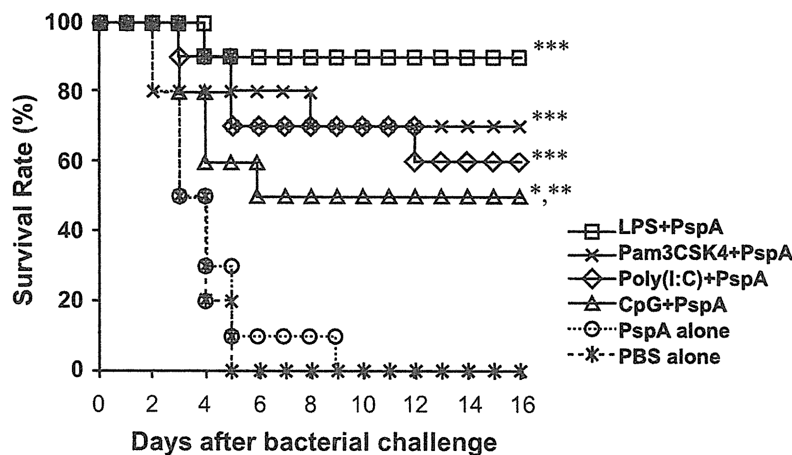

Figure 4: Survival of mice that were immunized nasally with a low dose of PspA plus each TLR agonist, a low dose of PspA alone and PBS alone after pneumococcal pneumonia. Immunized mice were intranasally challenged with 2 × 10^7 cfu of pneumococcal strain WU2, and the survival of the mice was monitored for 16 days. Results were examined by a Kaplan-Meier survival curve analysis for ten mice per group. *$p < 0.05$, when compared with mice that were nasally immunized PspA alone. **$p < 0.01$, when compared with mice that were nasally immunized PBS alone. ***$p < 0.001$, when compared with mice that were nasally immunized PspA alone or PBS alone.

significant difference was found in PspA-specific IgG1 levels among the sera of mice that were nasally immunized with PspA plus Pam3CSK4, Poly(I:C) or CpG 1826. No significant difference was found in PspA-specific IgG2a among sera of mice that were immunized nasally with PspA plus the TLR agonist. The mean ratios of PspA-specific IgG1 titers to PspA-specific IgG2a titers were 2.26 for LPS, 2.33 for Pam3CSK4, 1.68 for Poly(I:C), and 1.32 for CpG 1826, respectively.

IgG binding and C3 deposition on the bacterial surface

While no binding of mouse IgG was observed in the case of bacteria treated with sera from mice that were immunized nasally with PspA alone, the levels of mouse IgG found on bacteria that were treated with sera from mice immunized nasally with the PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 2A) was increased. The levels of mouse IgG were significantly higher in sera from mice that were immunized nasally PspA plus LPS compared to mice that were nasally immunized with PspA plus Pam3CSK4 ($p < 0.05$), PspA plus poly (I:C) or PspA plus CpG 1826 ($p < 0.01$). These levels of mouse IgG in serum from mice that were immunized nasally with PspA plus the TLR agonist closely corresponded with the levels of PspA-specific IgG induced in serum. The frequencies of C3 depositions were substantially increased in bacteria that had been pretreated with 10% and 20% sera from mice that were immunized nasally with PspA plus each of the TLR agonists, compared with those of mice that were immunized nasally with PspA alone (Figure 2B). No differences were found in the frequencies of C3 deposition on bacteria that were pretreated with sera from mice nasally immunized with PspA plus each TLR agonist.

Bacterial clearance from the lungs and the blood

The bacterial densities (mean ± S.D. for Log_{10} cfu / g) reached 6.83 ± 0.26 in the lung and 4.89 ± 0.3 in blood from mice that were immunized nasally with PspA alone at 24 h post-challenge (Figure 3A and 3B). Significant decreases were found in bacterial density in the lungs of mice that were immunized nasally with PspA plus either

LPS, Pam3CSK4, Poly(I:C) or CpG 1826 compared with mice that were immunized nasally with the PspA alone ($p < 0.0001$). No significant differences were found in the bacterial densities in the lung among mice immunized nasally with PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 3A). Significant decreases were also found in blood from mice that were immunized nasally with PspA plus either Pam3CSK4, Poly(I:C) or CpG 1826 compared with mice that were immunized nasally with the PspA alone ($p < 0.0001$). No bacteria were detected in blood samples from any of the mice that were immunized nasally with PspA plus LPS agonist. No significant differences were found among mice immunized nasally with PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826 (Figure 3B).

Protection by PspA plus each TLR vaccine against fatal pneumococcal pneumonia

As shown in Figure 4, the Kaplan-Meier analysis demonstrated significant protection as evidenced by the mean survival rate for mice that were immunized nasally with PspA plus each TLR agonist compared with mice that were immunized nasally with PspA alone or PBS alone. The survival rate was 90% for mice immunized nasally with the PspA plus LPS agonist ($p < 0.0001$ for PspA alone or PBS alone), 70% for mice immunized nasally with PspA plus Pam3CSK4 agonist ($p = 0.0008$ for PspA alone or $p = 0.0006$ for PBS alone), 60% for mice immunized nasally with PspA plus Poly(I:C) agonist ($p = 0.0005$ for PspA alone or $p = 0.0003$ for PBS alone), 50% for mice immunized nasally with PspA plus CpG 1826 agonist ($p = 0.0127$ for PspA alone or $p = 0.0062$ for PBS alone), respectively. No significant differences were found among mice that were immunized nasally with PspA plus LPS, Pam3CSK4, Poly(I:C) or CpG 1826.

Discussion

The findings reported herein provide a demonstration of the protective effects of the nasal vaccination of a low dose of PspA plus each TLR agonist against a fatal model of pneumococcal pneumonia with serotype 3 *S. pneumoniae* WU2. Nasal vaccination of a low dose of PspA plus each TLR agonist induced a high level of PspA-specific IgG in the serum and a low level of PspA-specific IgG in the airways of mice. The binding of PspA-specific IgG in sera resulted in an increase in C3 deposition on the bacterial surfaces. Subsequently, the bacterial densities in the lung tissues and blood were significantly decreased in mice that were immunized nasally with PspA plus TLR agonist, compared with the values for mice immunized nasally with a low dose of PspA alone. The reduction in bacterial densities in lung tissues could be explained by the sufficient extravasation of PspA-specific IgG into the alveolar space of mice that were immunized nasally with a low dose of PspA plus each TLR agonist [18]. Bacterial invasion into the blood circulation was readily suppressed by PspA-specific IgG in sera of mice immunized nasally with a low does of PspA plus each of the TLR agonists. The survival of infected mice that were immunized nasally with a low dose of PspA plus TLR agonist was significantly increased compared with those of mice that were immunized nasally with a low dose of PspA alone. These findings are in contrast with findings reported in our previous study, showing no significant difference in the survival of infected mice between nasal immunization with a high dose (2.5μg) of PspA plus TLR agonist and a high dose of PspA alone in a fatal pneumonia model using the WU2 strain [12]. The findings reported herein on the effect of a low dose of PspA plus TLR agonist also suggest possibilities for the development of a cost-effective PspA intranasal vaccine with the goal of preventing a fatal pneumonia.

We recently reported on the pivotal role of PspA-specific IgA on the bacterial clearance of a less virulent serotype 19F strain in the upper airway in a mouse model of bacterial colonization [19]. Since PspA-specific IgG was shown to be sufficient for protecting mice against a fatal bacteremic pneumonia caused by a virulent serotype 3 strain in this study, PspA-specific IgA may not be essential for invasive pneumococcal infections, such as bacteremic pneumonia.

While the rank orders of PspA-specific IgG induced in sera were LPS > Pam3CSK4 > Poly(I:C) and CpG 1826, no difference was found in the frequency of C3 deposition on bacterial surfaces in immune sera induced by a low dose of PspA plus each TLR agonist in this study. This discrepancy between the levels of PspA-specific IgG and the frequencies of C3 deposition may be explained by the similar ratios of PspA-specific IgG1 titers to IgG2a titers (1.32 to 2.33) among mice that were immunized nasally with a low dose of PspA plus each TLR agonist in this study, because the C3 binding activity of the IgG2a isotype is superior to those of other IgG isotypes [20]. Although antibacterial effects in the lung and blood were the highest in mice that were immunized nasally with a low dose of PspA plus LPS than those in mice immunized nasally with a low dose of PspA plus the other TLR agonists at 24 h post-infection, no significant difference was found in the survival rate of mice immunized nasally with a low dose of PspA plus each TLR agonist. This discrepancy may be explained by subsequent bacterial growth in the lungs and blood in mice that had been immunized nasally with a low dose of PspA plus LPS as well as mice immunized nasally with a low dose of PspA plus the other TLR agonists later than 24 h post-infection.

Despite similar C3 binding activities of immune sera, the rank orders for the survival rates of the immunized mice were LPS > Pam3CSK4 > Poly(I:C) and CpG 1826, and were in agreement with those of plasma levels of PspA-specific IgG reported in this study. Although LPS or Pam3CSK4 demonstrated a superior adjuvant effect among the TLR agonists tested in this study, these bacterial products are highly toxic to humans. In contrast, a synthetic analogue of a dsRNA compound, such as Poly (I:C) or Poly I:PolyC12 U (Ampligen[R]), or CpG ODN would be expected to be applicable as a safe mucosal adjuvant in humans [21,22].

