an exposed virulence factor found in virtually all pneumococcal strains [12,13]. Anti-PspA antibodies overcome the anticomplement effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [14,15]. Serum from humans immunized with PspA can passively protect mice against challenge with various pneumococcal strains [16]. Importantly, a recent study confirmed that the rabbit antibodies to PspA could mediate killing in the modified opsonophagocytosis killing assay [17].

PspA is composed of five domains: (i) a signal peptide, (ii) an α-helical highly charged (N-terminal) domain, (iii) a proline-rich region domain, (iv) a choline-binding domain, and (v) a short hydrophobic tail [18,19]. The α-helical domain of PspA has an antiparallel coiled-coil motif and is considered to be the most exposed part of the molecule [20]. The α-helical domain binds to protective monoclonal antibodies and inhibits killing of pneumococci by at least two host cationic peptides [21,22]. The proline-rich domain is composed of many repetitive sequences shared by other proline-rich domains making its inclusion important for achieving broad protection [23].

PspA proteins have been grouped into three families encompassing six different clades based on the C-terminal 100 amino acids of the α-helical region [24]. Family 1 comprises clades 1 and 2; family 2 comprises clades 3, 4 and 5; and family 3 comprises clade 6 [22,24]. Pneumococcal strains expressing family 1 or 2 PspA proteins constitute >96% of clinical isolates from patients with IPD [6,13,25]. Although different PspA proteins induce antibodies with different degrees of cross-reactivity in vitro and cross-protection of mice [26,27], our previous studies demonstrated that no single PspA construct can elicit complete protection against challenge by strains with all PspA clades and families [28]. To accommodate this variability, it was proposed that a combination of two PspA antigens, one from PspA family 1 and one from PspA family 2, should elicit protection against the vast majority of pneumococcal strains [29–31]. Thus, it is important to determine which PspA fragments show the broadest cross-reactivity. In this study, we prepared fusion proteins of three pairs of PspA molecules, and determined which provided the broadest cross-reaction with clinical isolates of *S. pneumoniae*.

2. Materials and methods

2.1. Pneumococcal strains

Six laboratory strains (all originally from patients), including BG9739 (serotype 4, PspA clade 1), D39 (serotype 2, PspA clade 2), WU2 (serotype 3, PspA clade 2), TIGR4 (serotype 3, PspA clade 3), EF5668 (serotype 4, PspA clade 4), and ATCC 6303 (serotype 3, PspA clade 5) were used to construct the fusion PspA proteins. These laboratory strains and a recent clinical isolate, KK1162 (serotype 3, PspA clade 4), were used for bacterial challenge. Sixty-eight clinical isolates, including KK1162 strain, from Japanese adult patients with IPD were also used [32]. These isolates were serotyped using agglutination assay, and their PspA clades were determined using a method published previously [32,33].

2.2. Construction of fusion PspA fragments

Our previous study demonstrated a significant protection against sepsis caused by WU2 strain (PspA clade 1) by immunization with full-length BG9739 derived PspA (clade 1) but only a weak protection against homologous challenge with BG9739 [28]. Therefore, we excluded PspA clade 1 derived from BG9739 strain from the fusion PspA proteins. In this study, we prepared the fusion proteins from three pairs of PspA clade 2 from family 1 and PspA clades 3, 4 and 5 from family 2. All cloning procedures were performed with *Escherichia coli* DH5α grown in Luria–Bertani medium (Sigma-Aldrich, St. Louis, MO) supplemented with kanamycin (30 μg/ml). DNA fragments encoding portions of the N-terminal regions (containing the α-helix domain and proline-rich region) of PspA clades 2 and 3 were amplified by PCR using strains D39 and TIGR4. The primers used in this procedure are available in Appendix 1. The resulting PCR products were digested with *Nde*I and *Eco*RI, and were ligated to the pET28a (+) vector (Novagen, Madison, WI), and the sequences were confirmed by DNA sequencing. The pET28a–PspA constructs digested with *Eco*RI and *Xho*I, and the resulting fragments, which encoded portions of the N-terminal regions of PspA clades 4, 5, or 2 were amplified by PCR using strains EF5668 (Accession no. U89711), ATCC6303 (Accession no. AF071820), or WU2 (Accession no. AF071814), respectively, and were ligated to the linearized vector. The fusion PspA proteins were obtained with primers that allowed the removal of the signal sequence. The fusion PspA2+4 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain (Accession no. AF071814) with the 5′ terminus of PspA clade 4 of EF5668 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA2+5 was constructed by fusing the 3′ terminus of PspA clade 2 of D39 strain with the 5′ terminus of PspA clade 5 of ATCC6303 strain, through the *Eco*RI ligated to pET28a–6 × His. The fusion PspA3+2 was constructed by fusing the 3′ terminus of PspA clade 3 of TIGR4 strain (Accession no. AE005672.3) with the 5′ terminus of PspA clade 2 of WU2 strain, through the *Eco*RI ligated to pET28a–6 × His.

2.3. PspA expression and purification

Competent *E. coli* BL21 (DE3) cells were transformed with pET28a (+) vectors containing the fusion PspA or the single PspA constructs. The recombinant proteins were purified and stored as described elsewhere [34].

