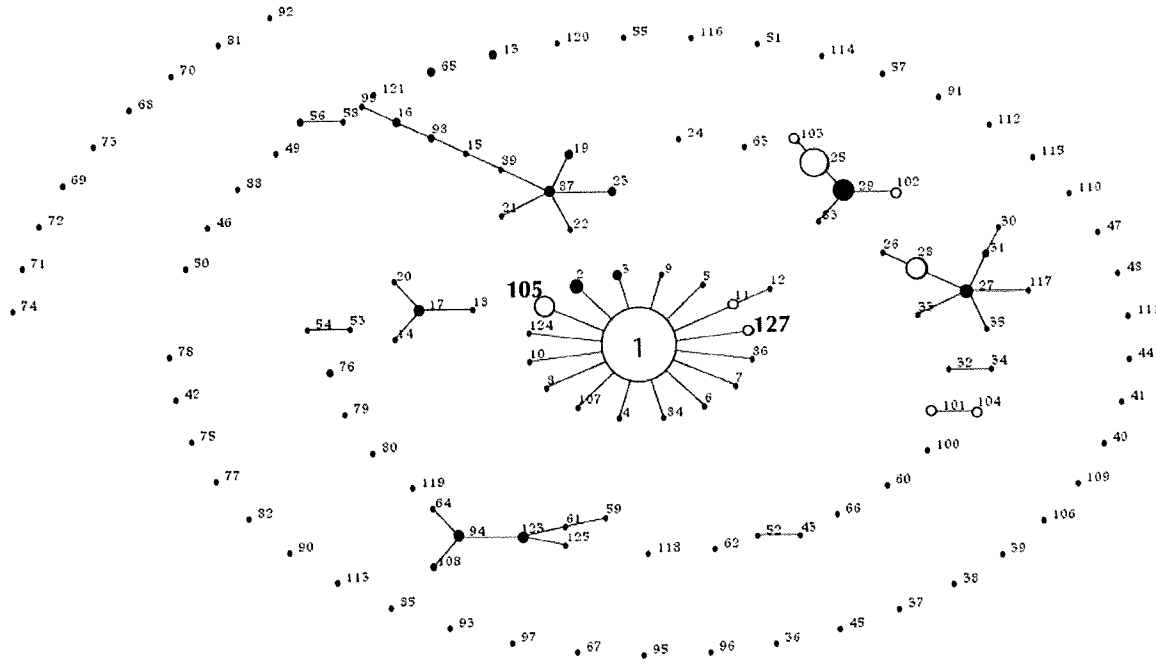
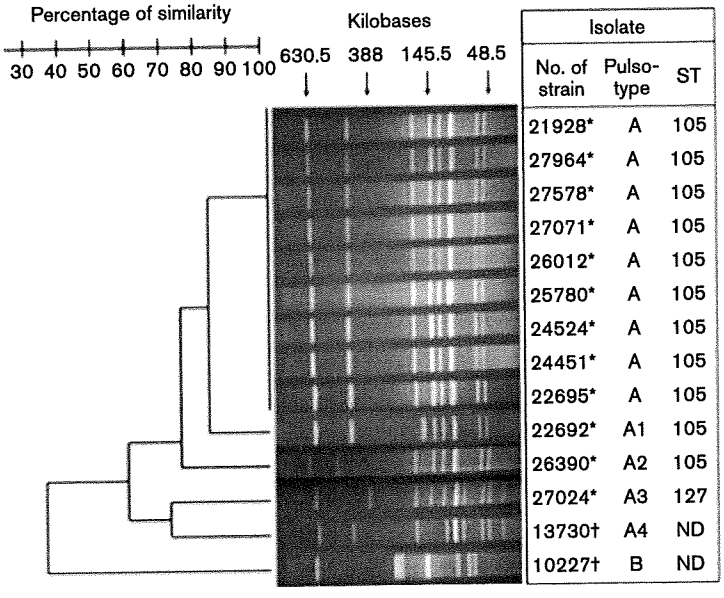


Fig. 2. Entire *S. suis* MLST database displayed as a single eBURST diagram. The primary founders of ST1 are located in the centre of the cluster, and subgroup founders are shown in closed circles, except for STs 1, 11, 25, 28, 101, 103, 104, 105 and 127. ST105 for 11 serotype 14 strains in this study and one strain of serotype 14 from Vietnam (Mai *et al.*, 2008) and ST127 for one strain of serotype 14 in this study are shown in grey circles. STs 1, 11, 25, 28, 101, 103 and 104 for human isolates of *S. suis* serotypes 2 and 14 in Thailand previously reported by Takamatsu *et al.* (2008) are shown in open circles. The size of each circle in the diagram corresponds to the abundance of the isolates of the ST in the input data.



Isolate		
No. of strain	Pulso-type	ST
21928*	A	105
27964*	A	105
27578*	A	105
27071*	A	105
26012*	A	105
25780*	A	105
24524*	A	105
24451*	A	105
22695*	A	105
22692*	A1	105
26390*	A2	105
27024*	A3	127
13730†	A4	ND
10227†	B	ND

Fig. 3. Dendrogram generated from PFGE profiles after *Sma*I digestion of 12 human isolates of *S. suis* serotype 14 obtained from humans living in Thailand and reference strains of serotype 14 (NIAH 13730) and serotype 1 (NIAH 10227). The numbers in the dendrogram indicate the percentage similarity. Arrows indicate molecular size; * indicates DMST (Department of Medical Science, Thailand) strain number; † indicates NIAH (National Institute of Animal Health) strain number.

similar to those previously reported for *S. suis* infections, except that there were no fatal cases. Clonal dissemination of serotype 14 with ST105 was demonstrated in 11 of the 12 isolates. To our knowledge, this is the first report of clonal dissemination of serotype 14 in humans. Because this serotype is becoming more common in human infections, continuous surveillance of this disease using the diagnostic tests, which include serotyping PCR and a coagglutination test, for both serotypes 2 and 14 should be required at hospital laboratories in South-East Asian countries.

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Intranasal immunization with a mixture of PspA and a Toll-like receptor agonist induces specific antibodies and enhances bacterial clearance in the airways of mice

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ABSTRACT

To develop an effective nasal vaccine for *Streptococcus pneumoniae*, the effects of a panel of Toll-like receptor (TLR) agonists in combination with pneumococcal surface protein A (PspA) on induction of PspA-specific antibodies and bacterial clearance were compared in mice. Mice were nasally immunized with 10 μg of TLR agonist (TLR 2–4 and 9) and 2.5 μg of PspA once per week for 3 weeks. Significantly increased levels of PspA-specific immunoglobulin G (IgG) and IgA in the airways and PspA-specific IgG in plasma were found in mice administered PspA plus each TLR agonist, compared with mice administered PspA alone. In a sub-lethal pneumonia model using a serotype 3 pneumococcal strain, bacterial density in the lungs of mice was significantly reduced in mice administered PspA plus each TLR agonist, compared with mice administered either PspA alone or phosphate-buffered saline alone 3 h after bacterial challenge. Similarly, enhanced bacterial clearance was found in the nasopharynx of mice administered PspA plus each TLR agonist 1 day after infection with a serotype 19F strain. Our data suggest that PspA-specific antibody induced by nasal immunization with PspA plus TLR agonist is capable of reducing the bacterial load in both the nasopharynx and lungs after challenge with pneumococci with different serotypes. Despite the skewed Th1/Th2 immune responses, the effects of nasal immunization with PspA plus each TLR agonist on bacterial clearances from the lungs 3 h after infection and from nasopharynx 1 day after infection in mice were equivalent.

1. Introduction

Streptococcus pneumoniae (*S. pneumoniae*) is a leading human pathogen causing diseases ranging from otitis media to pneumonia, bacteremia, and meningitis in children and adults. Although pneumococcal conjugate vaccine provides protective immunity against pneumonia as well as invasive disease in infants [1,2], polysaccharide-based vaccines are not ideal because they must include multiple polysaccharide serotypes and do not protect against strains with non-vaccine serotypes [3]. Previous investigators have examined several pneumococcal proteins as potential vaccine candidates with promising results [4–7]. One of these candidates, pneumococcal surface protein A (PspA) is a choline-binding protein tethered to the cell surface through its C-terminal choline-binding repeat region [4]. PspA is present on all pneumococcal strains, and anti-PspA antibody enhances bacterial clearance and induces cross-protection against infection from strains with different serotypes [8]. According to the mapping studies of the major cross-protective epitopes that reside in the ~100 amino acids of the α-helical region, PspAs have been divided into seven clades that constitute three families [9]. PspAs of families 1 and 2 are expressed by >98% of strains. Anti-PspA antibodies overcome the anti-complementary effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [10,11].

Nasal immunization is the most effective way to induce both mucosal secretory-IgA responses and systemic IgG responses [12]. An appropriate mucosal adjuvant is required to elicit an antigen-specific immune response in both mucosal and systemic compartments [13]. The Toll-like receptor (TLR) family is the best-studied family of pattern recognition receptors, and it recognizes

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a broad spectrum of pathogen-associated molecular patterns from different classes of microbes [14]. TLR ligands may stimulate dendritic cells (DC), thereby acting as an effective adjuvant to allow a DC-targeted protein to induce protective CD4 T cell responses at mucosal surfaces [13,14]. The balance of Th1/Th2 immune responses appears to be dependent on each TLR ligand [15]. Th1 immune responses augment IgG2a production, while Th2 immune responses enhance IgG1 and IgE production by B cells [16–18]. The pattern of IgG subclass response may affect the bacterial clearance afforded by such humoral immunity during infections. Two recent studies employing a PspA DNA vaccine [19] and a nasal lactococcal vaccine producing PspA [20] have suggested that the induction of a balanced IgG1/IgG2a response to PspA correlates with an increased protection against pneumococcal infections. Therefore, in this study, we examined the relationship between the Th1- or Th2-associated IgG isotype response and the enhanced bacterial clearance of *S. pneumoniae* from the airways after intranasal immunization using a mixture of PspA plus each TLR 2–4 or 9 agonist in mice.