In conclusion, the data presented here provide evidence to indicate that intranasal immunization with a vaccine containing a low dose of PspA plus each TLR agonist elicited a high level of systemic PspA-specific IgG, and was capable of preventing the development of fatal pneumococcal pneumonia in mice. An intranasal administration of each TLR agonist in combination with a low dose PspA significantly increased the survival rates of the infected mice in the following order: LPS > Pam3CSK4 > Poly(I:C) and CpG ODN 1826. This study confers an important insight regarding strategies for a cost-effective PspA protein-based vaccine against invasive pneumococcal infections.

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Genotypic Profile of Streptococcus suis Serotype 2 and Clinical Features of Infection in Humans, Thailand

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To examine associations between clinical features of *Streptococcus suis* serotype 2 infections in humans in Thailand and genotypic profiles of isolates, we conducted a retrospective study during 2006–2008. Of 165 patients for whom bacterial cultures of blood, cerebrospinal fluid, or both were positive for *S. suis* serotype 2, the major multilocus sequence types (STs) found were ST1 (62.4%) and ST104 (25.5%); the latter is unique to Thailand. Clinical features were examined for 158 patients. Infections were sporadic; case-fatality rate for adults was 9.5%, primarily in northern Thailand. Disease incidence peaked during the rainy season. Disease was classified as meningitis (58.9%) or nonmeningitis (41.1%, and included sepsis [35.4%] and others [5.7%]). Although ST1 strains were significantly associated with the meningitis category ($p<0.0001$), ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The ST1 and ST104 strains are capable of causing sepsis, but only the ST1 strains commonly cause meningitis.

Streptococcus suis, an emerging zoonotic pathogen, causes invasive infections in persons who are in close contact with infected pigs or contaminated pork-derived products (*1*). On the basis of capsular polysaccharides, 33 serotypes of *S. suis* have now been identified. Of these, serotype 2 is the most prevalent type in humans infected with this pathogen (*1,2*). Since the largest outbreak of human *S. suis* infection in 2005, in Sichuan Province, People's Republic of China (*3*), this disease has been increasingly recognized worldwide. The numbers of reported cases, especially in persons from Southeast Asian countries, have increased dramatically during past few years (*4*).

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In Thailand, at least 300 cases of *S. suis* infection in humans have been reported (*5–11*). Although an outbreak of *S. suis* infections was confirmed in Phayao Province during May 2007 (*9*), most cases in humans occur sporadically and are primarily located in the northern region of this country (*6–11*). A relatively low incidence of cases with *S. suis* serotype 14 has also been reported in this region (*12*). Although previous studies have reported high frequencies (59.0%–88.7%) of *S. suis* infections in persons in this area who ate raw pork products (*8–11*), the pathogenesis of this disease, including routes of transmission, is unclear.

The major clinical manifestations of the disease are bacterial meningitis and sepsis, but other manifestations have been reported (*1,4, 8,10,13*). Most cases of bacterial meningitis can be attributed to the hematogenic spread of invasive bacteria, but how circulating bacteria cross the blood–cerebrospinal fluid (CSF) barrier and cause meningitis is not clear (*14,15*). Furthermore, the overall clinical features of this disease have not been extensively and comprehensively investigated in Southeast Asian countries.

A variety of virulence factors associated with *S. suis* have been reported (*16–20*), but none have been proven to be essential for the host defense of this disease, except

the capsular polysaccharide (*19*). In serotype 2 isolates obtained during a previous outbreak in Sichuan, China, an ≈89-kb DNA fragment, which has been associated with a pathogenicity island (89K PAI), was identified (*21*). The 89K PAI fragment encodes a 2-compartment signal transduction system, SalK-SalR, which is required for full virulence (*22*).

We report the results of a retrospective study of the clinical features of 158 cases of human infection with *S. suis* serotype 2 and the molecular epidemiology of 165 *S. suis* serotype 2 isolates. The study objective was to demonstrate associations between the clinical features of disease caused by *S. suis* serotype 2 in persons in Thailand and the genotypic profiles of the isolates. The study was reviewed and approved by the Ethics Committees of Research Institute for Microbial Diseases, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

Methods

Isolate Identification

From January 2006 through August 2008, a total of 1,154 unidentified streptococcal isolates from blood or CSF were collected from hospitals in all 76 provinces of Thailand. Biochemical testing of these isolates, using API Strep (bioMérieux, Durham, NC, USA) and *S. suis*–specific and *S. suis* serotype 2– or 1/2–specific PCR (*12,23*), confirmed 165 isolates from 34 hospitals in 25 provinces as *S. suis*. The final serotype of all strains was confirmed by coagglutination tests that used rabbit antiserum (Statens Serum Institute, Copenhagen, Denmark).

Genotypic Profiles of Isolates

Multilocus sequence type (MLST) testing was performed as described by King et al. (*24*), with a modification for *mutS* as described by Rehm et al (*25*). MLST alleles and the resulting sequence type (ST) were assigned by using the *S. suis* MLST database (http://ssuis.mlst.net). eBURST was used to identify the clonal complexes for these 165 serotype 2 strains within *S. suis*, and the overall structure of the population was obtained through the MLST database (*26*). Virulence-associated genes (VAG), including extracellular released protein factor (*epf*), muramidase-released protein (*mrp*), and suilysin (*sly*), and variants of *mrp* or *epf* were determined by PCR as described by Silva et al. (27), with minor modifications. Presence of the 89K PAI fragment was determined by PCR as reported by Chen et al (*21*). Pulsed-field gel electrophoresis (PFGE) was performed as described (*28*), and the pulsotypes were assigned to clusters of isolates with >80% similarity from the dendrogram. The dendrogram representing the genetic relationships between the representative pulsotypes from 165 *S. suis* serotype 2 strains was drawn by using the Cluster 3.0 software program and examined by using the TreeView program as described (*12,29*).

Clinical Features of Cases

Of the 165 patients whose culture results were positive for *S. suis* serotype 2, medical records for 158 were retrospectively reviewed by physicians at local hospitals in Thailand. Medical records for the remaining 7 patients were not available. The clinical manifestations were mostly divided into 2 categories: meningitis and nonmeningitis. The meningitis category involved confirmed meningitis, bacteremic meningitis, and probable meningitis. All patients in the meningitis category had typical meningeal signs, such as neck stiffness, and acute disease onset. Although bacteremic meningitis was defined as a case in which both CSF and blood cultures were positive, confirmed meningitis was defined as a case with a positive CSF culture only, and probable meningitis was defined as a case with a positive blood culture only. The nonmeningitis category included the clinical manifestations of sepsis and sepsis with focal signs other than meningitis (septic arthritis or spondylodiscitis, infective endocarditis, and bacteremic pneumonia). Sepsis was defined as systemic inflammatory response syndrome and a positive blood culture (*30*), and septic arthritis or septic spondylodiscitis was defined as described (*31*). Diagnosis of infectious endocarditis was based on the Duke criteria (*32*). Septic shock was also defined as described (*33*).

Statistical Analyses

Comparisons of the clinical characteristics between fatal and nonfatal cases were analyzed by using the χ^2 test or Fisher exact test with Stata version 10.0 software (StataCorp, College Station, TX, USA). Patient ages and periods of hospital admission were tested for normality of the distribution using the Kolmogorov-Smirnov test and were compared by using the Student *t* test with SPSS version 11.0 software (SPSS Inc., Chicago, IL, USA). Data were considered significant at $p<0.05$.

Results

Genotypic Profiles of Isolates

Of the 165 *S. suis* serotype 2 isolates, 123 were isolated from blood and 42 from CSF. eBURST analysis based on MLST enabled classification of these strains into 4 ST complexes: the ST1, ST27, ST29, and ST104 complexes (Table 1). ST126, a novel ST, has a single locus variant from ST1. The largest cluster of 89K PAI–carrying strains was ST1 (n = 81, 49.1%), which had the *epf+/sly+/mrp+* genotype; these strains were isolated from blood and CSF. Another large cluster of non-89K PAI–carrying strains was

Table 1. Genotypic profiles of 165 clinical isolates of *Streptococcus suis* serotype 2, Thailand, January 2006–August 2008*

ST complex	ST	VAG†	Isolation site	89K PAI +	89K PAI –	No. (%) strains
1	1	*epf–/sly+/mrp+*	Blood	1	0	103 (62.4)
		epf+/sly+/mrp+	Blood	52	13	
			CSF	29	5	
		epf+/sly+/mrp^s	Blood	0	1	
			CSF	0	2	
	126	*epf+/sly+/mrp+*	Blood	1	0	3 (1.8)
			CSF	2	0	
27	28	*epf–/sly–/mrp+*	Blood	0	1	3 (1.8)
			CSF	0	2	
29	25	*epf–/sly–/mrp**	Blood	8	0	11 (6.7)
		epf–/sly–/mrp+	Blood	3	0	
	103	*epf–/sly–/mrp**	Blood	2	0	3 (1.8)
		epf–/sly–/mrp+	Blood	1	0	
104	104	*epf–/sly+/mrp–*	Blood	3	38	42 (25.5)
			CSF	0	1	
Total no. strains	NA	NA	NA	102	63	165 (100)

*ST, sequence type; VAG, virulence-associated gene; 89K PAI, an ≈89-kb pathogenicity island; CSF, cerebrospinal fluid; NA, not applicable.
†*mrp*^{s} and *mrp** are *mrp* variants that produce ≈750-bp and ≈1,800-bp fragments, respectively, by PCR (*23,34*).