2.4. Immunization of mice

Female C57/BL6j mice (6–8 weeks old) were purchased from CLA Japan. Mice were immunized subcutaneously three times at 7-days intervals with 0.1 μg of recombinant fusion PspA derivatives in lipopolysaccharide-free phosphate-buffered saline (PBS) (Sigma) in combination with 2.5 μg of TLR9 ligand adjuvants K3 CpG oligonucleotides (CpG ODNs) and 5 μg of aluminum hydroxide gel (AHG) (A gift from The Research Foundation for Microbial Diseases of Osaka University) or CpG ODNs alone (final volume of 200 μl per mouse). A subcutaneous route of immunization was chosen because our preliminary study demonstrated the levels of PspA-specific IgG in mice subcutaneously immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs were significantly higher than those in mice nasally immunized with 0.1 μg of PspA plus 2.5 μg of CpG ODNs (data not shown). CpG ODNs were prepared as described previously [35]. Because the PspA clade-specific IgG levels tended to be higher in mice immunized with each PspA fusion protein with CpG ODNs plus AHG than in those immunized with PspA fusion protein with CpG ODNs alone (see Appendix 2), we used the CpG ODNs plus AHG (define as the double adjuvants), for the immunization of mice with PspA fusion proteins in this study. These double adjuvants were safe in nonhuman primate models, and were applicable to humans [36]. Serum was collected from mice by retro-orbital bleeding 1 week after the third immunization. All animal experiments were approved by the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University, Japan (Permit Number: Biken-AP-H23-05-0).

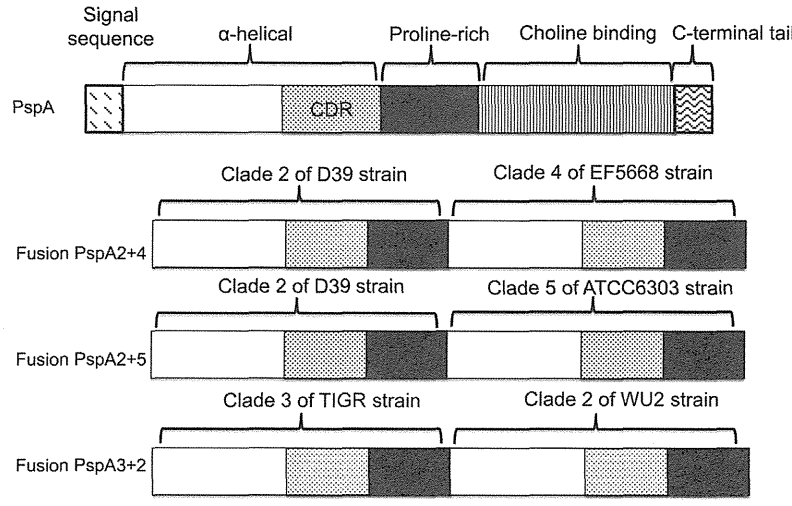

Fig. 1. Schematic diagram of PspA and three fusion PspA proteins. The entire PspA molecule containing the N-terminal α-helical domain, which contains the clade-defining region (CDR), the proline-rich region, the choline-binding domain, and the C-terminal tail (upper column). Each recombinant fusion protein is shown with its different composition (three lower rows).

2.5. Binding of PspA-specific IgG to pneumococcal strains by flow cytometry

Five pneumococcal strains for bacterial challenge and 68 clinical isolates were grown in blood agar plates overnight and then subcultured again on blood agar plates for 4–5 h. The bacteria were collected in PBS, harvested by centrifugation, and washed once with PBS. Ninety microliters of the bacterial suspension at a concentration of 1×10^8 colony-forming units (cfu)/ml in PBS was incubated with 10 μl of mouse antisera for 30 min at 37 °C. After incubation, the suspension was washed once with PBS, resuspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:100), and incubated for 30 min on ice. After the incubation, the bacterial suspension was washed twice with PBS and suspended in 500 μl of 1% formaldehyde. The samples were kept on ice in the dark until analyzed by flow cytometry using a BD FACSCalibur™ with CellQuest software (BD Sciences, San Jose, CA), and the percentage of fluorescent bacteria (>1 fluorescence intensity unit) in each group was determined. Sera from mice immunized with double adjuvants only were used as the negative controls.

2.6. Protection against pneumococcal challenge

The mice immunized with the PspA fusion protein plus double adjuvants were challenged intranasally with 2×10^7 cfu of strain BG9739 (clade 1), 2×10^7 cfu of strain WU2 (clade 2), 5×10^6 cfu of strain TIGR4, 2×10^7 cfu of strain KK1162 (clade 4), or 5×10^5 cfu of strain ATCC6303 (clade 5). Bacterial challenges were performed 2 weeks after the final immunization. Mortality was monitored for 2 weeks following pneumococcal challenge. The mice immunized with double adjuvants alone were used as a control.

2.7. Statistical analysis

Analysis of variance followed by an unpaired Mann–Whitney *U* test was used to evaluate differences in antibody titer. The percent binding by immune sera to each pneumococcal strain was compared by paired *t*-test. Survival rates were analyzed by the Kaplan–Meier log-rank test. All analyses were performed using GraphPad Prism Software (GraphPad software, La Jolla, CA). *p* values <0.05 were considered significant.