2. Material and method

2.1. Mice

Female C57BL/6 mice (6–8-week-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.

2.2. Bacterial strains

S. pneumoniae WU2 strain with serotype 3, expressing PspA belongs to family 1, clade 2 and is virulent in mice [21]. *S. pneumoniae* EF3030 strain with serotype 19F is a clinical isolate, expressing PspA belongs to family 1, clade 1, and is relatively avirulent in mice [22]. These strains were kindly provided by Dr. D.E. Briles, University of Alabama at Birmingham.

2.3. Recombinant PspA

PspA used for nasal immunization in this study was recombinant PspA/Rx1 (pUAB055) [5]. The recombinant plasmid pUAB055 containing the 0.9 kb *pspA* gene fragment inserted between the *pelB* leader sequence and the His-tag site in vector pET20b (a gift from Dr. S.K. Hollingshead, University of Alabama at Birmingham) was transformed into *E. coli* strain BL21 (DE3) for protein production. Rx1/PspA is of PspA family 1 (clade 2), which is the same family as both the WU2 strain and EF3030 strains. Induction with isopropyl-thio-β-D-galactopyranoside (Sigma, St. Louis, MO) resulted in production of 6× His-tagged recombinant PspA. The recombinant PspAs were purified by chromatography chelating-sepharose 4B pre-loaded with Ni^+ (GE Healthcare, Buckinghamshire, England) according to the manufacturer's instruction. The fraction containing PspA was loaded onto a gel filtration superdex-75 5/30 GL column (GE Healthcare) to further purify the PspA. Contaminated endotoxin was removed from the PspA preparation by using EndoTrap^R (Profos AG, Rosenberg, Germany). The purified PspA preparation was analyzed for the presence of endotoxin using a chromogenic *Limulus* lysate endopoint assay, QCL-1000^R (Cambrex, Walkersville, MD), and it contained 1.30 ng of LPS per 1 μg of PspA. To remove LPS extensively from the PspA preparations, we used another LPS removal column, ProteoSpin^R (Norgen, Thorold, Canada) and prepared the PspA with a lower concentration of LPS (0.048 ng of LPS per 1 μg of PspA).

2.4. Adjuvant

Pam3CSK4 is a synthetic tripalmitoylated lipopeptide that mimics bacterial peptides [23], and is recognized by the TLR2/TLR1 heterodimer. Poly(I:C) is a synthetic analog of double-stranded RNA, a TLR3 agonist [24]. Pam3CSK4, Poly(I:C), and Ultra Pure *Escherichia coli* K12 LPS, a TLR4 agonist, were purchased from InvivoGen (San Diego, CA). CpG DNA ODN1826 (TLR9 ligand, 5′-TCCATGACGTTCCTGACGTT-3′) was purchased from Hokkaido System Science (Sapporo, Japan) [25]. Each of these adjuvants was used in a dose of 10 μg for nasal immunization, because these TLR agonists demonstrated potent adjuvant effects at this dose in mouse experiments [24–26].

2.5. Nasal immunization

Mice were immunized three times at weekly intervals intranasally with 12 μl of phosphate-buffered saline (PBS) containing 10 μg of each TLR agonist and 2.5 μg of PspA, 2.5 μg of PspA alone or 12 μl of PBS alone on day 0, days 7 and days 14. On days 21, mice were euthanized to obtain plasma, bronchoalveolar lavage fluid (BALF) and nasal wash (NW). A dose of 2.5 μg of PspA was employed for nasal immunization in this study, as nasal immunization with this dose of PspA plus 10 μg of each TLR agonist induces PspA-specific antibodies in the airways. A dose of PspA alone for nasal immunization, therefore, contained 3.25 ng of LPS. After removing the mandible, the nasal cavity was gently flushed from the posterior opening of the nose with 1 ml of PBS [27]. The NW flushing out from the anterior openings of the nose was collected. BALF was obtained by irrigation with 1 ml of PBS using of a blunted needle inserted into the trachea after tracheotomy [28].

2.6. PspA-specific antibody assays

PspA-specific antibody titers of IgG, IgG1, IgG2a or IgA in plasma, BALF and NW were determined by ELISA as previously described [28]. The coefficient variation (CV) of the levels of PspA-specific IgG, IgG1, IgG2a or IgA was also determined.

PspA was used as the coating antigen (1 μg/ml). 100 μl of sample was added to each well, followed by incubation at 37 °C for 30 min. The plate was washed, and then reacted with 100 μl of alkaline phosphatase-conjugated goat anti-mouse IgA, IgG, IgG1 or IgG2a (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.

2.7. Pneumonia model

To determine the effects of nasal immunization with PspA plus each TLR agonist, *S. pneumoniae* WU2 strain at a dose of 2.0×10^6 cfu suspended in 30 μl of sterile saline was intranasally administered to both immunized and untreated mice 2 weeks after the last immunization. The 2-week interval between the last immunization and the bacterial challenge was kept to avoid the influence of each TLR agonist on pulmonary defense, as some TLRs are involved in the innate immune response to *S. pneumoniae* [29–31]. The lungs were removed aseptically from mice that had been euthanized with pentobarbital at 3 h, 6 h and 12 h post-bacterial challenge. The lung tissue was homogenized in 9 ml of sterile saline per gram of lung tissue prior to culturing and quantitative bacterial cultures of lung tissue were performed on horse blood agar. The detection limit of bacterial culture of the lung tissue was 10^3 cfu/g. The survival rate after intranasal challenge with 2.0×10^6 cfu of the WU2 strain was 100%.

2.8. Nasal carriage model

S. pneumoniae EF3030 strain at a dose of 3×10^5 cfu in suspended 30 μl of sterile saline was similarly intranasally administered to both immunized and untreated mice 2 weeks after the last immunization. One or 6 days after bacterial challenge, NW was obtained as described above, and a quantitative bacterial culture of the NW was performed.

2.9. Statistics

Statistical analyses were performed using one-way ANOVA and multiple comparison methods by Fisher's LSD. Data were considered to be statistically significant if the *P*-values were less than 0.05. All data were expressed as mean ± S.D.

3. Results

3.1. PspA-specific IgG and IgG isotypes in plasma

Nasal administration of PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826 significantly increased the levels of PspA-specific IgG in the plasma, compared with administration of PspA alone ($P < 0.05$, Fig. 1A). No differences were found in the levels of PspA-specific IgG among mice nasally administered PspA plus each TLR agonist. The CV of the levels of PspA-specific IgG by PspA plus each TLR agonist was much smaller than that induced by PspA alone.

Since the preparation of PspA after removal of LPS with Endotrap contained LPS (3.25 ng per 2.5 μg of PspA), PspA-specific IgG might be elicited by the adjuvant effect of the residual LPS. We then compared the levels of PspA-specific IgG in between the plasma of mice nasally administered 2.5 μg of PspA preparations containing either 3.25 ng of LPS or 0.12 ng of LPS. No significant differences were found in the levels of PspA-specific IgG in plasma of mice after nasal immunization with two different PspA preparations (data not shown). These data suggest the residual LPS did not contribute to the induction of PspA-specific IgG in plasma as an adjuvant, and PspA itself could induce PspA-specific IgG in plasma.

To assess whether each TLR agonist induces either a Th1- or a Th2-associated IgG isotype response, plasma samples were analyzed for PspA-specific IgG1 and IgG2a isotypes (Fig. 1B). Nasal administration of PspA plus Pam3CSK4, Poly(I:C) or LPS significantly increased the levels of PspA-specific IgG1 in plasma, while PspA-specific IgG1 increased to a lesser extent in plasma of mice nasally administered PspA plus CpG1826. The IgG1 levels differed significantly between mice administered PspA plus either Pam3CSK4, Poly(I:C) or LPS and mice administered PspA plus CpG1826 ($P < 0.05$, Fig. 1B). Furthermore, PspA-specific IgG1 levels were significantly higher in mice administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826 than in mice administered PspA alone ($P < 0.05$). In contrast, mice nasally administered PspA plus either Poly(I:C) or CpG1826 demonstrated significant increases in the levels of PspA-specific IgG2a in plasma, compared with mice administered PspA plus either Pam3CSK4, LPS or PspA alone ($P < 0.01$). The CV of the levels of PspA-specific IgG1 in plasma of mice nasally administered PspA plus each TLR agonist was much smaller than that of mice nasally administered PspA alone. In contrast, the CV of the levels of PspA-specific IgG2a induced by either PspA plus each TLR agonist, except for Poly(I:C), or PspA alone was large in plasma.

3.2. PspA-specific IgG and IgA in BALF and NW

Although the levels of PspA-specific IgG were negligible in the BALF and NW of mice given PspA alone, the levels of PspA-specific IgG were significantly greater in the BALF (Fig. 2A) and NW (Fig. 3A) of mice nasally administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826 than in mice nasally administered PspA alone ($P < 0.05$). A PspA-specific IgG1 response was found in the BALF of mice administered PspA plus either Pam 3CSK4, Poly(I:C), LPS or CpG1826 (Fig. 2C). In contrast, significant increases of PspA-specific IgG2a were also found in the BALF of mice administered PspA plus either Poly(I:C) or PspA plus CpG1826, compared with mice administered PspA plus either Pam3CSK4 or LPS or PspA alone ($P < 0.05$, Fig. 2C). However, PspA-specific IgG2a was rarely detected in the BALF of mice administered PspA plus either Pam3CSK4 or