ST104, which had the *epf–/sly+/mrp–* genotype (n = 39, 23.6%); most of these strains (n = 38) were isolated only from blood. ST103, ST104, and ST126 were found only in isolates from humans in Thailand.

PFGE of Isolates

Of the 165 serotype 2 strains, PFGE analyses identified 20 pulsotypes (Figure 1, panel A). Analysis of the dendrogram for these 20 pulsotypes revealed at least 16 clusters (I to XVI) (Figure 1, panel B). Although 5 pulsotypes of A were identified for the ST1 and ST126 strains, 2 major pulsotypes (A [n = 32] and A1 [n = 43]), A1 (n = 43), and A4 (n = 3) were grouped in 1 cluster. Pulsotype A2 (n = 21), which consisted of ST1 strains lacking the 89K PAI fragment, was classified into a distinguished cluster. PFGE showed diverse DNA patterns for strains ST25 and ST103. ST25 strains were classified into 5 clusters of I, II, III, IV, and VIII. ST103 strains were classified into 3 clusters of VI, XIV, and XV. Three ST28 strains lacking 89K PAI exhibited the unique DNA pattern of pulsotype D; these were classified into cluster XVI. Although 4 pulsotypes (H, H1, H2, and H3) were identified for ST104 strains, 2 major pulsotypes (H [n = 29] and H1 [n = 11]) in ST104 strains were classified into cluster VII. Collectively, clusters X and XI for ST1 and ST126 strains and cluster VII for ST104 strains accounted for the major 3 clusters found for cases in Thailand.

Geographic and Seasonal Distribution

Of the 165 isolates, 136 (82.4%) were from the northern region, 19 (11.5%) from the central region, 7 (4.2%) from the northeast region, and 3 (1.8%) from the eastern region (Table 2; Figure 2, panel A). No strains were isolated from the southern region. The dates of isolation suggest that human cases occur more frequently during the rainy season, June–August of each year (Figure 2, panel B).

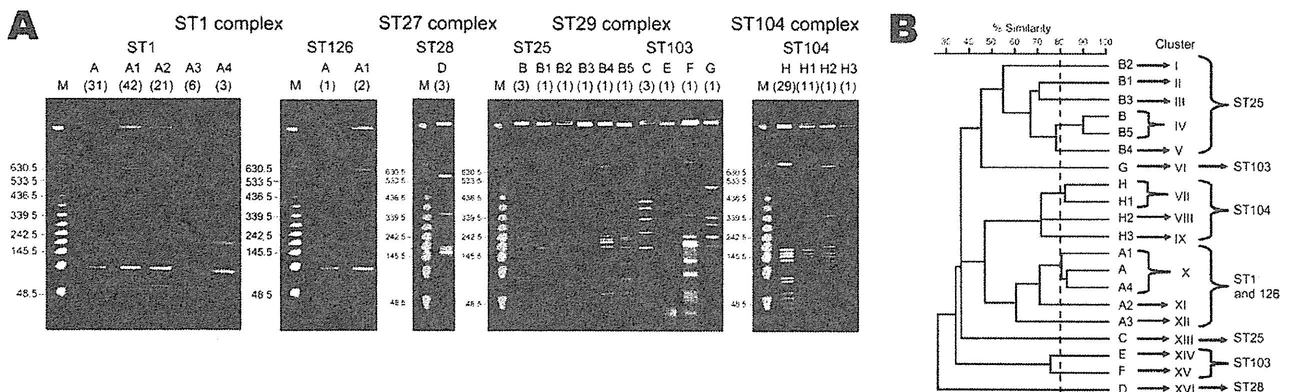

Figure 1. A) Pulsed-field gel electrophoresis profiles of 165 human isolates of *Streptococcus suis* serotype 2, after *Sma*I digestion. Numbers of isolates are indicated in parentheses below pulsotype numbers. B) Dendrogram generated from the pulsed-field gel electrophoresis profiles. ST, sequence type.

Table 2. Distribution of sequence types of 165 clinical isolates of *Streptococcus suis* serotype 2, by region, Thailand

Sequence type	North	Northeast	East	Central	South
1	85	6	1	11	0
25	11	0	0	0	0
28	3	0	0	0	0
103	1	0	1	1	0
104	33	1	1	7	0
126	3	0	0	0	0
Total	136	7	3	19	0

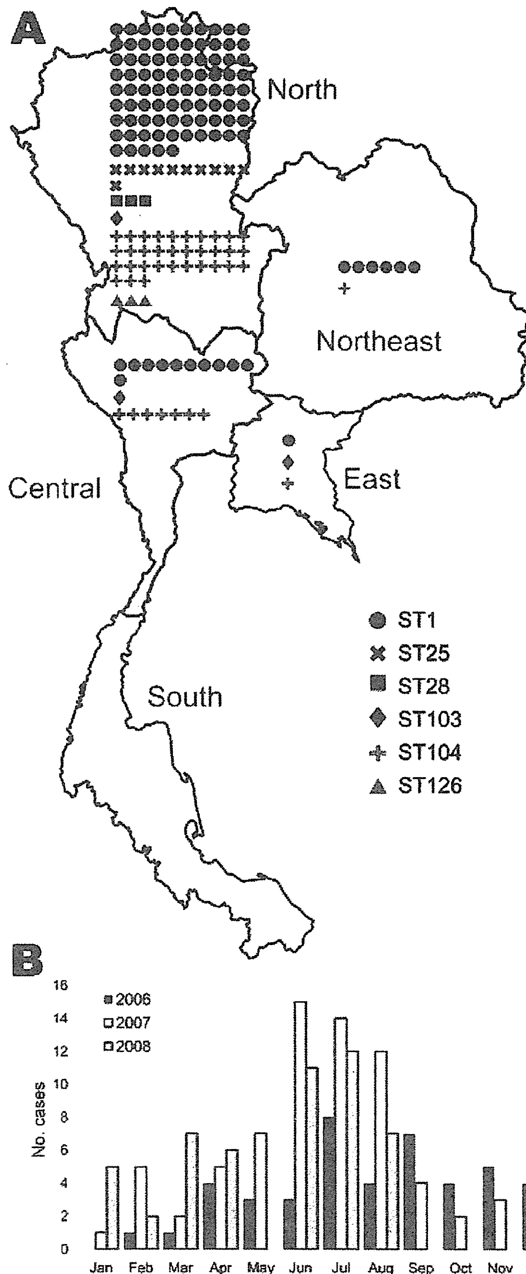

Figure 2. Distribution and sequence types (STs) of 165 human isolates of *Streptocoocus suis* serotype 2, January 2006–August 2008, Thailand. A) Regions of isolation; B) monthly distribution of isolations.

Clinical Features of Cases

The clinical features of the 158 human cases of *S. suis* serotype 2 infection are summarized in Table 3. The median age (range) of the 155 patients for whom age was known was 55.0 (18–93) years; 72.8% were male. No cases in children were identified in this study. All 158 patients had been hospitalized; median duration (range) of hospitalization for the 158 patients was 11 (1–45) days; 15 (9.5%) patients died. No significant differences were found between the fatal and nonfatal cases with respect to patient age or period of admission.

The meningitis category (n = 93) included 22 cases of confirmed meningitis, 44 cases of bacteremic meningitis, and 27 cases of probable meningitis (Figure 3). The nonmeningitis category (n = 65) included sepsis with focal signs other than meningitis (n = 9) and sepsis (n = 56). Sepsis with focal signs other than meningitis included septic arthritis (n = 5), infective endocarditis (n = 3), and bacteremic pneumonia (n = 1). Of the 15 fatal cases, 8 were assigned to the meningitis category (probable meningitis [n = 6], meningitis [n = 1], bacteremic meningitis [n = 1]), 6 cases were sepsis, and 1 case was infective endocarditis (Table 3). Although the cases of bacteremic meningitis were significantly associated with a nonfatal outcome ($p = 0.043$), the probable meningitis cases were significantly associated with a fatal outcome ($p = 0.013$). The combined frequencies for the recent consumption of raw pork products and exposure to pigs were 39.9%. None of the clinical signs or possible risk factors, including recent exposure to pigs or raw pork products, or alcohol abuse, was significantly associated with a fatal outcome. Of the 158 patients, 154 parenterally received antimicrobial drugs, such as ceftriaxone, and data concerning antimicrobial drug treatment were not available for 4. Corticosteroids, such as dexamethasone, were used for only 4 patients.