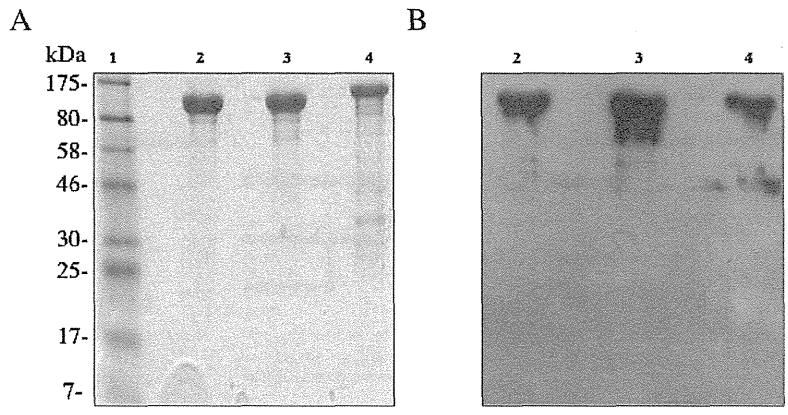

Fig. 2. Characterization of three purified fusion PspA proteins by SDS–PAGE (A) and Western blot analysis (B). The proteins were subjected to SDS–PAGE and detected by direct staining with Coomassie brilliant blue. Lane 1, standard molecular weight markers; lane 2, PspA2+4; lane 3, PspA2+5; 4, lane PspA3+2. The values on the left are molecular sizes in kilodaltons. Mouse antiserum against PspA recombinant protein (clade 2) was used for Western blot analysis. Lane 2, PspA2+4; lane 3, PspA2+5; lane 4, PspA3+2.

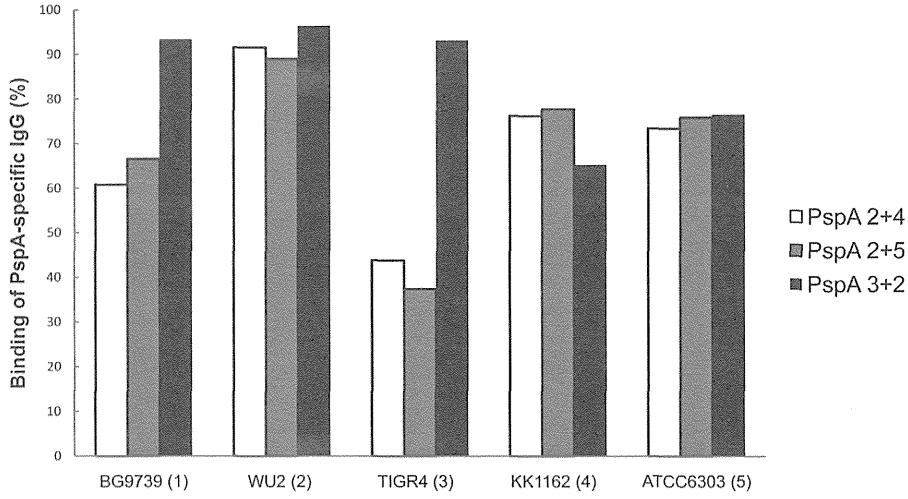

Fig. 3. The binding of PspA-specific IgG by antisera with PspA fusion proteins to the challenge strains with different clades. The mean percentages of fluorescent bacteria positive for IgG binding by antisera from mice immunized with PspA2+4, PspA2+5, or PspA3+2 in combination CpG ODNs plus AHG (double adjuvants) are shown for five pneumococcal strains with PspA clades 1–5 used in the challenge experiments. The numbers in parentheses represents the PspA clade.

3. Results

A schematic diagram of PspA and the three PspA fusion proteins constructed from PspA families 1 and 2 are shown in Fig. 1. The purified recombinant fusion proteins were electrophoresed on sodium dodecyl sulfate–polyacrylamide (SDS–PAGE) gels and evaluated by Coomassie blue staining (Fig. 2A) and by Western blotting using mouse anti-PspA/Rx1 sera (PspA/Rx1 and PspA/D39 are identical clade 2 PspA molecules) (Fig. 2B).

PspA-specific IgG binding >60% was found in antiserum raised by PspA2+4 or PspA2+5 plus double adjuvants for the challenge strains expressing PspA clades 1, 2, 4, and 5, but not for the strain expressing clade 3 (Fig. 3). By contrast, PspA-specific IgG binding > 60% was found for the challenge strains expressing all five PspA clades in antiserum raised by PspA3+2 plus double adjuvants.

For the challenge with the bacterial strain BG9739 with PspA clade 1, the survival rate was greater in mice immunized with PspA3+2 plus double adjuvants ($p < 0.01$) compared with mice