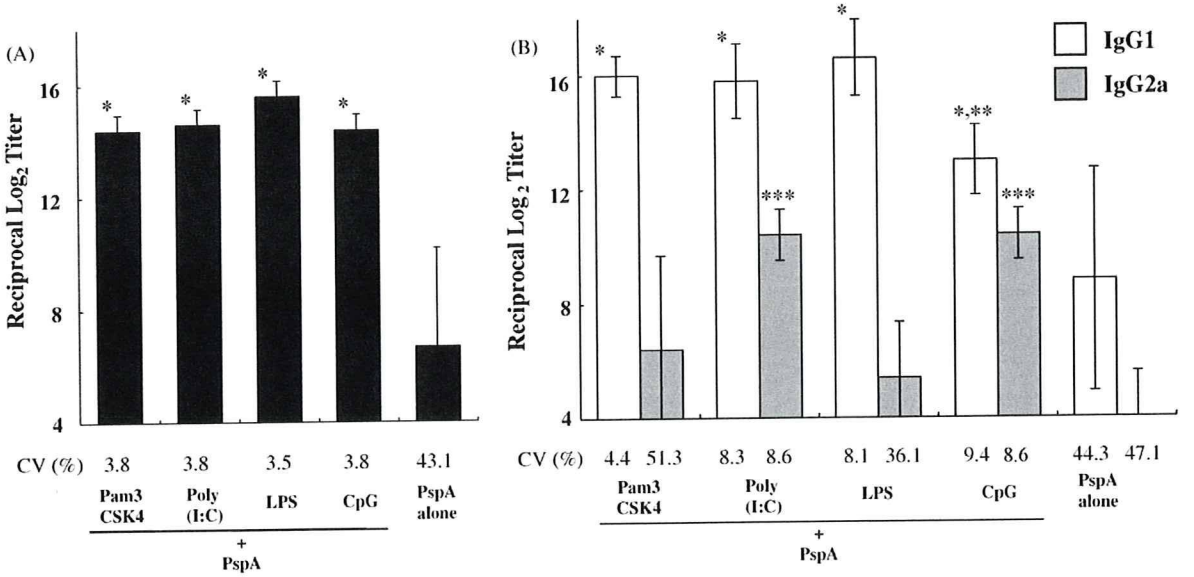

Fig. 1. Induction of PspA-specific IgG (closed bar) (A), PspA-specific IgG1 (open bar) and IgG2a (gray bar) (B) in plasma by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain plasma, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered PspA alone; **$P < 0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, Poly(I:C) or LPS; ***$P < 0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, LPS or PspA alone.

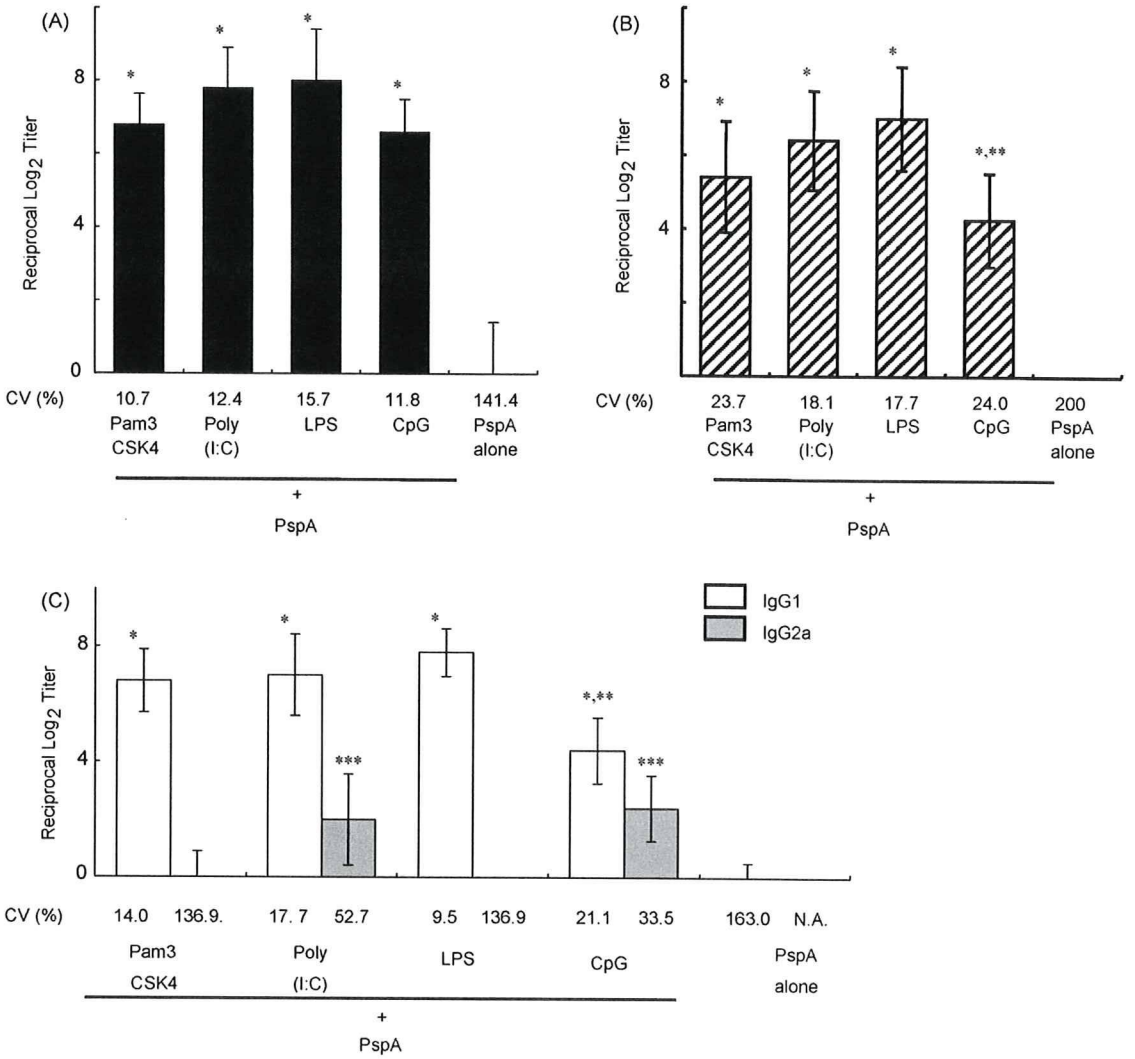

Fig. 2. Induction of PspA-specific IgG (closed bar) (A), IgA (hatched bar) (B) and PspA-specific IgG1 (open bar) and IgG2a (gray bar) (C) in bronchoalveolar lavage fluid (BALF) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times weekly with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain BALF and NW, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; N.A., not available; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. *$P < 0.05$, when compared with mice nasally administered PspA alone; **$P < 0.05$, when compared with mice nasally administered PspA plus either Pam3CSK4, Poly(I:C) or LPS; ***$P < 0.05$, when compared with nasally administered PspA plus either Pam3CSK4, LPS or PspA alone.

LPS or PspA alone. Mice nasally administered PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826 demonstrated significant increases in the levels of PspA-specific IgA in the BALF (Fig. 2B) and NW (Fig. 3B), compared with mice nasally administered PspA alone ($P < 0.05$). The levels of PspA-specific IgA were significantly lower in the BALF of mice administered PspA plus CpG1826 than in mice administered PspA plus either Poly(I:C) or LPS ($P < 0.05$). The CV of the levels of PspA-specific IgG or IgA induced in the BALF by PspA plus each TLR agonist was similarly much smaller than that induced by PspA alone. A similar tendency of the CV was found in NW.

3.3. Bacterial clearance from the lungs

At 3 h post-nasal challenge with a sub-lethal dose of serotype 3 WU2 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lungs reached to 6.0 ± 0.4 and 6.0 ± 0.3 in mice nasally administered PspA alone and PBS alone, respectively (Fig. 4A). No significant difference was found between these two groups. In contrast, significant decreases were found in bacterial density in the lungs of mice nasally administered PspA plus Pam3CSK4, Poly(I:C), LPS or CpG1826, compared with mice nasally administered either PspA alone or PBS alone ($P < 0.05$). No significant differences were found in the bacterial density among mice nasally administered PspA plus each TLR agonist. At 6 h post-nasal challenge with the same dose of WU2 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lungs remained unchanged at 6.3 ± 0.4 for mice administered PBS alone (Fig. 4B). In contrast, significant decreases were found in the bacterial density in the lungs of mice nasally administered either PspA plus each TLR agonist or PspA alone, compared with mice administered PBS alone ($P < 0.05$). No significant difference was found in the bacterial density among mice nasally administered either PspA plus each TLR agonist or PspA alone. At 12 h post-nasal challenge, the bacterial density (mean ± S.D. for Log_{10} cfu/g) in the lung declined to 4.7 ± 0.7 in mice administered PBS alone (Fig. 4C). In contrast, bacteria were not detected in the lungs of mice nasally administered either PspA plus each TLR agonist or PspA alone. No bacteria were detected in the blood of any mice examined at 3 h, 6 h and 12 h post-nasal challenge.

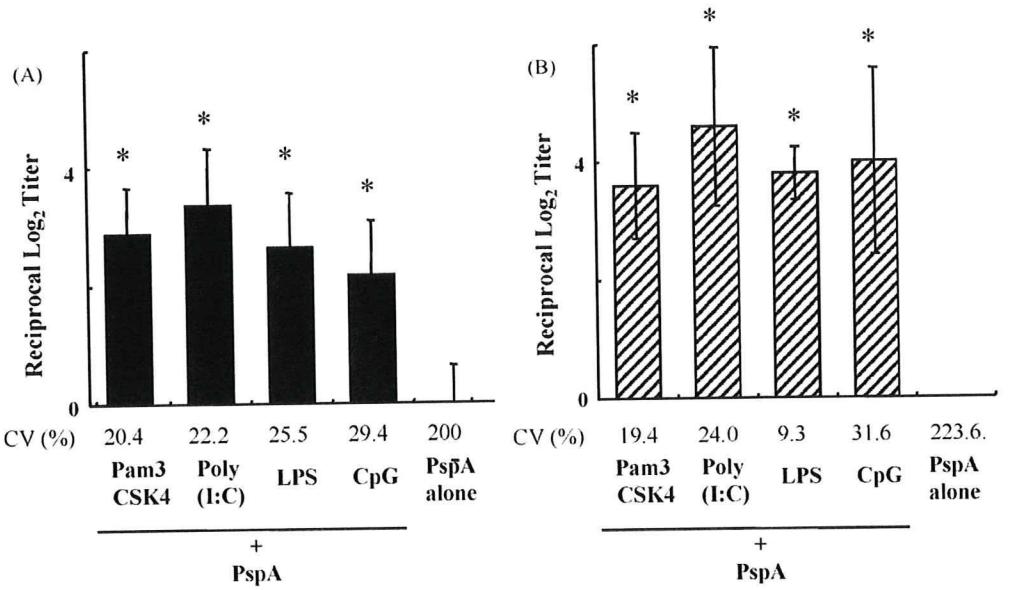

Fig. 3. Induction of PspA-specific IgG (closed bar) (A) and IgA (hatched bar) (B) in nasal wash (NW) by intranasal immunization with either PspA plus each TLR agonist or PspA alone. Mice were nasally immunized three times at weekly intervals with 10 μg of TLR agonist and 2.5 μg of PspA. One week after the final immunization, mice were euthanized to obtain BALF and NW, and PspA-specific antibody titers were determined using ELISA. The results are expressed as means ± S.D. for six mice per group. CV, coefficient of variation; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. $^{*}P < 0.05$, when compared with mice nasally administered PspA alone.