Clinical Features and Genotype Profiles

The distributions of STs for the 158 human isolates for the meningitis and nonmeningitis categories are shown in Table 4. Although the ST1 strains were significantly associated with the meningitis category ($p<0.0001$), the ST104 strains were significantly associated with the nonmeningitis category ($p<0.0001$). The VAG profile of *epf+/sly+/mrp+*, which was dominant in the ST1 strains,

Table 3. Demographic and clinical features of 158 human cases of *Streptococcus suis* serotype 2 infections, Thailand, January 2006–August 2008*

Characteristic	All, n = 158	Fatal, n = 15; 9.5%	Nonfatal, n = 143; 90.5%	p value
Demographic				
Male sex, %	72.8	66.7	73.4	0.386
Mean (median) age, y†	56.6 (55.0)	53.9 (52.5)	57.0 (56.0)	0.264
Period of admission, d, mean (median)	12.5 (11)	10.1 (6)	12.9 (12)	0.737
Meningitis category, no. (%) cases				
Confirmed meningitis	22 (13.9)	1 (6.7)	21 (14.7)	0.348
Bacteremic meningitis	44 (27.8)	1 (6.7)	43 (30.1)‡	0.043
Probable meningitis	27 (17.1)	6 (40.0)	21 (14.7)§	0.013
Nonmeningitis category, no. (%) cases				
Septic arthritis	5 (3.2)	0	5 (3.2)	1
Infective endocarditis	3 (1.9)	1 (6.7)	2 (1.4)	0.905
Bacteremic pneumonia	1 (0.6)	0	1 (0.7)	1
Sepsis	56 (35.4)	6 (40.0)	50 (35.0)	0.698
Signs and symptoms, no. (%) cases				
Diarrhea	28 (17.1)	5 (33.3)	23 (16.1)	0.1
Hearing loss	34 (21.5)	4 (26.7)	30 (21.0)	0.409
Altered consciousness	35 (22.2)	4 (26.7)	31 (21.7)	0.434
Shock	9 (5.7)	2 (13.3)	7 (4.9)	0.205
Possible risk factors, no. (%) cases				
Recent consumption of raw pork products	52 (32.9)	5 (33.3)	47 (32.9)	0.589
Recent exposure to pigs	11 (7.0)	2 (13.3)	9 (6.3)	0.28
Alcohol abuse	33 (21.0)	5 (33.3)	28 (19.6)	0.178

*Statistical analyses were performed by using the χ^2 or Fisher exact test.
†Ages were not available for 3 patients.
‡One case of bacteremic meningitis was associated with pneumonia.
§Two cases of probable meningitis were associated with spondylodiscitis.

was also significantly associated with the meningitis category (p<0.0001). The VAG profile of *epf–/sly+/mrp–*, which was observed only in the ST104 strains, was also significantly associated with the nonmeningitis category (p<0.0001). Because the largest cluster of 89K PAI–carrying strains was associated with the VAG profile of *epf+/sly+/mrp+*, the presence of 89K PAI was also significantly associated with the meningitis category (p<0.0001). None of the genotypic profiles that included STs, VAG, and presence of 89K PAI were significantly associated with fatal or nonfatal outcomes (data not shown).

Discussion

Our finding that isolated *S. suis* serotype 2 strains peaked during the rainy season of 2006–2008 confirmed conclusions reached in previous small-scale studies

Table 4. Genotypic features of *Streptococcus suis* serotype 2 as risk factor for meningitis*

Feature	Clinical category, no. (%) strains			p value
	All, n = 158	Meningitis, n = 93	Nonmeningitis, n = 65	
Sequence type				
1	98 (62.0)	73 (78.5)	25 (38.5)	<0.0001†
104	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
25	11 (7.0)	7 (7.5)	4 (6.2)	0.478
28	3 (1.9)	2 (2.2)	1 (1.5)	0.632
103	3 (1.9)	2 (2.2)	1 (1.5)	0.655
126	3 (1.9)	2 (2.2)	0	0.201
VAG profile				
epf+/sly+/mrp+	97 (61.4)	72 (79.6)	25 (35.4)	<0.0001†
epf+/sly+/mrp^s	3 (25.3)	3 (3.2)	0 (0)	0.201
epf–/sly+/mrp–	40 (25.3)	6 (6.5)	34 (52.3)	<0.0001‡
*epf– /sly–/mrp**	10 (6.3)	6 (6.5)	4(6.2)	0.607
epf–/sly–/mrp+	7 (4.4)	5 (5.3)	2 (3.1)	0.392
epf–/sly+/mrp+	1 (1.0)	1 (1.1)	0 (0)	1
89K PAI profile, 89K PAI+	98 (62.0)	70 (75.3)	28 (43.1)	<0.0001†

*Statistical analyses were performed by using the χ^2 or Fisher exact test. VAG, virulence-associated gene; 89K PAI, ≈89-kb pathogenicity island.
†Significant association with the meningitis category.
‡Significant association with the nonmeningitis category.

conducted in northern Vietnam and Hong Kong (*35,36*). The predominant distribution of these isolates in northern Thailand is also in accordance with previous reports (*6–11*). However, why no human cases were identified in southern Thailand remains uncertain. A recent study from Hong Kong reported heavy contamination of *S. suis* in raw pork meat at local supermarkets or wet markets; therefore, a hot and humid climate may facilitate the growth of *S. suis* in raw pork products in those markets (*36*) and increase the risk for *S. suis* infections in humans in northern Thailand. The finding of no cases in children suggests that the routes of transmission are associated with adult behavior.

A recent study from northern Thailand, based on 20 human isolates collected during 1998–2002, reported that the most common isolates of *S. suis* serotype 2 were ST25 (40%), followed by ST1 (15%) and ST103 (15%) (*34*). By contrast, the MLST and PFGE results in this study clearly demonstrated that ST1 strains with major pulsotypes of A, A1 and A2, and ST104 with major pulsotypes of H and H1 were currently circulating in the same region of Thailand during 2006–2008. Collectively, these data suggest dynamic replacement of STs from ST25 to ST1 and ST104 among serotype 2 strains during recent years in this region.

Although *S. suis* serotype 2 has been reported to be the most frequent cause of bacterial meningitis in adults in Vietnam (*13,35*), other clinical manifestations, such as sepsis and infectious endocarditis, have also been found to be common in Thailand (*6,8,11*). Of the 158 human cases in the study reported here, ≈60% were assigned to the meningitis category and ≈35% were sepsis. Other clinical manifestations, including infective endocarditis, were rare. The findings reported here demonstrate significant associations between the ST1 strains and the meningitis category and between the ST104 strains and the nonmeningitis category. These findings indicate that both the ST1 and ST104 strains cause bacteremia and sepsis but that the ST1 strains are more likely to cross the blood–CSF barrier and subsequently result in meningitis. Because ≈80% of the cases in the meningitis category were caused by strains with ST1, as evidenced by a VAG profile of *epf+/sly+/mrp+* and 89K PAI, these genotypic profiles of *S. suis* serotype 2 may favor bacterial survival and multiplication in the bloodstream, which would result in high levels of bacteremia, crossing of the blood–CSF barrier, and invasion of the meninges and the central nervous system (*15*). Our PFGE data showed that the pulsotype A1 found in serotype 2 strains with ST1 was identical to pulsotype 11 of serotype 2 strains with ST1 from Vietnam and pulsotype I of the serotype 2 strains with ST1 from Hong Kong (*13,28*). These isolates from Vietnam and Hong Kong were associated with a VAG profile of *epf+/sly+/mrp+*, and the strains from Vietnam were also the cause of meningitis in adults. A unique DNA pattern of pulsotype D, classified into cluster XVI, was found for 3 strains with ST28 isolated from nonfatal cases in this study. Previous studies also reported 1 nonfatal case caused by the ST28 strain from Thailand and Japan (*34,38*).

Associations for bacteremic meningitis cases with nonfatal outcomes and probable meningitis cases with fatal outcomes contrasted strikingly in this study. Of 6 fatal cases of probable meningitis, 2 were caused by ST1, 2 by ST25, and 2 by ST104 strains. The extent to which the virulence of each ST strain contributed to these deaths remains uncertain. Another possible explanation may be a frequent involvement of critically ill patients, for whom lumbar puncture was not possible; these patients had probable meningitis and typical meningeal signs, acute disease onset, and positive blood culture only.

Because the clinical charts were retrospectively reviewed and the etiologic diagnosis of *S. suis* infection might not have been readily reported to the attending physicians during the hospitalization of the patients in this study, the extent of investigations of clinical manifestations, possible risk factors, and causes of death might have been limited. Because different physicians were involved in the assessment of different patients in this study, the possibility of misdiagnosis for clinical categories cannot be completely excluded even though meningeal signs and acute disease onset are clinical indicators of meningitis.

In conclusion, this study of the clinical features of 158 cases of *S. suis* seotype 2 infection in humans in Thailand showed that the disease occurs sporadically in adults and results in a mortality rate of ≈9.5%; the major clinical manifestations include meningitis and sepsis. MLST analyses of 165 isolates from humans indicated that the major STs were ST1 followed by ST104. Although both ST1 and ST104 strains cause sepsis, it is likely that only the ST1 strain causes meningitis. Further studies are needed to elucidate the pathogenesis of the human *S. suis* infections that are prevalent in Southeast Asian countries.