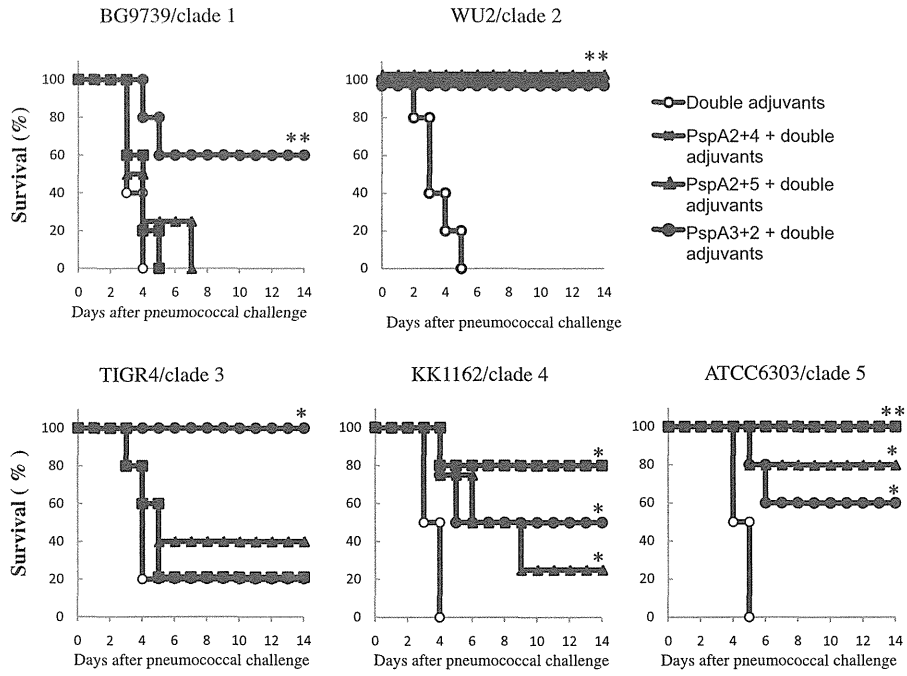

Fig. 4. Protective effects of immunization with fusion PspA proteins against pneumococcal challenge in mice. Mice were immunized subcutaneously with PspA2+4 (closed squares), PspA2+5 (closed triangles), or PspA3+2 (closed circles) in combination with CpG ODNs plus AHG (double adjuvants) or double adjuvants alone (open circles) three times at 1-week intervals. Two weeks after the last immunization, the immunized mice were challenged intranasally with pneumococcal strains with PspA clades 1–5. Mortality was monitored for 2 weeks. Eight to 10 mice per group were examined in each challenge experiment using pneumococcal strain with five different clades. $^{*}p < 0.05$ (vs double adjuvants alone), $^{**}p < 0.01$ (vs double adjuvants alone).

Table 1
Serotypes and PspA clades of 68 isolates from adults with invasive pneumococcal disease.

Serotype	No. strain	No. strain				
		Family 1		Family 2		
		Clade 1	Clade 2	Clade 3	Clade 4	Clade 5
1	1	1				
3	10	9		1		
4	4			4		
6A	2		1			1
6B	10	7		3		
6C	1		1			
7F	2			2		
9V	1			1		
10A	3	3				
11A	2				2	
12F	1			1		
14	5	5				
15A	1				1	
15B	1			1		
16	1			1		
18B	1	1				
18C	1	1				
19A	3			3		
19F	3	1		2		
20	1	1				
22F	3	3				
23A	1				1	
23F	5					5
33	1	1				
34	1	1				
35	2				2	
38	1		1			
Total 68 (100%)		34 (50%)	3 (4%)	19 (28%)	6 (9%)	6 (9%)

immunized with double adjuvants alone (Fig. 4). By contrast, the survival rate did not differ between mice immunized with PspA2+4 or PspA2+5 plus double adjuvants compared with mice immunized with double adjuvants alone. For the bacterial challenge with the WU2 strain with PspA clade 2, the survival rate was significantly higher in mice immunized with PspA2+4, PspA2+5, or PspA3+2 plus double adjuvants ($p < 0.01$) compared with mice immunized with double adjuvants alone. For the bacterial challenge with the TIGR4 strain with PspA clade 3, the survival rate was significantly higher in mice immunized with PspA3+2 plus double adjuvants ($p < 0.05$) compared with mice immunized with double adjuvants alone. The survival rate did not differ between mice immunized with PspA2+4 or PspA2+5 plus double adjuvants compared with mice immunized with double adjuvants alone. In the case of challenge with clade 4 and 5 strains, all three PspA fusion vaccines showed significant protection compared with mice immunized with double adjuvants alone ($p < 0.01$ or $p < 0.05$). These data indicate that immunization with the PspA3+2 vaccine conferred significant protection of mice against pneumococcal challenge by all of the strains expressing PspA clades 1–5. The other two PspA fusion proteins failed to elicit protection against two of the challenge strains (PspA clades 1 and 3).

The distribution of serotypes and PspA clades of 68 clinical isolates from adult patients with IPD are shown in Table 1. The major serotypes were serotype 3 (15%) and 6B (15%), followed by serotypes 14 (7%) and 23F (7%). The major PspA clades were clade 1 (50%) and clade 3 (28%), followed by clade 4 (9%), clade 5 (9%), and clade 2 (4%). All the clinical isolates belonged to PspA clades 1–5, which is in agreement with previous studies [6,13,25].

The binding of PspA-specific IgG in antiserum raised by PspA2+4, PspA2+5, or PspA3+2 plus double adjuvants was examined for the 68 clinical isolates (Fig. 5). The binding of PspA-specific IgG for clade 3 strains ($n = 19$) in antiserum raised by PspA3+2 was significantly higher than in that raised by PspA2+4 or PspA2+5 ($p < 0.05$). By contrast, the binding of PspA-specific IgG for clade 5 strains ($n = 6$) in antiserum raised by PspA3+2 was significantly lower than that by PspA2+4 ($p < 0.05$) or PspA2+5 ($p < 0.05$). No significant difference was found in the binding of PspA-specific IgG for 34 clade 1 strains, three PspA clade 2 strains, or six PspA clade 4 strains between the three types of antiserum raised by PspA2+4, PspA2+5, or PspA3+2.