3.4. Bacterial clearance from the nasopharynx

One day after nasal challenge with 3×10^5 cfu of serotype 19F EF3030 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/ml) in NW reached to 5.21 ± 0.26 and 5.08 ± 0.11 in mice administered both PspA alone and PBS alone, respectively (Fig. 5A). No significant difference was found between these two groups. In contrast, significant decreases were found in the bacterial density of mice nasally administered PspA plus either Pam3CSK4, Poly(I:C), LPS or CpG1826, compared with mice nasally administered PspA alone ($P < 0.05$). No significant differences were found in the bacterial density among mice nasally administered PspA plus each TLR agonist. Six days after challenge with 3×10^5 cfu of the EF3030 strain, the bacterial density (mean ± S.D. for Log_{10} cfu/ml) in NW declined to 4.78 ± 0.29 and 4.69 ± 0.29 for mice administered both PspA and PBS alone, respectively (Fig. 5B). No significant difference was found

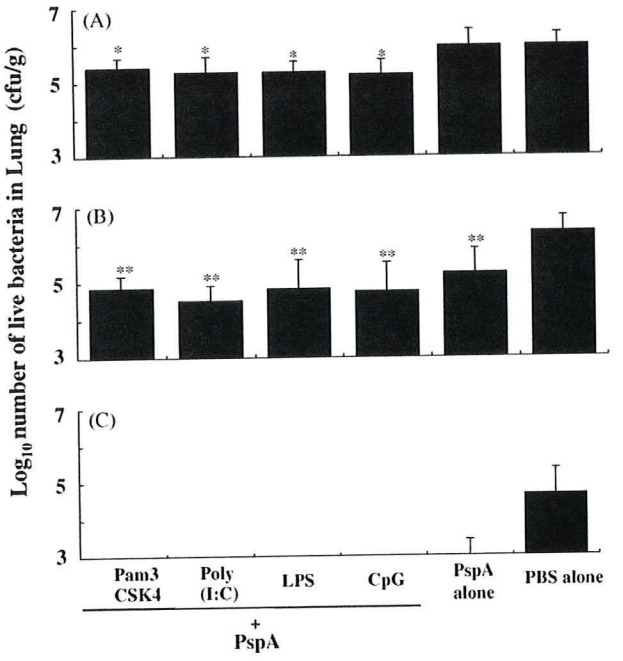

Fig. 4. The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the lung tissue at 3 h (A), 6 h (B) and 12 h (C) post-challenge with *S. pneumoniae* WU2 strain. A dose of 2×10^6 cfu/mouse was nasally administered to mice previously immunized with either PspA plus each TLR agonist, PspA alone or PBS alone. Mice were euthanized to obtain the lung tissues from infected mice at indicated time-points after bacterial challenge, and quantitative bacterial cultures of lung tissue were performed. Values represent the Log_{10} cfu/g (mean ± S.D.) for six mice per group. CV, coefficient of variation; N.A., not available; LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. $^{*}P < 0.05$, when compared with mice nasally administered either PspA alone or PBS alone; $^{**}P < 0.05$, when compared with mice nasally administered PBS alone.

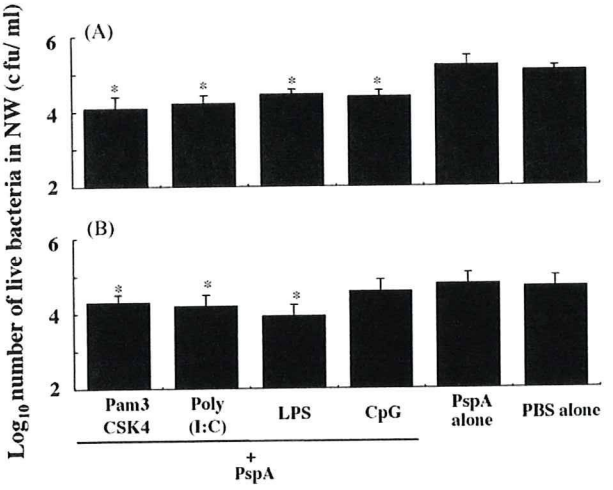

Fig. 5. The effect of intranasal immunization by PspA plus each TLR agonist on the bacterial densities in the nasopharynx 1 day (A) and 6 days (B) after challenge with *S. pneumoniae* EF3030 strain. A dose of 3×10^5 cfu/mouse was nasally administered to mice previously immunized with either PspA plus each TLR agonist, PspA alone or PBS alone. Mice were euthanized to obtain the nasal wash (NW) from infected mice at indicated time-points after bacterial challenge, and a quantitative bacterial culture of NW was performed. Values represent the Log_{10} cfu/ml (mean ± S.D.) of for six mice per group. LPS, *E. coli* K12 LPS; CpG, CpG DNA ODN1826. $^{*}P < 0.05$, when compared with mice nasally administered either PspA alone or PBS alone.

between these two groups. Similarly, significant reductions were detected in the bacterial density of mice nasally administered either PspA plus Pam3CSK4, Poly(I:C) or LPS, but not CpG1826, compared with mice nasally administered either PspA or PBS alone ($P < 0.05$). No significant differences were detected in bacterial density among mice nasally administered either Pam3CSK4, Poly(I:C) or LPS.

4. Discussion

In the present study, nasal immunization with PspA plus each TLR agonist, such as either Pam3CSK4, Poly(I:C), LPS or CpG1826, induced PspA-specific IgA and IgG in the airways as well as PspA-specific IgG in systemic circulation of mice. In contrast, nasal administration of PspA alone induced PspA-specific IgG in plasma, but neither PspA-specific IgA nor IgG in the airways. Therefore, we confirmed that each TLR agonist was an effective nasal adjuvant for the PspA antigen.

The concentrations of PspA-specific IgG in both BALF and plasma and PspA-specific IgA in both BALF and NW increased similarly in mice administered PspA plus each TLR agonist. Furthermore, Pam3CSK4 and LPS induced Th2-associated IgG isotype responses, while Poly(I:C) and CpG 1826 induced Th1- and Th2-associated IgG isotype responses. Previous studies also reported that both CpG motifs and Poly(I:C) induced a Th1 response: our data are consistent with these reports [26,32]. Moreover, the previous reports on the Th2 immune response induced by agonists of either TLR2 (Pam3Cys) or TLR4 (*lpxL1 mutant* LPS), are consistent with the results we obtained using either Pam3CSK4 or LPS [26,33].

It is of interest to determine whether the PspA-specific antibody induced in the airway by nasal immunization of PspA plus agonist of TLR has a protective role against pneumococcal infection. Arulanandam et al. demonstrated that intranasal immunization with PspA plus interleukin-12 (IL-12) induced the concentrations of PspA-specific IgG1, IgG2a and IgA in both plasma and BALF of mice, compared with administration of PspA alone [34]. Because IL-12 activates Th1 and NK cells to induce IFN-γ, the production of both Th1- and Th2-associated IgG isotypes specific for PspA were found in this study. Furthermore, the authors found that immune sera raised by PspA plus IL-12 augmented opsonophagocytic activity against *S. pneumoniae*. This response was primarily attributable to IgG2a and, to a lesser extent, IgA, although this assay evaluated antibody-mediated opsonophagocytic activity without complement. Because PspA-specific antibodies overcome the anti-complementary effects of PspA [11], in the presence of a complement, they likely mediate the efficient opsonophagocytic killing of *S. pneumoniae*.

In our sub-lethal pneumonia model using a serotype 3 WU2 strain, the significant reduction in bacterial density in the lungs of mice nasally administered PspA plus each TLR agonist at 3 h, but neither at 6 h nor at 12 h, post-infection, was associated with induction of PspA-specific IgA and IgG in the airways. No reduction of bacterial density in the lungs of mice nasally administered PspA alone at 3 h post-infection may also be explained by a negligible level of PspA-specific IgG2a and a low level of PspA-specific IgG1 in the plasma of these mice. By contrast, no differences were found in the bacterial density in the lungs of mice nasally administered PspA plus each TLR agonist nor in mice administered PspA alone at 6 h and 12 h post-nasal challenge. These findings may be explained by the extravasation of PspA-specific IgG into the alveolar space of mice given PspA alone during the progression of lung inflammation at 6 h or 12 h post-nasal challenge [35], as a relatively low, but detectable level of PspA-specific IgG was measured in the plasma of these mice after nasal immunization. A previous study demonstrated that the induction of PspA IgG1, followed by IgG2b, but not IgG2a, by oral immunization with PspA plus cholera toxin could provide protective immunity in mice [36]. Although the opsonophagocytic activity of PspA IgG1 has not been evaluated, PspA-specific IgG1 primarily induced in plasma of mice nasally administered PspA alone should transfer from plasma to the alveolar space and act as an opsonic antibody at 6 h and 12 h post-infection. Because an influx of neutrophils occurs in the lungs within several hours after bacterial challenge in mice [37], PspA-specific IgG is likely to enhance complement fixation on the surface of bacterium [11]. Thus, opsonophagocytic killing is enhanced by accumulation of neutrophils in the lung parenchyma.