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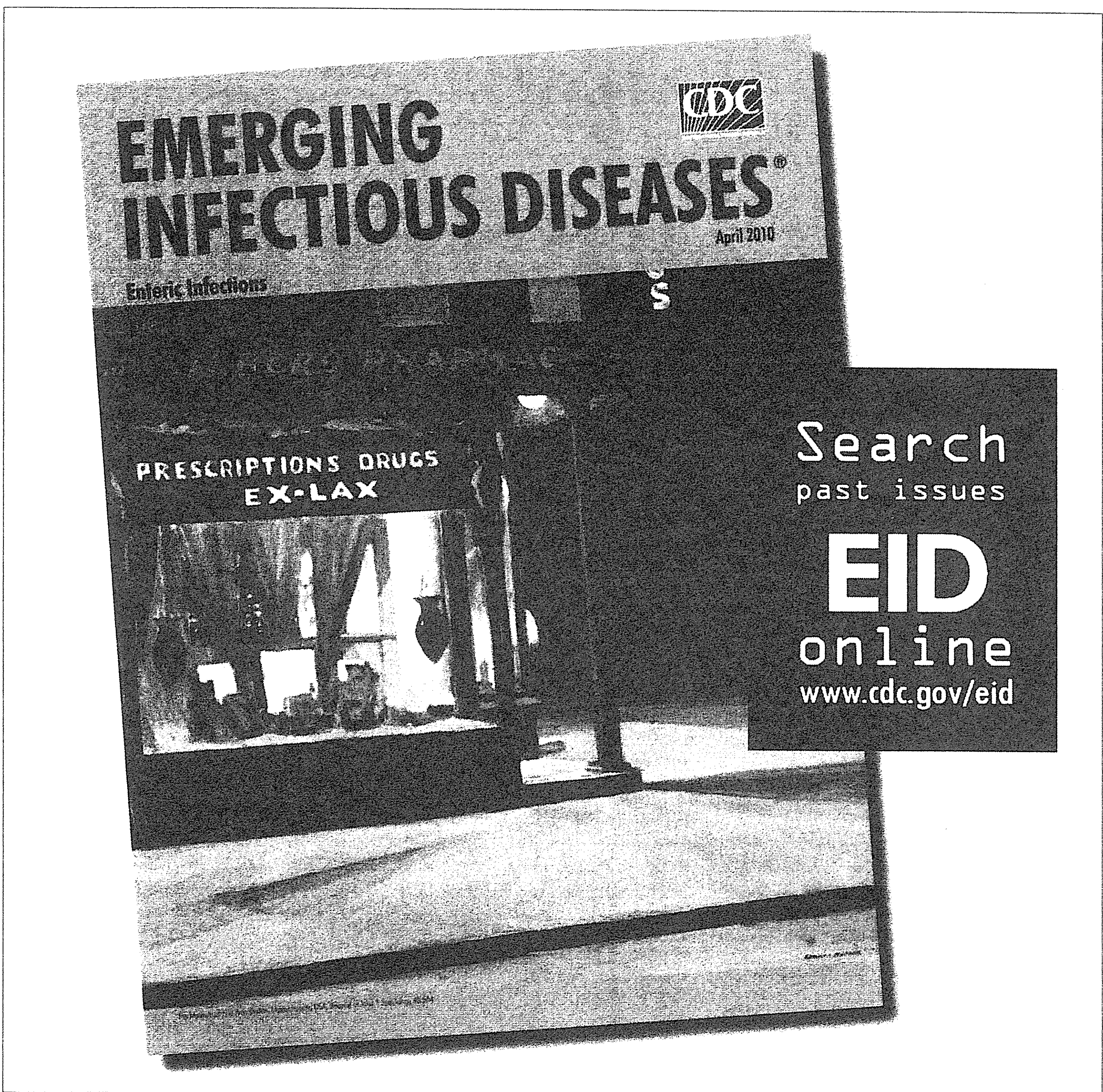

Case Report

Sepsis and spontaneous bacterial peritonitis in Thailand

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In June, 2007, a 66-year-old man (case 1), an alcohol misuser with alcoholic liver cirrhosis who habitually ate raw pork, was referred to Uttaradit Hospital, northern Thailand. He had a fever and massive ascites. His leucocyte count was 4·4×10⁹/L, and total bilirubin and albumin concentrations were 23·7 mg/L, and 26 g/L, respectively. Polymorphonuclear leucocyte count of ascitic fluid was 4·1×10⁸/L and culture was positive despite a negative blood culture. This patient was diagnosed with spontaneous bacterial peritonitis,[1] and successfully treated with ceftriaxone. Testing of this isolate with the API 20 Strep Kit (BioMérieux, Marcy l'Etoile, France) suggested *Streptococcus equi* subspecies *zooepidemicus* with 91·8% identification. However, there was 99% similarity of the 16S rDNA sequence with known *S suis* strains. Confirmation that this isolate belonged to this species was further supported by a positive reaction for *S suis*-specific PCR amplification of the *S suis* 16S rRNA gene.[2]

In May, 2007, a 62-year-old woman (case 2), with liver cirrhosis who had had repeated episodes of spontaneous bacterial peritonitis in 2006, was admitted to Phetchabul Hospital, northern Thailand, with a fever. Physical examination showed cutaneous jaundice and ascites. Her leucocyte cell count, total bilirubin, and albumin were 15·1×10⁹/L, 108·2 mg/L, and 18 g/L, respectively. Culture of ascitic fluid was negative, blood culture was positive, and she was diagnosed with sepsis. The isolate was identified as *S suis* by the API 20 Strep Kit. This patient also improved on treatment with ceftriaxone.

The isolates from these two cases were confirmed by a co-agglutination test as serotype 5 for case 1 and serotype 24 for case 2 (table), and were assigned to the novel sequence types by multilocus sequence typing.[2]

We report the first human cases of *S suis* infection with serotypes 5 and 24. *S suis* is a zoonotic pathogen that can cause invasive infections in human beings who consume raw pork products or are in close contact with infected pigs.[3] Although serotype 2 is the most prevalent in human beings, cases with serotypes 1, 4, 14, and 16 have been reported.[2–4] In Thailand between 2006 and 2008, 179 human isolates of *S suis* were collected from sterile sites eg, blood, cerebrospinal fluid. Of these isolates, 165 (92·2%) were serotype 2, and 12 (6·7%) were serotype 14.[2] The differential diagnosis of our two cases includes melioidosis and leptospirosis. Bacterial translocation has an important role in the pathogenesis of spontaneous bacterial peritonitis in cirrhosis, and the most common pathogens are enterobacteriaceae.[1] Previous reports on human infections after recent consumption of raw pork products suggested that the gastrointestinal tract is a major route of entry in cases of *S suis* infections in Thailand and Vietnam.[2,3,5] In this region, the occurrence of spontaneous bacterial peritonitis through bacterial translocation of *S suis* after consumption of raw pork products is possible in patients with liver cirrhosis. A similar case of spontaneous bacterial peritonitis caused by serotype 16 strain of *S suis* in a patient with alcoholic liver cirrhosis was reported from Vietnam.[4] Although the isolation rates for serotypes 5 and 24 are low (2/179 cases; 1·1%), *S suis*-specific PCR is recommended for identification of streptococcal isolates from sterile sites, and a serious caution against eating raw pork products should be given to patients with liver cirrhosis, especially in southeast Asian countries.

	Case 1	Case 2
Source of isolate	Ascites	Blood
Clinical diagnosis	Spontaneous bacterial peritonitis	Sepsis
Comorbid illness	Alcholic liver cirrhosis	Liver cirrhosis
Identification with API 20 Strep	*S equi* subspecies *zooepidemicus*	*S suis*
S suis-specific PCR	Positive	Positive
Sequencing of 16S rRNA gene	*S suis* (99%)	*S suis* (99%)
Serotype	5	24
Multilocus sequence typing	ST181	ST221

ST: sequence type.

Table: **Microbiological features of two human cases of *Streptococcus suis* infection**

Contributors

Patient care: AB, SS. Microbiology: AK, WN, SC, YA. Serotyping: MG. Study coordination: SD. Writing: PS, DS, KO. Written consent to publish was obtained.

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The Nasal Dendritic Cell-Targeting Flt3 Ligand as a Safe Adjuvant Elicits Effective Protection against Fatal Pneumococcal Pneumonia[▽]

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We have previously shown that a pneumococcal surface protein A (PspA)-based vaccine containing DNA plasmid encoding the Flt3 ligand (FL) gene (pFL) as a nasal adjuvant prevented nasal carriage of *Streptococcus pneumoniae*. In this study, we further investigated the safety and efficacy of this nasal vaccine for the induction of PspA-specific antibody (Ab) responses against lung infection with *S. pneumoniae*. C57BL/6 mice were nasally immunized with recombinant PspA/Rx1 (rPspA) plus pFL three times at weekly intervals. When dynamic translocation of pFL was initially examined, nasal pFL was taken up by nasal dendritic cells (DCs) and epithelial cells (nECs) but not in the central nervous systems, including olfactory nerve and epithelium. Of importance, nasal pFL induced FL protein synthesis with minimum levels of inflammatory cytokines in the nasal washes (NWs) and bronchoalveolar lavage fluid (BALF). NWs and BALF as well as plasma of mice given nasal rPspA plus pFL contained increased levels of rPspA-specific secretory IgA and IgG Ab responses that were correlated with elevated numbers of $CD8^+$ and $CD11b^+$ DCs and interleukin 2 (IL-2)- and IL-4-producing $CD4^+$ T cells in the nasal mucosa-associated lymphoid tissues (NALT) and cervical lymph nodes (CLNs). The *in vivo* protection by rPspA-specific Abs was evident in markedly reduced numbers of CFU in the lungs, airway secretions, and blood when mice were nasally challenged with *Streptococcus pneumoniae* WU2. Our findings show that nasal pFL is a safe and effective mucosal adjuvant for the enhancement of bacterial antigen (Ag) (rPspA)-specific protective immunity through DC-induced Th2-type and IL-2 cytokine responses.