4. Discussion

In this study, we have demonstrated >60% binding of PspA-specific IgG in the antiserum raised in mice by PspA2+4 or PspA2+5 to four challenge strains expressing clades 1, 2, 4, and 5, but low binding of PspA-specific IgG to the strain expressing clade 3 (Fig. 3). By contrast, >60% binding of PspA-specific IgG in antiserum raised in mice by PspA3+2 was found to all five challenge strains expressing PspA clades 1–5. Immunization with PspA3+2 provided significant protection against pneumococcal challenge by these five strains expressing clades 1–5, but PspA2+4 or PspA2+5 protected mice against only three of the strains expressing clades 2, 4 and 5 in this study (Fig. 4). Therefore, it may be speculated that the binding of PspA-specific IgG closely correlates with the protective effects of PspA fusion protein against pneumococcal challenge in mice. These findings are supported by a recent report on the ability of opsonophagocytic killing and protection of mice against pneumococcal infection by human antiserum to PspA [17]. Only one exception for this speculation is that no protection was found against pneumococcal challenge by the clade 1 strain BG9739 (serotype 4) in mice immunized with PspA2+4 or PspA2+5 plus double adjuvants despite of >60% binding of PspA-specific IgG in antiserum raised by PspA2+4 or PspA2+5 for this clade 1 strain. One possible reason for the inefficient immunization with PspA2+4 or PspA2+5 in mice infected with BG9739 strain may be the presence of serotype 4 capsular polysaccharide. Our previous study demonstrated that the difficulty in protecting against serotype 4 strains was eliminated when mice were immunized with a homologous PspA of the same PspA family [37]. However, only weak protection against infection with strain BG9739 was observed by immunization of mice with the homologous PspA clade 1 [28]. Therefore, it remains uncertain whether immunization with PspA2+4 or PspA2+5 plus double adjuvants did not protect against pneumococcal challenge by the clade 1 strain BG9739 in mice.

No differences were found in the binding of PspA-specific IgG to the clinical isolates belonging to the major clade 1 ($n = 34$) and the two minor clades 2 ($n = 3$) and 4 ($n = 6$) between the types of antiserum raised by the three PspA fusion proteins. For the clinical isolates belonging to the second major clade 3 ($n = 19$), antiserum raised by PspA3+2 demonstrated the greatest binding between the three types of antiserum raised by the PspA fusion proteins (Fig. 5). These findings are in agreement with those showing the binding of PspA-specific IgG to the TIGR4 strain expressing clade 3 for the three types of antiserum raised by each PspA fusion protein (Fig. 3). However, antiserum raised by PspA3+2 demonstrated the lowest binding to six clinical isolates belonging to the minor clade 5 between three types of antiserum raised by each PspA fusion protein. Collectively, PspA3+2 appears to be advantageous in terms of its cross-reactivity with clinical isolates and cross-protection against pneumococcal challenge in mice compared with the other two PspA fusion proteins.

Darrieux et al. reported that immunization with fusion proteins containing fragments of PspA from families 1 and 2 provided cross-protection against pneumococcal strains from families 1 and 2 in mice [30]. The fusion proteins containing PspA clade 1 and PspA clade 3 or 4 fragments provided significant protection against the A66.1 strain (PspA clades 1, and 2), but the protection against strains from clades 3 and 4 was of borderline significance. In another