The effect of PspA plus each TLR agonist to reduce bacterial density in the nasopharynx of mice continued for 6 days after pneumococcal challenge, except for PspA plus CpG1826, in a nasopharyngeal colonization model using a serotype 19F EF3030 strain. Similar levels of PspA-specific IgG and IgA in the NW of mice nasally administered PspA plus each TLR agonist cannot explain the lack of bacterial reduction found only in mice nasally administered PspA plus CpG1826 at 6 days post-challenge. Since we previously reported the discrepancy between the level of serotype-specific IgG and opsonophagocytic functions in certain host conditions [38], the functional assays of PspA-specific IgG or IgA induced by PspA plus each TLR agonist may explain a lack of bacterial reduction found only in mice nasally administered PspA plus CpG1826 at 6 days post-infection. Further studies on the time-course of the levels of PspA-specific IgG and IgA after infection also are required.

Our data suggest that the PspA-specific antibody induced in the airway by nasal immunization with PspA plus each TLR agonist reduced the density of bacterial colonization in the upper airways of mice. A previous study also reported that intranasal immunization with PspA plus cholera toxin B subunit (CTB) induced a salivary IgA response to PspA and decreased nasopharyngeal carriage in mice [39]. However, reduction in the nasaopharyngeal carriage was greater following nasal immunization with PsaA, which is an adhesin of pneumococci, than after immunization with PspA plus CTB [5]. Another study also reported that nasal immunization with PspC, which is a paralog of PspA that is also termed CbpA, plus CTB also reduced nasopharyngeal carriage in CBA/N mice at 7 days post-bacterial challenge [40]. In an infant rat model, PspC was shown to act as a cell surface adhesin and to play a major role in nasopharyngeal colonization [41]. PspA, therefore, may also play some role in bacterial adherence in the nasopharynx of mice, although opsonophagocytic killing of *S. pneumoniae* by PspA-specific antibodies cannot be ruled out.

The complement-fixing ability of the IgG2a isotype on the bacterial surfaces is higher than other IgG isotypes [42], and PspA-specific antibodies may mediate the complement-dependent opsonophagocytic killing of *S. pneumoniae*. Therefore, Th1-associated immune responses to PspA are expected to be more efficacious for preventing pneumococcal infections, as previously reported [19,20]. However, the effects on bacterial clearance by nasal immunization with PspA plus Poly(I:C) or CpG1826, which showed a balanced IgG1/IgG2a immune response to PspA, were comparable to those by nasal immunization with PspA plus either Pam3CSK4 or LPS, which showed a predominant induction of PspA-specific IgG1 in the present study. Although the function of PspA-specific IgA remains unknown, it may play a role in bacterial clearance of the airways as PspA-specific IgG play an important role [5,20,21].

Since bacterial products, such as Pam3CSK4 and LPS, are highly toxic to humans, non-toxic TLR4 agonist, such as monophosphoryl lipid A (MPL) or *lpxL1* mutant LPS, may have clinical use as a mucosal adjuvant [13,43]. PolyI:PolyC_{12}U (Ampligen^{R}), which exhibits greatly reduced toxicity and is being used in humans, can act as a mucosal adjuvant similar to Poly(I:C) for the influenza virus [44,45]. A previous study also reported that nasal administration of CpG 1826 did not induce any local or systemic tissue damage or inflammation in mice [46]. Therefore, CpG ODN may be used as a

safe mucosal adjuvant in humans. Because the antibacterial effects of nasal immunization with PspA plus a TLR agonist were evident in the present study, the combination of a safe TLR agonist and PspA has potential clinical application as a nasal pneumococcal vaccine.

The mucosal immune system in respiratory and alimentary tracts regulates immune responses to pathogenic and commensal bacteria, and quiescently maintains the mucosal surface [47]. This review suggests the presence of a multivalent mucosa-associated regulatory system of unique mononuclear cells in the upper airways, including NALT DCs which can induce antigen-specific immune responses, although the phenotype of NALT DC has not been determined. It is conceivable that soluble TLR agonists administered with PspA may have distinct mode of distribution within the mucosa. In particular, efficiency of cellular up-take by, and the resultant activation of, the antigen presenting cells including the DCs for soluble TLR agonists may be quite different from 'endogenous' TLR agonists existing as a compartment of commensal microbes, normally restricted on mucosal surface niche. This distinct delivery mode for antigens may explain, in part, why PspA-specific antibodies were induced in the airway by nasally administered PspA plus each TLR agonist, but not by PspA alone in this study.

Pivotal but complex roles of innate immune receptors in the induction of adaptive immune responses (immunogenicity) have only recently been revealed. In fact, some innate immune receptors such as RIG-I-like receptors (RLRs) and NOD-like receptors (NLRs) have also been shown to be involved in the immunogenicity of vaccines. For example, Poly(I:C), dsRNA ligand for both TLR3 and melanoma-associated gene 5 (MDA5), works as an adjuvant mainly via MDA5, and to lesser extent, TLR3 [48]. On the other hand, although influenza A virus stimulates both TLR7 and RIG-I for innate immune activation, only the TLR7–MyD88 pathway was required for the protective adaptive immune response in mice [49]. Moreover, NLRs that sense microbial and self-derived danger particle (or crystal) molecules in the cytosol [50]. Aluminum hydroxide (alum), which is a widely used adjuvant in human vaccines, stimulates the signaling of NLR pathways for a humoral adaptive immune response [51]. Alum-mediated adjuvant activity, however, remains to be controversial [52]. Taken together, activations of TLR, RLR, or NLR on antigen presenting cells including DCs by microbial stimuli seem to have non-redundant roles in inducing the following adaptive immune responses to co-administered antigens. Presumably, Pam3CSK4 and LPS trigger activation of TLR 2 and 4 on NALT DCs, respectively. Similarly, Poly(I:C) triggers activation of both MDA5 in cytoplasm and TLR3 in endosome, and CpG 1826 activates TLR9 in endosome of NALT DCs. Therefore, nasal administration of each TLR agonist, in combination with PspA, works as potent mucosal adjuvants for induction of PspA-specific antibodies in the airways.

In conclusion, the induction of PspA-specific IgA and IgG was associated with enhanced bacterial clearance of pneumococcal strains with different serotypes from the nasopharynx and lungs of mice nasally administered PspA plus each TLR agonist. Despite the difference in the Th1- and Th2-associated IgG isotype responses among TLR agonists, bacterial clearances from the lungs at 3 h post-infection in a pneumonia model, and from nasopharynx in a colonization model at 1 day post-infection, were equivalent in mice after nasal immunization with PspA plus each TLR agonist.

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キノロン耐性B群連鎖球菌の臨床的・疫学的特徴と耐性機序の解析

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Key words：　GBS，キノロン耐性，血清型

はじめに

B群β溶血性連鎖球菌(Group B *Streptococcus*：GBS) は，新生児の肺炎，敗血症および髄膜炎などの起因菌であり，さらに成人，特に高齢者において重篤な感染症を引き起こす病原体である[1]．今回我々は当院におけるキノロン耐性 GBS 検出例を解析し，疫学的・臨床的特徴を明らかにするとともに，耐性機構の解析を行い，発生・伝播経路に関する考察を行った．

I　材料および方法

被検菌株は1999年1月～2005年8月の間に，当院検査部に起因菌検索を目的に提出された各種臨床材料由来 GBS821 株を対象とした．菌種同定は，血液寒天培地でβ溶血性を示したグラム陽性球菌でカタラーゼ陰性，レンサ球菌同定用ラテックス試薬 (streptex) でB群に凝集した株をGBSとした．薬剤感受性試験は微量液体希釈法で MIC を測定した．Ofloxacin (OFLX) と Levofloxacin(LVFX)の MIC が8μg/ml以上の株をR（耐性）と判定した．

さらに2005年10月から2006年12月までに提出された臨床材料(膣分泌物34件，尿11件，呼吸器由来検体29件，血液・膿6件)から分離されたGBS80株について，疫学的解析を行った．血清型はB群溶血性レンサ球菌型別用免疫血清「生研」(デンカ生検) の抗血清を用いて判定した．また，キノロン耐性関連領域にある *gyrA* 遺伝子と *parC* 遺伝子について表1に示すprimerを設定し，PCR反応を行い得られた増幅産物について遺伝子配列を決定し，変異の有無を調べた．

II　結　果

1. GBSのキノロン耐性率年次推移

GBS のキノロン耐性率年次推移を図 1 に示す．1999年から2002年にかけて耐性率6%前後を推移していたが，2003年には15%を超え，2005年には26.3%に達した．また，当院でのLVFX購入量も，キノロン耐性率と平行して上昇し，2002 年に比べ2005年には約20%増加していた．

表1　Primerの塩基配列

Primer		Sequence(5' →3')
GyrA	Upper primer	GAC AAG TGA AAT GAA AAC GAG
	Lower primer	CGC TCC ATT GAC TAA TAA ATT AGG
ParC	Upper primer	TGG AAA AAT AAT ACT AAG CCA CTC
	Lower primer	TCA TCA AAA TTC CAC GCA AAG

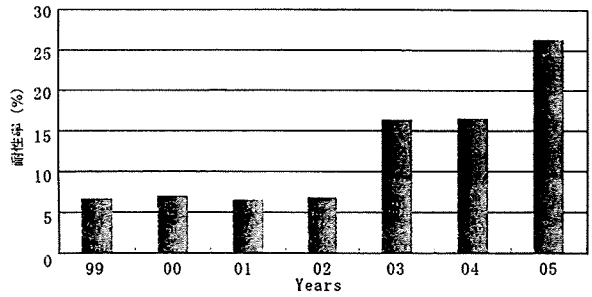

図1　GBSのキノロン耐性率年次推移

2. GBS の MIC 分布

GBS のキノロン薬に対する MIC 分布を図 2 に示す．OFLX のレンジは 0.125～≧16 μg/ml でピークは 2 μg/ml，LVFX のレンジは 0.5～≧8 μg/ml でピークは 1 μg/ml であった．MIC が 8 μg/ml 以上を示したキノロン耐性株は 98 株(11.9%)であった．