Streptococcus pneumoniae is a leading human pathogen causing diseases ranging from otitis media to pneumonia, bacteremia, and meningitis. This bacterium, commonly termed the pneumococcus, can result in an estimated 1.6 million deaths per year worldwide, more than half of which are young children in developing countries (2). Although pneumococcal capsular polysaccharide and pneumococcal protein-capsular conjugate vaccines can provide protective immunity against pneumonia and invasive diseases in adults and infants, a strong need still exists for a new generation of effective vaccines for the prevention of all potential *S. pneumoniae* infections. In this regard, the multivalent polysaccharide vaccines do not provide protection against strains with nonvaccine serotypes (28, 41). Of importance, pneumococcal surface protein A (PspA) has been extensively investigated as a candidate vaccine antigen (Ag) to prevent pneumococcal infection (5, 37). For instance, PspA-specific antibody (Ab) enhances bacterial clearance and induces cross-protection against infection with strains of different serotypes (4, 31). Further, previous studies have demonstrated that PspA-specific Abs overcome the anticomplementary effect of PspA, allowing increased complement activation and C3 deposition on PspA-bearing bacteria (27, 30).

Nasal immunization has been shown to preferentially induce Ag-specific Ab responses in the respiratory tract (20) and other mucosal lymphoid tissues (10, 25, 26). To induce maximal levels of Ag-specific immune responses in both mucosal and systemic lymphoid tissue compartments, it is often necessary to use a mucosal adjuvant (16, 22, 39). Although native cholera toxin and related *Escherichia coli* enterotoxin are potent mucosal adjuvant for enhancement of Ag-specific immune responses, their application for human use is not warranted since they can cause diarrhea or Bell's palsy (6, 23, 29). Moreover, these toxins are known to migrate into and accumulate in the olfactory tissues when given nasally (40). In this regard, our previous studies demonstrated that nasal application of a DNA plasmid (pFL) containing the gene of the Flt3 ligand (FL), which is a kind of cytokine, preferentially expanded $CD8^+$ dendritic cells (DCs) and subsequently induced Ag-specific mucosal immune responses mediated by interleukin 4 (IL-4)-producing $CD4^+$ T cells when mice were nasally administrated ovalbumin with pFL as the mucosal adjuvant (19). Further, a combination of nasal pFL and CpG oligonucleotides as a

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double DNA adjuvant enhanced mucosal and systemic immune responses via induction of plasmacytoid DCs as well as $CD8^+$ DCs in mucosal compartments (11, 17). Nasal administration of an adenovirus vector encoding FL cDNA also showed enhancement and maintenance of long-term immunity (17, 32).

In this study, we examined the safety and effectiveness of nasal pFL as a mucosal adjuvant for the induction of functional bacterial Ag (recombinant PspA [rPspA])-specific Ab responses for protection against *S. pneumoniae* infection in the lower respiratory tract. Our findings show that nasal rPspA plus pFL adjuvant successfully elicits protective immunity in both the upper and lower respiratory tracts by enhancing mucosal DC-mediated Th2-type and IL-2 cytokine responses without detectable cytokine-mediated inflammation.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 mice (6 to 8 weeks old) were purchased from Charles River Japan (Kanagawa, Japan) and used in this study. Upon arrival, these mice were transferred to microisolators, maintained in horizontal laminar flow cabinets, and provided sterile food and water as part of a specific-pathogen-free facility at Osaka University (Suita, Japan), and all experiments were conducted in accordance with the guidelines provided by Osaka University. All of the mice used in these assays were free of bacterial and viral pathogens.

rPspA and adjuvants. Endotoxin-free rPspA was purified by chromatography on a chelating Sepharose 4B column preloaded with Ni^+ (GE Healthcare, Piscataway, NJ) from *Escherichia coli* BL21(DE3) carrying pUAB055, which comprised the first 302 of the 588 amino acids of PspA/Rx1, including all of the α-helical region and some of the proline-rich region (3). The plasmid pORF9-mFlt3L (pFL) consists of the pORF9-mcs vector (pORF) plus the full-length murine FL cDNA gene (InvivoGen, San Diego, CA). These plasmids were purified using the Gene Elute endotoxin-free plasmid kit (Sigma-Aldrich, St. Louis, MO) (19). The Limulus amebocyte lysate assay (BioWhittaker, Walkersville, MD) resulted in <0.1 endotoxin unit of lipopolysaccharide (LPS) per 1 μg of plasmids or rPspA.

Nasal immunization and sample collection. Mice were immunized three times at weekly intervals nasally with 6 μl/nostril phosphate-buffered saline (PBS) containing 5 μg of rPspA and 50 μg of pFL as a mucosal adjuvant. As controls, mice were immunized nasally with 50 μg of pORF (empty plasmid) and 5 μg of rPspA under anesthesia. In some experiments, mice were administered pFL (50 μg), pORF (50 μg), rPspA (1 μg or 5 μg), native cholera toxin (nCT) (1 μg), or PBS alone under anesthesia. Plasma, nasal washes (NWs), and bronchoalveolar lavage fluid (BALF) were obtained as described previously (36).

Dynamic translocation of pFL. On 12 h or 7 days after mice were nasally given pFL (50 μg) alone, mononuclear cells were isolated from nasal mucosa-associated lymphoreticular tissues (NALT) and nasal passages (NPs) as described previously (14, 19), and NALT and NP dendritic cells (DCs) were purified by the AutoMACS cell sorter (Miltenyi Biotec, Auburn, CA) using anti-CD11c monoclonal Ab (MAb) microbeads (19). Further, nasal epithelial cells (nECs) and olfactory nerves and epithelium (ON/E) were isolated from nasal passages and olfactory bulbs, respectively (14, 40). In brief, cells from the nasal mucosa and olfactory bulb were prepared by gentle teasing through stainless screens and were subjected to discontinuous gradient centrifugation using 40% and 55% Percoll. Cells on the surface of the 40% layer were used as nECs and ON/E. To further confirm the presence of nECs and ON/E, the size and granularity of cells were determined by using flow cytometry. DNA was then extracted from NALT, NP-DCs, nECs, and ON/E, and the ampicillin resistance gene (858 bp) contained in the pFL plasmid was detected by a primer-specific PCR method. The sense primer was 5′-CCA ATG CTT AAT CAG TGA GGC-3′, and the anti-sense primer was 5′-ATG AGT ATT CAA CAT TTC CGT GTC G-3′. The PCR products were separated by electrophoresis in 2% agarose gels and visualized by UV light illumination following ethidium bromide (0.5 mg/ml) staining (19).

Analysis of FL protein synthesis. Twelve hours after nasal administration of pFL (50 μg), empty plasmid (50 μg), rPspA alone, or PBS, DCs from NALT and NPs and nECs and ON/E were purified aseptically as described above and were then cultured for 48 h (2×10^6 cells/ml) in complete medium. The concentrations of FL protein secreted into the medium were determined by FL-specific enzyme-linked immunosorbent assay (ELISA) (Quantikine M mouse Flt3 ligand ELISA kit; R & D Systems, Minneapolis, MN). Mice were next nasally immunized weekly for three consecutive weeks with rPspA (5 μg) plus pFL (50 μg) or pORF (50 μg), rPspA alone (5 μg), or PBS, and 1 week after the last immunization, the FL protein in nasal washes (NWs) and bronchoalveolar lavage fluid (BALF) was determined by FL-specific ELISA (R & D Systems).

Detection of inflammatory cytokines in mucosal secretion. In order to determine inflammatory cytokines by nasal application of pFL, NWs and BALF were collected 5 days after the nasal administration of pFL (50 μg), pORF (50 μg), rPspA (1 μg or 5 μg), or native cholera toxin (1 μg). Next, the mucosal secretion samples were subjected to ELISA specific to IL-1β, IL-6 (R & D Systems), and tumor necrosis factor alpha (TNF-α) according to the manufacturer's instructions (eBioscience, San Diego, CA).

rPspA-specific Ab assays. In order to examine mucosal and systemic immune responses to Ag, rPspA-specific IgA and IgG antibody (Ab) levels in plasma, NWs, and BALF were determined by ELISA on day 7 after the last immunization, as described previously (18, 19, 32). Briefly, 96-well Falcon microtest assay plates (BD Biosciences, Oxnard, CA) were coated with 1 μg/ml of rPspA in PBS. After incubating serial dilutions of samples, horseradish peroxidase-conjugated goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b, IgG3, or IgA (Southern Biotechnology Associates Inc., Birmingham, AL) was added to wells. The color reaction was developed for 15 min at room temperature. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution that gave an optical density at 415 nm (OD_{415}) of 0.1 greater than the background level. Further, mononuclear cells obtained from spleen, NALT, cervical lymph nodes (CLNs), mediastinal lymph nodes (MeLNs), NPs, and lungs were subjected to an enzyme-linked immunospot (ELISPOT) assay in order to determine the numbers of Ag-specific Ab-forming cells (AFCs) (18, 19). In brief, mononuclear cells in the spleen, NALT, CLNs, and MeLNs were isolated aseptically by a mechanical dissociation method using gentle teasing through stainless steel screens as described previously (14). For isolation of mononuclear cells from NPs, a modified dissociation method was used based upon a previously described protocol (18). Mononuclear cells from lungs were isolated by a combination of an enzymatic dissociation procedure with collagenase type IV (0.5 mg/ml; Sigma-Aldrich) followed by discontinuous Percoll (Amersham Biosciences, Arlington Heights, IL) gradient centrifugation.