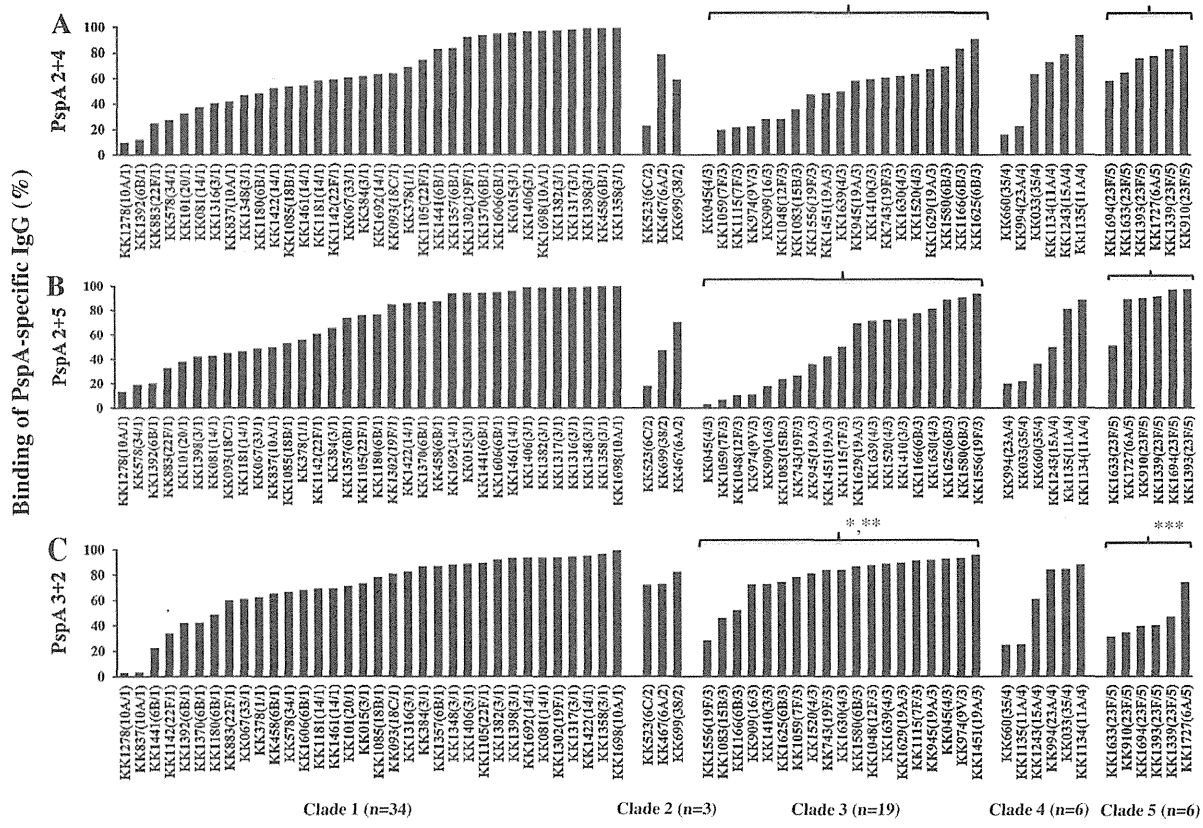

Fig. 5. Comparison of PspA-specific IgG binding by antisera with PspA2+4 (A), PspA2+5 (B), or PspA3+2 (C) in combination with CpG ODNs plus AHG (double adjuvants) to 68 pneumococcal isolates (34 for clade1, three for clade 2, 19 for clade 3, six for clade 4, and six for clade 5). The serotypes and PspA clades are shown in parentheses after the strain names. $^{*}p < 0.01$ (vs PspA2+4), $^{**}p < 0.05$ (vs PspA2+5), $^{***}p < 0.05$ (vs PspA2+4 or PspA2+5).

study, these authors reported that antiserum against fusion protein PspA1+4 demonstrated strong cross-reactivity with PspA clades 1 and 5 but low cross-reactivity with PspA clade 2 or 3 [29]. Consequently, Darrieux et al. failed to demonstrate significant protection against pneumococcal challenge by strains with PspA clades 1–5, although they demonstrated limited cross-protection by immunization with the fusion proteins containing fragments of PspA from families 1 and 2.

A limitation of our study is that we generated and examined only three PspA fusion proteins, which contained one clade each from PspA families 1 and 2. Another limitation is that the binding of PspA-specific IgG was assessed in a small number of clinical isolates from adult patients with IPD.

The antiserum raised by PspA3+2 demonstrated relatively weak binding capacity to the clinical isolates expressing PspA clade 5 in this study. Further studies are required to generate the other types of PspA fusion proteins that can induce PspA-specific IgG with a high affinity to strains expressing PspA clades 5, as well as to strains expressing PspA clade 1–4. In addition, immunization with PspA2+4 or PspA2+5 provided better protection than PspA3+2 against bacterial challenge of clade 4 or clade 5 strain in this study. Therefore, the combined immunization with PspA3+2 with PspA2+4 or PspA2+5 simultaneously or sequentially may have the potential to improve the breadth of immunity against pneumococcal isolates.

In conclusion, immunization of mice with PspA3+2 induced antiserum exhibiting a high binding capacity to the clinical isolates expressing PspA clades 1–4, but not clade 5. Among the three PspA fusion proteins examined in this study, PspA3+2 was found to be advantageous over the other two PspA fusion proteins because PspA3+2 induced a broad range of cross-reactivity with clinical isolates and afforded a cross-protection against pneumococcal challenge in mice.

Author contributions

K.O., Y.A., K.J.I., K.U. and K.T. conceived and designed the experiments. Z.P. and Y.A. performed the experiments. Z.P. and D.T. analyzed the data. K.O., Z.P., Y.A., and D.E.B. wrote the paper.

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

We thank Michiyo Hayakawa for technical assistance. This work was supported by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation and by the Biomedical Cluster Kansai project, which is promoted by the Regional Innovation Cluster Program and is subsidized by the Japanese Government; and by research grants from the Ministry of Health, Labor and Welfare of Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.07.108.

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A Bivalent Vaccine Based on a Replication-Incompetent Influenza Virus Protects against *Streptococcus pneumoniae* and Influenza Virus Infection

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ABSTRACT

***Streptococcus pneumoniae* is a major causative pathogen in community-acquired pneumonia; together with influenza virus, it represents an important public health burden. Although vaccination is the most effective prophylaxis against these infectious agents, no single vaccine simultaneously provides protective immunity against both *S. pneumoniae* and influenza virus. Previously, we demonstrated that several replication-incompetent influenza viruses efficiently elicit IgG in serum and IgA in the upper and lower respiratory tracts. Here, we generated a replication-incompetent hemagglutinin knockout (HA-KO) influenza virus possessing the sequence for the antigenic region of pneumococcal surface protein A (PspA). Although this virus (HA-KO/PspA virus) could replicate only in an HA-expressing cell line, it infected wild-type cells and expressed both viral proteins and PspA. PspA- and influenza virus-specific antibodies were detected in nasal wash and bronchoalveolar lavage fluids and in sera from mice intranasally inoculated with HA-KO/PspA virus, and mice inoculated with HA-KO/PspA virus were completely protected from lethal challenge with either *S. pneumoniae* or influenza virus. Further, bacterial colonization of the nasopharynx was prevented in mice immunized with HA-KO/PspA virus. These results indicate that HA-KO/PspA virus is a promising bivalent vaccine candidate that simultaneously confers protective immunity against both *S. pneumoniae* and influenza virus. We believe that this strategy offers a platform for the development of bivalent vaccines, based on replication-incompetent influenza virus, against pathogens that cause respiratory infectious diseases.**