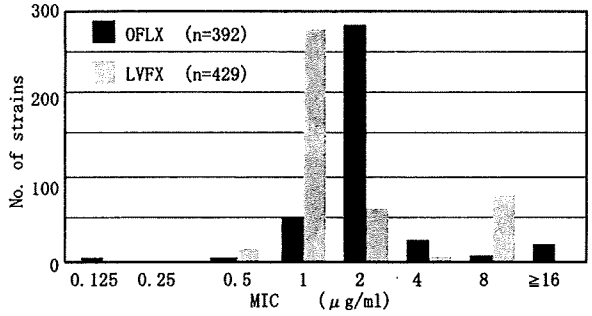

図2　GBSのキノロン薬に対するMIC分布

3. 年齢・男女別分離頻度

年齢・男女別キノロン耐性 GBS の分離頻度を図 3 に示す．男女比は男 31 名(42.5%)，女 42 名（57.5%）であった．年齢別では，新生児 11 名（14%）と，60 歳以上 33 名（46%）からの分離頻度が多く，全体の半数近くを占めた．高齢者では，基礎疾患（糖尿病，尿路疾患，悪性腫瘍など）を持ち，キノロン薬の長期・頻回投与を受けていた．

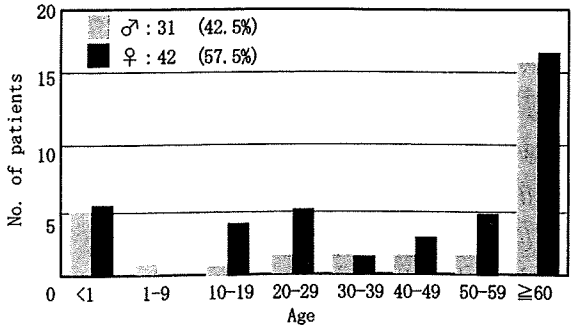

図3　年齢・男女別キノロン耐性GBSの分離頻度

4. 新生児と母親から検出した GBS

通常，小児や妊婦にはキノロン薬は投与されない．しかし，新生児の臨床的背景をみると，11 例中 7 例で GBS 感染症を認めた．キノロン耐性を示した新生児の母親の約半数から GBS を検出した．また，これらの家庭環境を調べたところ，67%が 3 世代同居であった．

5. 血清型とキノロン耐性

GBS80 株の血清型を図 4 に示す．Ⅳと 7271 型を除く全ての型に分かれた．特に多かったのは Ib 型 32.5%，次いでⅤ型 16.3%，NT6，Ia 型 11.3%であった．血清型陰性は 15%であった．

次に材料別血清型を図 5 に示す．膣分泌物では Ib，Ⅴ型，呼吸器検体では Ia，Ib 型が優位であった．

キノロン耐性 GBS は 20 株分離され，すべての検査材料から分離された．この内，19 株 95%が血清型 Ib 型であった．

6. キノロン耐性遺伝子

キノロン耐性遺伝子のアミノ酸変異を表 2 に示す．全ての耐性株において *gyrA* 遺伝子 81 番アミノ酸がセリン(TCA)からイソロイシン（TTA）に，*parC* 遺伝子 79 番アミノ酸がセリン（TCC）からフェニルアラニン（TTC）に，81 番アミノ酸がイソロイシン（ATC）からイソロイシン（ATT）にアミノ酸変異していた．

表2　キノロン耐性遺伝子のアミノ酸変異

	gyrA (n=36)	*parC* (n=19)	
	81番ｱﾐﾉ酸	79番ｱﾐﾉ酸	81番ｱﾐﾉ酸
感受性株	Ser (TCA)	Ser (TCC)	Ile (ATC)
耐性株	Leu (TTA)	Phe (TTC)	Ile (ATT)

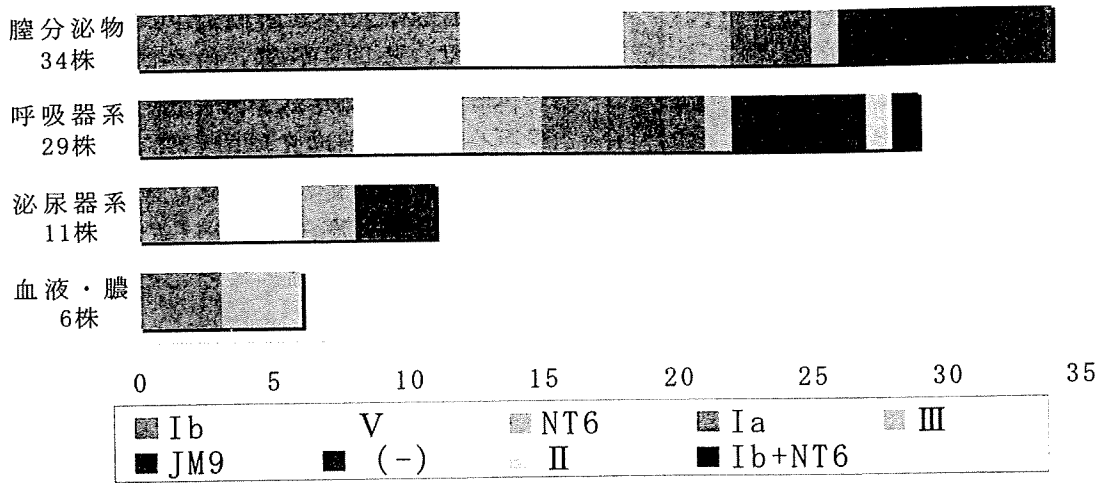

図5　材料別血清型（n=80）

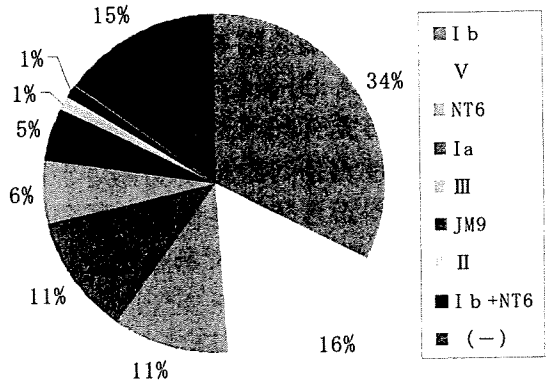

図4　GBSの血清型（n=80）

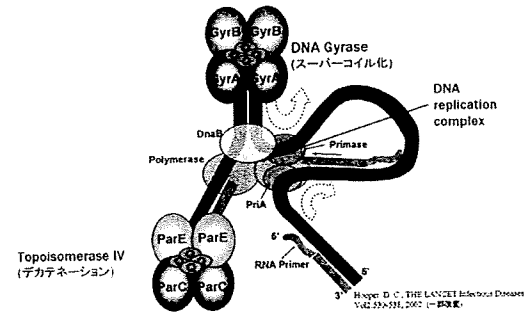

図6　β溶血性レンサ球菌におけるキノロン系薬剤耐性機構[1]

7. キノロン耐性GBS検出患者の臨床背景

母親と新生児では7例中5例，泌尿器系（膀胱癌やリンパ腫を基礎に持つ高齢者）では4例中2例，血液・膿（髄膜炎，敗血症，腸腰筋膿瘍の高齢者）では3例全てキノロン耐性 GBS が検出された．キノロン投与歴のない母親と新生児では，71%の高い割合でキノロン耐性GBSが検出された．

Ⅲ　考　察

1. 発生・伝播経路

キノロン耐性 GBS の伝播経路として，尿路疾患・血液疾患等の基礎疾患を持つ高齢者のキノロン薬の長期・頻回投与に伴い，キノロン耐性株が選択的に生き残ったと考えられる．通常，新生児や妊婦にはキノロン薬は投与されない．だが，母児の両方からキノロン耐性 GBS が検出される背景には，高齢者との同居等による接触感染によって伝播されたと考えられる．

さらに，海外では高率に分離される血清型株が，同一あるいは近縁のクローンであるという報告がされている．1998 年，Elliott らは，当時アメリカで急激に増加した血清型Ⅴ型の GBS について遺伝子解析をした結果，大部分が近縁のクローンであったと報告している[2]．

2. 薬剤耐性機序

キノロン系抗菌薬の標的酵素は DNA ジャイレース（gyrase）と DNA トポイソメラーゼ IV（topo IV）である．DNA ジャイレースは，DNA の複製，修復，転写，組み換えに関与する酵素であり，*gyrA*, *gyrB* 遺伝子に支配されている．Topo IVは DNA 複製後の二本鎖 DNA の分離に関与するといわれ，細菌の生存に必須な酵素であり，*parC*, *parE* 遺伝子に支配されている．

染色体上の *gyrA* (*parC*) と *gyrB* (*parE*) 遺伝子の変異により，キノロン薬耐性化決定領域とされる部位のアミノ酸に変異が起きる．また，内膜上に存在する薬物排出ポンプの亢進，標的酵素へのキノロンの結合を阻害するプラスミド性のタンパクの存在が考えられる[3]．

3. PK/PD

近年，抗菌薬の効果を最大限に発揮するために，薬物動態（pharmacokinetics：PK）と薬力学（pharmacodynamics：PD）を組み合わせ，最適かつ，耐性菌の出現を抑制するような投与法の試みがなされている[5]．

PK/PD パラメーターからみると，キノロン系抗菌薬は濃度依存性抗菌薬であり，1日1回投与で血中濃度を上げたほうが，効果は期待できる．米国では 500mg または 750mg を1日1回経口投与しているのに対し，日本では，1回 200mg を1日3回経口投与している．ガイドラインでみる限り，日本の使い方では1回量が少ない傾向が認められる．

しかし，日本人が欧米人と同量のキノロン薬を服用すれば，小柄な日本人が副作用の発現率は高くなる．現実的には，日本人には日本の薬用量できちんと治療できるケースもあるとされる[6]．