Flow cytometric analysis. To characterize the phenotype of DCs, aliquots of mononuclear cells (0.2×10^6 to 1.0×10^6 cells) were isolated from various lymphoid compartments 1 week after the last immunization with rPspA plus pFL or pORF. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD11b, CD8, or B220 MAbs (BD Biosciences). In some experiments, mononuclear cells were incubated with phycoerythrin (PE)-labeled anti-mouse I-A^b, CD11c, CD40, CD80, or CD86 MAbs (BD Biosciences) and biotinylated anti-mouse CD11c MAbs (BD Biosciences), followed by CyChrome-streptavidin. These samples were then subjected to flow cytometry analysis (FACSCalibur; BD Biosciences) for cell subset analysis (19).

rPspA-specific $CD4^+$ T cell responses and cytokine-specific ELISA. $CD4^+$ T cells from lungs, CLNs, and spleen were purified using an automatic cell sorter (AutoMACS) system (Miltenyi Biotec) as described previously (18, 19). The purified $CD4^+$ T cell fraction (>97% $CD4^+$ and >99% viable) was resuspended in RPMI 1640 (Sigma-Aldrich) supplemented with HEPES buffer (10 mM), L-glutamine (2 mM), nonessential amino acid solution (10 μl/ml), sodium pyruvate (10 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), gentamicin (80 μg/ml), and 10% fetal calf serum (FCS) (complete medium; 4×10^6 cells/ml) and cultured in the presence of T cell-depleted, complement- and mitomycin-treated splenic Ag-presenting cells taken from nonimmunized, normal mice with or without 2 μg/ml rPspA. To assess rPspA-specific T cell proliferative responses, an aliquot of 0.5 μCi of tritiated [^{3}H]TdR (PerkinElmer Japan Co., Ltd., Japan) was added during the final 18 h of incubation, and the amount of [^{3}H]TdR incorporation was determined by scintillation counting (19). The culture supernatants were collected on day five and analyzed using gamma interferon (IFN-γ)-, IL-2-, IL-4-, IL-5-, IL-6-, and IL-10-specific ELISA kits (eBioscience). The detection limit for each cytokine was as follows: 15 pg/ml for IFN-γ, 2 pg/ml for IL-2, 4 pg/ml for IL-4, IL-5, and IL-6, and 30 pg/ml for IL-10.

Pneumococcal infection. Mice were nasally challenged with a serotype 3 *S. pneumoniae* strain (WU2) with a mucoid phenotype at a dose of 1.8×10^7

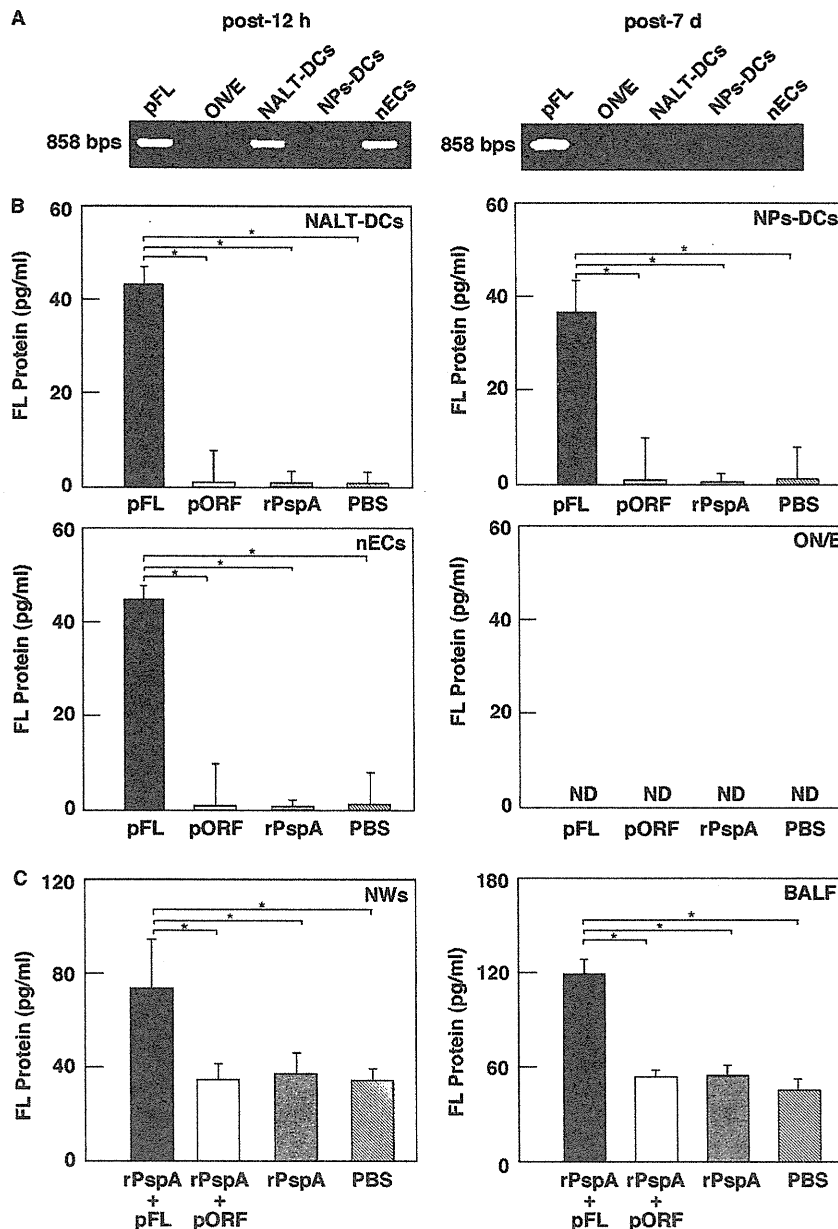

FIG. 1. (A to C) Translocation of FL plasmid after nasal administration of pFL (A), FL protein production by nasal DCs and epithelial cells (B), and expression of the FL protein in mucosal secretions (C). (A) Twelve hours (left) or 7 days (right) after nasal application of pFL (50 μg), the DNA samples were extracted from 1.0×10^5 (each) cells of the olfactory nerve and epithelium (ON/E; lane 2), NALT-DCs (lane 3), NPs-DCs (lane 4), and nasal epithelial cells (nECs; lane 5). In order to show the presence of plasmid in these cell populations, the ampicillin resistance gene (858 bp) contained in pFL was detected by PCR using specific primers. pFL (0.1 μg) was employed as a positive control (lane 1). (B) Mice were nasally administered pFL (50 μg; black column), pORF (50 μg; white column), rPspA (5 μg; shaded column), or PBS (hatched column). Twelve hours later, NALT-DCs, NPs-DCs, nECs, and ON/E were isolated and cultured (2×10^6 cells/ml, respectively) for 48 h in complete medium. The concentration of FL protein secreted in medium was measured by FL-specific ELISA. The values shown are the means ± SEM for 30 mice for each group and a total of three experiments. *, $P < 0.05$ compared with results for the mouse group given pORF, rPspA, or PBS. (C) Mice were nasally immunized weekly for three consecutive weeks with rPspA (5 μg) plus pFL (50 μg; black column) or pORF (50 μg; white column), rPspA alone (5 μg; shaded column), or PBS (hatched column). One week after the last immunization, NWs and BALF (100 μl, respectively) were collected and subjected to FL-specific ELISA. The values shown are the means ± SEM of data for 30 mice for each group and a total of three experiments. *, $P < 0.05$ compared with results for mouse group given pORF, rPspA, or PBS.

CFU (20 μl). Forty-eight hours after the bacterial challenge, the lungs were removed aseptically and homogenized in 9 ml of sterile saline per gram of lung tissues. NWs and blood were collected as described above. Bacterial colonies were counted by plating lungs, NWs, and blood (50 μl, respectively) on horse blood agar (BD Biosciences), followed by incubation at 37°C overnight. The detection limit of bacterial culture was 10^2 CFU/g. The 50% lethal dose was calculated to be 2.5×10^6 CFU.

Statistical analysis. Each result is expressed as the mean ± 1 standard error of the mean (SEM). All mouse groups were compared with control mice using an unpaired Mann-Whitney U test by using the Statview software program