IMPORTANCE

***Streptococcus pneumoniae* and influenza viruses cause contagious diseases, but no single vaccine can simultaneously provide protective immunity against both pathogens. Here, we used reverse genetics to generate a replication-incompetent influenza virus carrying the sequence for the antigenic region of pneumococcal surface protein A and demonstrated that mice immunized with this virus were completely protected from lethal doses of infection with either influenza virus or *Streptococcus pneumoniae*. We believe that this strategy, which is based on a replication-incompetent influenza virus possessing the antigenic region of other respiratory pathogens, offers a platform for the development of bivalent vaccines.**

Streptococcus pneumoniae is a Gram-positive aerobic bacterial species for which there are more than 90 serotypes based on the chemical and serological features of its capsular polysaccharides. *S. pneumoniae* is a common cause of community-acquired pneumonia, and its colonization of the nasopharynx always precedes infections such as otitis media, sinusitis, pneumonia, and meningitis (1–4). Pneumococcal carriage is an important source of the horizontal spread of this pathogen within the community, because pneumococcal diseases do not occur without preceding nasopharyngeal colonization (1).

The pneumococcal conjugate vaccine can induce serotype-specific antibodies in children and is thought to reduce the nasopharyngeal carriage of vaccine-type pneumococci in children (5, 6). The introduction in 2000 of the seven-valent pneumococcal conjugate vaccine for children in the United States younger than 2 years, as well as children aged 2 to 4 years in a high-risk category, was effective, dramatically reducing the incidence of invasive pneumococcal disease (7, 8). However, although several studies have demonstrated the protective efficacy of pneumococcal conjugate vaccines, they are ineffective against invasive pneumococcal disease caused by serotypes that are not included in the vaccine. Therefore, efforts are ongoing to develop a vaccine that is effective regardless of serotype. Several proteins that are expressed on the

Received 29 April 2014 Accepted 2 September 2014
Published ahead of print 10 September 2014
Editor: D. S. Lyles
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doi:10.1128/JVI.01205-14

surface of the bacteria, such as choline-binding protein A and pneumococcal surface adhesin A, are considered attractive antigens for a new vaccine (1, 2, 9, 10). Among them, pneumococcal surface protein A (PspA) is thought to be particularly promising. PspA is found in all clinical *S. pneumoniae* isolates (11). Some studies have demonstrated that antibodies against PspA neutralize the anticomplement effect of PspA, which results in clearance of the bacteria by depositing complement C3 on the bacterial surface (12, 13). Moreover, anti-PspA antibodies have also been shown to prevent infection from strains with different serotypes (14). We previously reported that mice immunized with recombinant PspA protein in combination with polyinosinic-poly(C) [poly(I·C)], a Toll-like receptor (TLR) agonist, as an adjuvant were completely protected against secondary pneumococcal pneumonia after influenza virus infection (15). Moreover, in human trials, intramuscular immunization with the recombinant PspA protein induced cross-reactive antibodies to heterologous PspA (14).

Influenza virus also causes serious respiratory infections, and inactivated and live-attenuated influenza vaccines are approved for prophylaxis against influenza. Although inactivated vaccines are highly safe and induce IgG in serum, they cannot elicit secretory IgA at the mucosal surface of the respiratory tract, where influenza virus replicates. Intranasal administration of live-attenuated vaccines, which carry mutations that lead to temperature sensitivity and viral attenuation, induces not only IgG in serum but also IgA at the mucosal surface. However, live-attenuated vaccines are not recommended for children under the age of 2, adults aged 50 or over, immunocompromised patients, or pregnant women (16–18). To overcome these limitations, efforts are ongoing to develop an ideal influenza vaccine that is highly safe and induces secretory IgA at the mucosal surface of the respiratory tract.

Recently, we (19) and others (20, 21) demonstrated that replication-incompetent influenza viruses that lack a functional hemagglutinin (HA) segment can induce virus-specific humoral and cellular immunity and provide protective immunity against a lethal dose of infection with influenza virus. Given that such viruses replicate efficiently in HA-expressing cell lines, this system could be used to generate bivalent vaccines in which the antigen gene of another respiratory pathogen is introduced into the HA gene. To assess this possibility, here we generated an HA knockout (KO) PspA virus as a bivalent vaccine candidate, possessing the PspA antigen gene instead of the HA gene, and examined its immunogenicity and vaccine efficacy against both influenza virus and *S. pneumoniae* in mice.

MATERIALS AND METHODS

Cells. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) containing 5% of newborn calf serum (NCS). Human embryonic kidney 293T (HEK293T) cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum. MDCK cells expressing HA (HA-MDCK) were established by cotransfection with plasmids for the expression of HA derived from influenza virus A/Puerto Rico/8/34 (PR8) and puromycin *N*-acetyltransferase as previously described (19). HA-MDCK cells were cultured in MEM containing 5% NCS and 5 μg/ml puromycin dihydrochloride (Nacalai Tesque).

Preparation of virus and bacteria. PR8 was generated by using reverse genetics (22) and propagated in MDCK cells at 37°C. Forty-eight hours after infection, the supernatants were harvested and stored at −80°C until use. *S. pneumoniae* strain WU2 with serotype 3 and strain EF3030 with serotype 19F, which are virulent and relatively avirulent in mice, respectively (23, 24), were grown in Todd-Hewitt Broth (Becton, Dickinson and Company) supplemented with 0.5% yeast extract (THY) to mid-log phase and washed twice with Dulbecco's phosphate-buffered saline (PBS) without $CaCl_2$ and $MgCl_2$ (Sigma-Aldrich). The bacteria were then suspended in THY containing 10% glycerol, aliquoted, and stored at −80°C until use.