キノロン薬は，PK/PD により適正に使用すれば有効性を確保できると同時に，耐性菌の出現を抑制することも可能である．

結語

キノロン耐性 GBS は，近年増加傾向にあり，その血清型の多くは Ib で，侵襲性感染症を発症している．これは同一または近縁のクローンが市中で広まっていると推測される．今後，キノロン耐性 GBS の Ib 型について分子疫学的解析を行い，感染経路の解明が必要とされる．

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血液培養から分離された肺炎球菌の莢膜血清型と薬剤感受性および臨床背景の検討

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及川　信次[1)]　吉田　敦[1-3)]　奥住　捷子[2)]　家入　蒼生夫[1,3)]

Key words：肺炎球菌，莢膜血清型，ワクチン

はじめに

肺炎球菌(*Streptococcus pneumoniae*)はヒトの上気道に生息し，小児の中耳炎や髄膜炎、高齢者肺炎などの呼吸器感染症を引き起こす重要な起炎菌である[1,2]．

近年、ペニシリン耐性株による難治症例や劇症型症例が多数報告され問題となっている．

今回我々は、当院において過去５年間に血液培養から分離された肺炎球菌の莢膜血清型と薬剤感受性および臨床背景についてretrospective に考察を加えたので報告する．

I　材料，方法および結果

材料は 2003 年 1 月から 2007 年 3 月までの期間，当院検査部で血液培養から肺炎球菌が分離された 25 症例を対象とした．そのうち莢膜血清型が検索できたのは 16 例，臨床背景の詳細が確認できたのは 20 例であった．

薬剤感受性試験は日本化学療法学会標準法に準拠した微量液体希釈法で PenicillinG(PCG)，Ampicillin(ABPC)，Cefazolin(CEZ)，Cefotiam(CTM)，Cefotaxime(CTX)，Cefditren(CDTR)，Fromoxef(FMOX)，Panipenem(PAPM)，Erythromycin(EM)，Clindamycin(CLDM)，Levofloxacin(LVFX)，および Vancomycin (VCM)の MIC を測定した．

莢膜血清型は肺炎球菌莢膜血清型別用免疫血清「生研」(デンカ生研)の抗血清を用いて判定した．

死亡群と生存群の検査値(WBC，CRP，Creatinine)の比較は Mann-Whitney's Utest を用いて危険率 5%で有意差検定をおこなった．

1．年齢別・男女別分離頻度

年齢別・男女別の分離頻度を図 1 に示す．肺炎球菌が分離された 25 症例中，男性が 18 名と多く 72%を占めた．

年齢別では 9 か月から 90 歳と幅広く分布し，平均 53.8 歳であった．70 歳代が最も多く 8 例，次いで 50 歳代が 6 例，10 歳未満は 4 例であった．

2．肺炎球菌の莢膜血清型

肺炎球菌の莢膜血清型の結果を図 2 に示す．血清型は 8 種類に分類され，23 型が最も多く 4 株，次いで 3 型，12 型が 3 株，4 型が 2 株であった．

今回、当院で分離された肺炎球菌の莢膜血清型 8 種類はすべて 23 価ワクチンに含まれていた．また，ペニシリン耐性株は 23 型と 6 型に認めた．

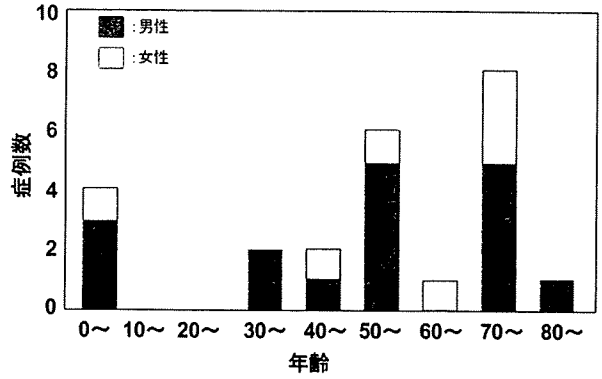

図1 年齢別・男女別患者数

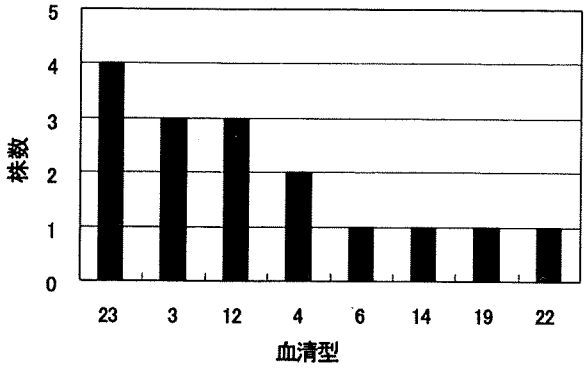

図2 肺炎球菌の莢膜血清型(n=16)

3. 薬剤感受性成績

薬剤感受性成績を表1に示す.

PCGのMIC$_{90}$は1μg/mlで感受性率は72%であった. ペニシリン耐性を示した28%の内訳は, PRSP1株, PISP6株であった. 他のペニシリン系, セフェム系薬剤は80%以上の感受性率を示し, 12薬剤の中で最も低いMIC$_{90}$はPAPMの0.06μg/mlであった. また, PAPM, VCMの感受性率は100%であった. LVFXは1株のみ耐性を示したが, 感受性率は96%と高い割合であった.

表1 薬剤感受性成績(n=25)

薬剤	MIC$_{90}$	感受性率	薬剤	MIC$_{90}$	感受性率
PCG	1	72.0%	FMOX	1	84.0%
ABPC	2	96.0%	PAPM	0.06	100%
CEZ	2	84.0%	EM	>4	32.0%
CTM	4	80.0%	CLDM	>4	56.0%
CTX	0.5	92.0%	LVFX	2	96.0%
CDTR	0.25	100%	VCM	0.5	100%

4. 投与された抗菌薬

臨床背景の詳細が確認できた20例の投与された抗菌薬を図3に示す. ほぼすべての症例で2系統以上の抗菌薬が投与されていた. 重複例が多く, カルバペネム系薬剤が14例と最も多かった. 次いでペニシリン系7例, テトラサイクリン系6例, セフェム系5例であった. カルバペネム系薬剤の内訳はMEPMが8例, PAPMが4例, IPM, BIPMがそれぞれ1例であった.

5. 肺炎・DICの有無

肺炎球菌性肺炎は20例中13例65%に認められた. 入院時すでに呼吸困難を訴える症例が9例, 両側性の肺炎像を呈する症例が5例, そのうち3例は誤嚥性を伴っていた. DICは20例中8例40%に認められた.

6. 基礎疾患と死亡例の関係

基礎疾患と死亡例の関係を図4に示す. 臨床症例20例のうち, 基礎疾患は18例85%に認められた. 最も多かった基礎疾患はCOPD・気管支喘息などの呼吸器疾患で7例, 次いで肝疾患と悪性腫瘍の5例であった. 肝疾患では5例すべて, また基礎疾患のない症例でも1例死亡していた.

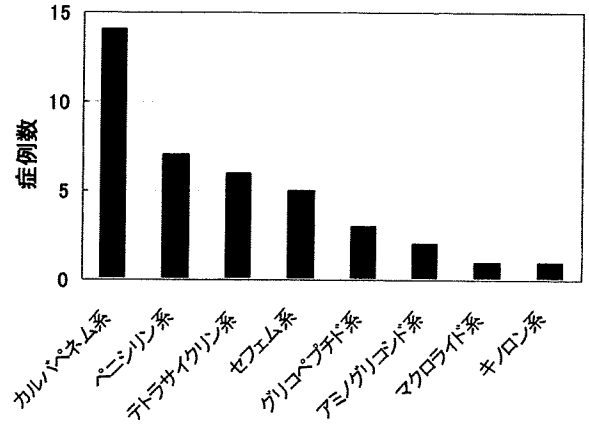

図3 投与された抗菌薬

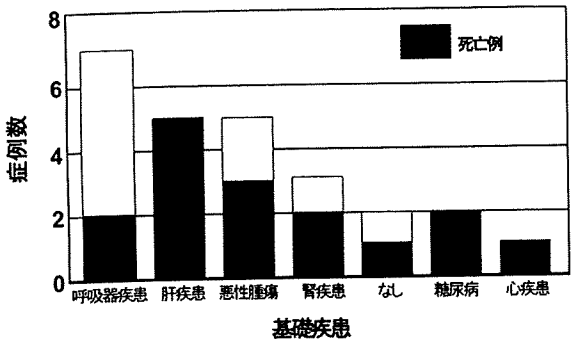

図4 基礎疾患と死亡例の関係

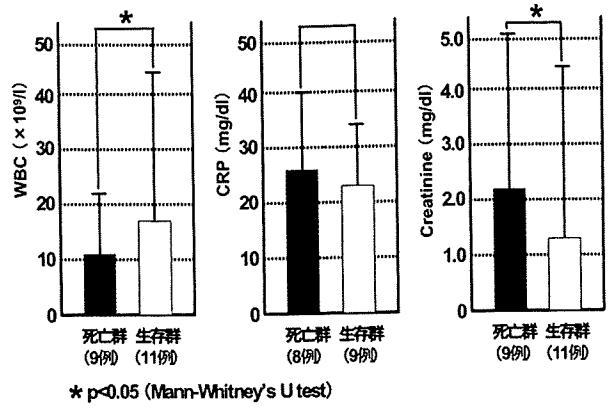

図5 死亡群と生存群の検査値比較

7．死亡群と生存群の検査値比較

死亡群と生存群の検査値の比較を図5に示す．データは血液培養が提出された日と同日の結果である．白血球は死亡群が有意に低く，白血球数 2.0×10^9/l 以下の症例が2例あった．CRP は死亡群の平均値が高い結果となったが，生存群と有意な差は認めなかった．Creatinine は死亡群が有意に高い結果となった．

8．転帰と死亡に至る時間

転帰は 20 例中，死亡が 9 例 45%，軽快が 10 例 50%，髄膜炎から運動機能と言語機能に障害が残った後遺症が 1 例 5%であった．死亡 9 例のうち，本菌による敗血症が死因と考えられた症例は 6 例あり，そのうち 5 例は血液培養採取後 72 時間以内に死亡という急激な転帰をとっていた．