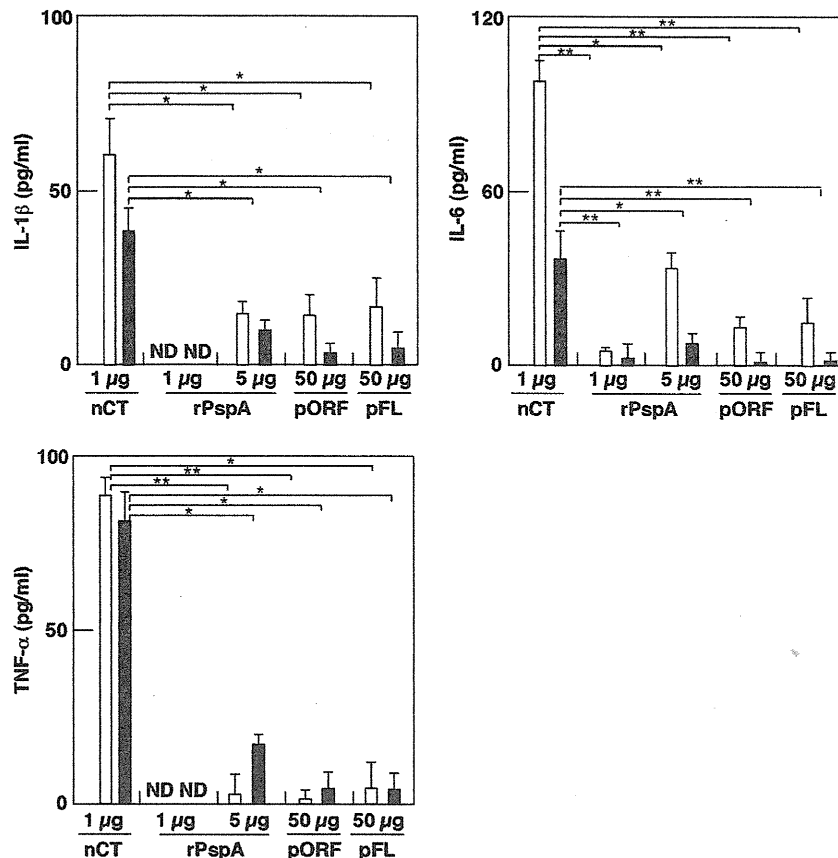

FIG. 2. Inflammatory cytokine production in NWs (white column) and BALF (black column). Mice were nasally administered native cholera toxin (nCT) (1 μg), rPspA (1 or 5 μg), pORF (50 μg), or pFL (50 μg). Five days later, NWs and BALF were collected and subjected to IL-1β-, IL-6-, and TNF-α-specific ELISA. The values shown are the means ± SEM of data for 30 mice for each group and total of three experiments. *, $P < 0.05$; **, $P < 0.01$ (compared with results for mouse group given nCT).

(Abacus Concepts, Cary, NC), designed for Macintosh computers, with Bonferroni's correction. P values of <0.05 or <0.01 were considered significant.

RESULTS

Tracking plasmid expression and FL protein synthesis. In order to examine safety of pFL for nasal application, we initially traced plasmid-specific ampicillin resistance gene expression by nasal DCs, nECs, and the ON/E. DCs from NALT and NPs, as well as nECs, possessed the ampicillin resistance gene 12 h after nasal administration of pFL (Fig. 1A, left). Of interest, on 7 days after nasal pFL application, the ampicillin resistance gene was detected only in nECs (Fig. 1A, right). Further, NALT-DCs, NP-DCs, and nECs of mice given nasal pFL produced significantly elevated levels of the FL protein compared with those of mice given nasal pORF (empty plasmid), rPspA alone, or PBS (Fig. 1B). In addition, nasal application of the combination of rPspA and pFL resulted in FL protein production comparable to that with nasal application of pFL alone (data not shown). However, FL protein synthesis in mice given nasal rPspA plus pORF was at essentially the same level as that seen in mice given pORF or rPspA alone (data not shown). Thus, NWs and BALF from mice given nasal pFL plus rPspA contained significantly higher levels of FL than those from mice given nasal pORF plus rPspA, rPspA alone, or PBS only (Fig. 1C). On the other hand, of importance, no plasmid-specific genes were essentially detected in the ON/E of mice given nasal pFL (Fig. 1A). Thus, the culture supernatants of ON/E did not contain detectable levels of the FL protein (Fig. 1B). These results show that pFL is largely present in nasal DCs and nECs but not in the ON/E and suggest that pFL on nECs may maintain production of the FL protein.

Nasal pFL induces lower levels of inflammatory cytokines than nCT. Although pFL was not taken up by the central nervous system, it is important to show that FL produced in the nasal cavity does not induce inflammatory responses. In this regard, the levels of IL-1β, IL-6, and TNF-α production in NWs and BALF were examined 5 days after nasal administration with rPspA, pORF, native cholera toxin (nCT), or pFL. The levels of inflammatory cytokine synthesis in NWs and BALF of mice given nasal pFL were essentially the same as or lower than that of mice given nasal rPspA or pORF alone (Fig. 2). Similarly, nasal application of rPspA plus pFL resulted in low levels of inflammatory cytokine production which were similar to those seen in pFL alone (data not shown). Conversely, nasal nCT induced markedly high levels of these inflammatory cytokines (Fig. 2). These results show that nasal pFL application does not elicit unnecessary inflammatory responses in the nasal mucosa.

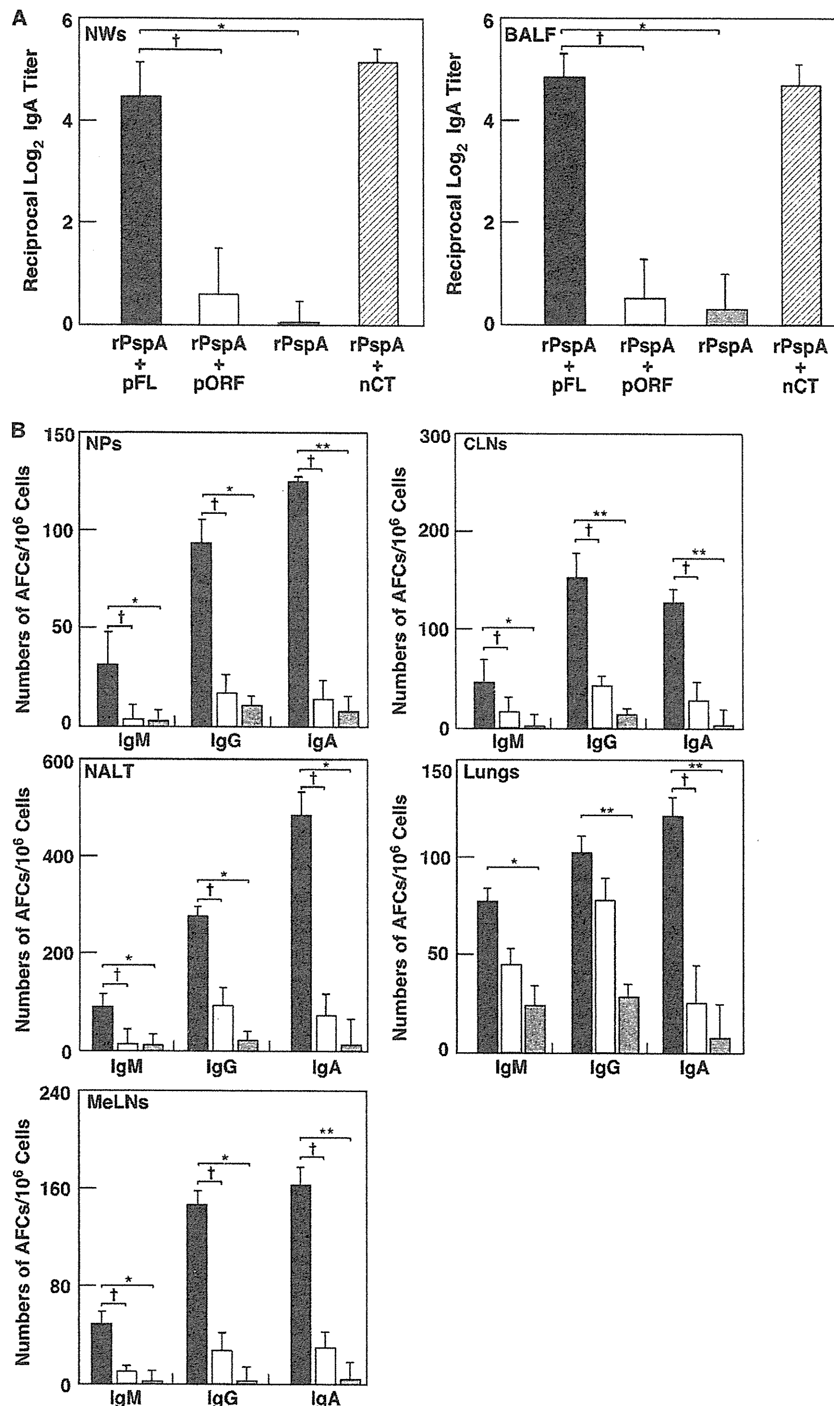

FIG. 3. Mucosal immune responses to rPspA in external secretions and mucosal lymphoid tissues. C57BL/6 mice were nasally immunized three times at weekly intervals with rPspA (5 μg) plus pFL (50 μg; black column) or pORF (50 μg; white column), rPspA alone (shaded column), or rPspA (5 μg) plus nCT (1 μg; hatched column). (A) Seven days after the last immunization, the levels of rPspA-specific IgA Abs in NWs and BALF were determined by rPspA-specific ELISA. (B) Seven days after the last immunization, mononuclear cells isolated from NPs, CLNs, NALT, lungs, and MeLNs were subjected to ELISPOT assay to determine the numbers of Ag-specific IgM, IgG, and IgA Ab-forming cells (AFCs). The values shown are the means ± SEM (n = 20). *, $P < 0.05$; **, $P < 0.01$ (compared with mouse group given rPspA alone). †, $P < 0.05$ (compared with mouse group given rPspA plus pORF).

Induction of rPspA-specific Ab responses in mucosal and systemic tissues of mice given rPspA plus pFL. We next examined whether nasal administration of pFL as a mucosal adjuvant would enhance rPspA-specific Ab responses. Giving mice nasal rPspA plus pFL resulted in significantly increased levels of rPspA-specific IgA Ab responses in NWs and BALF compared with results for mice given nasal rPspA plus pORF or rPspA Ag alone (Fig. 3A). The levels of rPspA-specific IgA