9．死亡例の詳細

死亡例の詳細を表2に示す．男性で肝臓に関わる基礎疾患を有し，腎機能の低下をきたしている症例が死亡するケースが多かった．また多くは気管内挿管による人口呼吸管理がされていたが，多臓器不全，ショック状態，DIC などを伴い死亡に至っていた．莢膜血清型は様々で死亡との関連性はなかった．

表2 死亡例の詳細

症例	年齢・性別	基礎疾患	Creatinine (mg/dl)	莢膜血清型	臨床背景
1	44・M	無し	0.70	19型	収縮性心外膜炎に心室性頻脈を合併
2	79・M	肝硬変・肝癌	1.41	23型	気管内挿管，多臓器不全
3	78・M	胃癌・水腎症	1.36	6型	肺炎は改善，原疾患にて死亡
4	56・M	肝硬変・肝癌	4.12	4型	腎不全，DIC，ショック状態
5	80・M	糖尿病・心不全	1.48	3型	気管内挿管，ショック状態
6	47・M	肝硬変・糖尿病	2.24	14型	気管内挿管，ショック状態，DIC，肝不全
7	38・M	気管支喘息	0.94	3型	気管内挿管，多臓器不全，DIC，透析
8	54・M	アルコール性肝障害	5.31	23型	気管内挿管，多臓器不全，DIC
9	73・F	慢性C型肝炎	1.46	12型	多臓器不全，DIC，両側足壊死

Ⅱ　考察

今回，肺炎球菌感染症に対して選択された抗菌薬はカルバペネム系薬剤が圧倒的に多かった．これはペニシリン耐性株に対しても現有の抗菌薬のなかで最も優れた抗菌力を有しているためであると思われる[3]．しかし，ペニシリン感受性であることが判明した場合は速やかに狭域スペクトルの抗菌薬に変更する必要がある．

高齢者や基礎疾患を有する患者に発症した劇症型の肺炎球菌性肺炎では適切な抗菌化学療法を行なっても予後不良であるため，肺炎球菌ワクチンによる感染予防が重要であると示唆された．

肺炎球菌ワクチン(ポリサッカライドワクチン)とは，肺炎球菌の莢膜多糖体成分でできた成分ワクチンである[4]．肺炎球菌は莢膜を有し，莢膜多糖体は 90 種類の血清型に分けられるが，そのうち 23 種類の血清型の莢膜多糖体を含有している(含有血清型：1，2，3，4，5，6B，7F，8，9N，9V，10A，11A，12F，14，15B，17F，18C，19A，19F，20，22F，23F，33F)[5]．これらの血清型は，疫学的成績と血清型間の交差免疫反応を基に決定されたものであり，わが国で 1980~83 年に行なわれたサーベイランスでは臨床分離株の 79%をカバーしていた[6]．

1997 年の Centers for Disease Control and Prevention(CDC)の勧告では，65 歳以上の高齢者，2 歳以上の慢性心疾患・呼吸器疾患・糖尿病・脾臓摘出者には有効性が疫学的，臨床的に確立しているため積極的なワクチン接種が強く勧められている[7]．

本邦でも 1988 年に 23 価肺炎球菌ワクチン(ニューモバックス)が認可されたが，65 歳以上の高齢者の接種率はまだ 4%に満たない．米国における接種率の 70%と大きな隔たりがある．その背景には感染予防のための健康保険適応がなく脾臓摘出患者，脾臓不全患者のみが保健適応となっていることや自費で 6000 円から 9000 円の負担があること，さらに接種は 1 回限定という要因があると考えられる．

今後，高齢者人口は増加し続ける見込みであり，これまで以上に安全で有効性の高いワクチンを簡便に，しかも安価で投与できるように高齢者のワクチン戦略を推進していく必要がある．

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グラム陽性球菌

Streptococcus suis, *Streptococcus dysgalactiae*

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要旨　近年本邦では *Streptococcus suis*（豚レンサ球菌）や *Streptococcus dysgalactiae*（G群，C群レンサ球菌）による侵襲性感染症の報告が増加している．前者では職業関連感染として関心が高くなっており，後者では菌側の病原因子について研究が進んでいる．今後これらemerging infectionに関するサーベイランスシステムの強化，臨床像の多施設解析，ならびに重点的な基礎研究が求められる．

はじめに

レンサ球菌（*Streptococcus*）では，近年，*Streptococcus suis* や *Streptococcus dysgalactiae* の感染例の報告が増加し，動物の感染症と思われていた，ないしはほとんど病原性を発揮しないと思われていたレンサ球菌が，侵襲性の重症感染症を起こすことが明らかになってきた．本稿ではこの2菌についてemerging infectionの側面に注目し，検査上・臨床上の特徴を解説した．

■*S. suis*（豚レンサ球菌）感染症

S. suis は元来豚や牛，馬が保菌するレンサ球菌であり，本邦における豚での保菌率は30%以上に上る．全く無症状で経過する例がほとんどであるが，一部では髄膜炎や敗血症，肺炎，心内膜炎などを起こす．保菌しているこれらの家畜やその肉に直接接触することで人への感染が生じる．莢膜多糖体の違いにより35の血清型に分けられるが，豚でも人でも最も多い血清型は2型である．ランスフィールド分類ではR，S，T型に属する．

1. 臨床検査上の注意

ヒツジ血液寒天培地上では通常α溶血を示し（ウマ血液寒天ではβ溶血），灰色で小さな（0.5～1mm）コロニーを形成する．ムコイドを産生することもある．グラム染色では，しばしば陽性双球菌としてみえる（単一ないしは短い鎖としてみえることもある）．同定には以下の4つの性状が参考になる．①VP（Voges-Proskauer）反応：陰性，②サルシンからの糖産生（+），③トレハロースからの糖産生（+），④6.5%NaCl中での発育なし[1]．しかしながら生化学的性状は多様なので，同定キットや自動機械のみでなく，血清型判定やPCR法による遺伝子増幅（16Sリボゾーマル RNA や菌種特異的領域を対象とする）を併用するのがよい．なお検査材料から直接 *S. suis* の遺伝子を検出した報告はほとんどない．

一方でELISAや蛍光抗体法を用いた抗体検査も開発されている．

表1　本邦における *S. suis* 感染症の報告

症例	報告年	地域	年齢・性別	分離箇所	血清型	臨床像	職業・感染の背景
1	1994	不明	50歳代M	血液		敗血症で死亡	飲食店従業員．豚の内臓を串に刺す時，誤って手に刺した
2	2003	長崎	58M	髄液	2	髄膜炎．難聴が残った	養豚業
3	2003	新潟	47M	髄液	2	髄膜炎，硬膜外膿瘍．難聴が残った	食肉加工業者．素手で豚を解体
4	2004	新潟	47F	髄液		髄膜炎，多関節痛．難聴が残った	症例3と同じ職場．素手で豚を解体
5	2006	新潟	56M	髄液		髄膜炎，敗血症性ショック，DIC，難聴，骨髄膿瘍	豚解体業．素手で作業
6	2006	石川	57F	血液		敗血症，髄膜炎，眼内炎．難聴が残った	串焼き屋店員．指に外傷
7	2006	不明	56F	血液		敗血症，DIC，関節炎，眼内炎	豚の内臓（生）に触れた
8	2007	宮崎	63M	血液		敗血症性ショック，紫斑	農家
9	2008	千葉	50M	血液	2	感染性心内膜炎(脊椎炎，肺塞栓合併)	酪農業．哺乳時に子牛に咬まれる

2. 疫学的特徴

人での報告は，1968年のデンマークの例が最初であるという．以後現在までにヨーロッパ，アジアから700例以上が報告された．これらの多くは散発例であったが，98年，99年には中国江蘇省で，2005年には同四川省で集団発生が生じた．特に四川省の例は大規模で，204人が感染し，38人が死亡している．一方，本邦では1994年以降，現在まで9例の報告がある（表1）[2~4]．

人への感染は，主に豚との直接接触や，豚肉の加工による．豚の口内や鼻に手を入れたり，手指に傷がある例も多い（表1）．このため養豚業者，屠殺業者，食肉加工業者，獣医での罹患のリスクが高く（1,500倍），職業関連感染の一面も持っている．

3. 臨床的特徴

髄膜炎を生じることが最も多く，次いで敗血症の頻度が高い（表2）．髄膜炎は，高熱，頭痛，悪寒，嘔吐，めまいといった症状で始まり，項部強直や意識障害をきたすが，*S. suis* では難聴，点状出血，関節痛，顔面神経麻痺，強い筋肉痛，斑状出血，発疹，横紋筋融解症がみられたとの報告がある[1,5]．一方，敗血症では，しばしば敗血症性ショックを生じ，皮下出血やDIC，多臓器不全を高率に合併する（表3）．

4. 診断，治療と予後

症状・所見から診断するのは難しくないが，病歴（職業・曝露）や微生物検査から *S. suis* 感染症と絞り込めるかどうかが鍵になる．微生物検査上誤りやすいのは viridans group streptococci であり，これが血液と髄液からともに検出された場合には，病歴の確認と菌種の同定を行う必要がある．抗菌薬として最も有効なのは penicillin G（PCG）で，中等症までであれば比較的有効だが，重症になるとPCGの増量と他の抗菌薬との併用が必要になる．敗血症性ショックになると抗菌薬では限界の場合もある．髄膜炎での死亡率は7%といわれ，難聴を残す頻度が高い（54～64%）．

5. なぜ最近報告例が目立つのか，病原性の変化はあるのか？

S. suis の宿主への侵入と感染症の発症機序については，不明の部分が多い．病原性の発揮に重要なものとして，莢膜やストレプトリジンO類似の溶血毒がある．さらに宿主の細胞のフィブロネクチン，アルブミン，免疫グロブリンG受容体に結合して，免疫による攻撃を逃れるともいわれている．現在，菌の病原因子の変化についてゲノムレベルで解析が行われつつあるが，主に関心の高まり，診断能力の向上が，報告が増えている要因として考えられている．