Plasmid construction. For viral RNA (vRNA) expression, plasmids containing the cDNAs of PR8 genes between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator (referred to as PolI plasmid) were generated. To generate plasmids that express the PspA antigenic region or green fluorescence protein (GFP) from the HA segment, we utilized the packaging signal of the HA segment of influenza virus (25). Plasmids pPolI-HA(9)PspA(80) and pPolI-HA(9)GFP(80) were constructed to replace the PolI plasmid that encoded the HA segment of PR8. These plasmids contained the 3′ HA noncoding region, 9 nucleotides that correspond to the HA-coding sequence at the 3′ end of the vRNA followed by the PspA antigenic region of the Rx1 strain (serotype 2) (amino acid positions 32 to 333), or the GFP-coding sequence, 80 nucleotides that correspond to the HA-coding sequence at the 5′ end of the vRNA, and lastly the 5′ HA noncoding region. The sequences were determined to ensure that no unwanted mutations were introduced. Primer sequences are available upon request.

Plasmid-driven reverse genetics. To generate the viruses that possess the HA segment encoding the PspA antigenic region (HA-KO/PspA virus) or GFP (HA-KO/GFP virus), we used plasmid-driven reverse genetics as described previously (22). Briefly, pPolI-HA(9)PspA(80) or pPolI-HA(9)GFP(80) and the remaining 7 PolI plasmids were cotransfected into HEK293T cells together with eukaryotic protein expression plasmids for PB2, PB1, PA, NP, and wild-type HA derived from PR8 by using the TransIT-293 transfection reagent (Mirus). Forty-eight hours after transfection, the supernatants containing the HA-KO/PspA virus or the HA-KO/GFP virus were harvested and propagated once in HA-MDCK cells at 37°C for 48 h in MEM containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (0.8 μg/ml) and 0.3% bovine serum albumin (BSA; Sigma-Aldrich). Cell debris was removed by centrifugation at 2,100 × *g* for 20 min at 4°C, and the supernatants were stored at −80°C until use. The virus titers were determined by counting cells expressing PspA or GFP by immunostaining or fluorescence observation, respectively, after a plaque assay using HA-MDCK cells.

Immunofluorescence assay. MDCK and HA-MDCK cells were infected with PR8 or HA-KO/PspA virus at a multiplicity of infection (MOI) of 0.0001. Thirty-six hours after infection, the cells were washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After permeabilization with PBS containing 0.2% Triton X-100, the cells were incubated with a mouse anti-PspA antiserum and with rabbit antiserum against influenza virus (A/WSN/33). Goat anti-mouse IgG Alexa Fluor 488 and anti-rabbit IgG Alexa Fluor 594 (molecular probes) served as secondary antibodies. Cells were observed by means of confocal microscopy (Nikon).

Immunization and protection test. Seven-week-old female C57BL/6 mice (Japan SLC) were intranasally inoculated with 10^5 PFU of HA-KO/PspA virus (in 50 μl) twice, with a 2-week interval between the inoculations. As control groups, age-matched female C57BL/6 mice were intranasally inoculated with 10^5 PFU of HA-KO/GFP virus (in 50 μl) or medium on the same schedule. Two weeks after the final vaccination, six mice per group were euthanized to obtain sera, bronchoalveolar lavage fluids (BALF), and nasal wash samples. Also 2 weeks after the final vaccination, mice were challenged with 100 times the 50% mouse lethal dose (MLD_{50}) of virus PR8. Eight mice per group were monitored for survival and body weight changes for 14 days after PR8 challenge. Lungs and nasal turbinates from three mice per group were collected on days 3 and 6 after challenge to determine virus titers. Virus titers were determined in MDCK cells. In addition, 2 weeks after the final vaccination, mice were intranasally challenged with 3 MLD_{50} (equivalent to 2×10^7 CFU) of *S. pneu-*

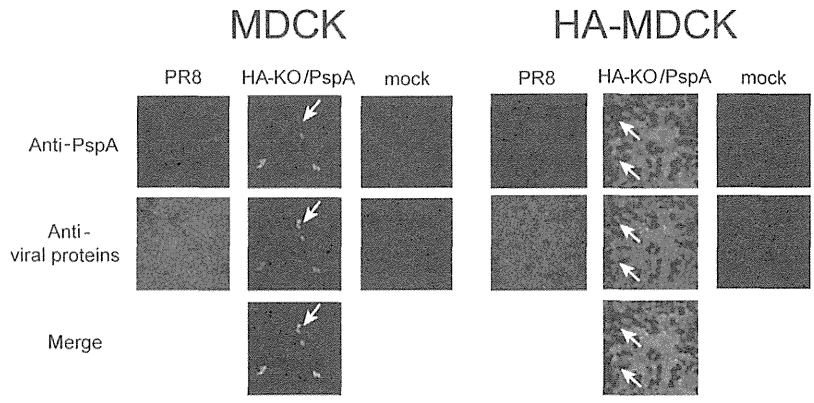

FIG 1 Expression of the PspA antigenic region and viral proteins in cells infected with PR8 or HA-KO/PspA virus. MDCK and HA-MDCK cells were infected with the indicated virus at an MOI of 0.0001, and an immunofluorescence assay was performed 36 h postinfection. PspA (green) and viral proteins (red) were detected by anti-PspA and anti-WSN antibodies, respectively. White arrows indicate cells that express the viral proteins but not the PspA protein.

moniae strain WU2. Ten mice per group were monitored for survival for 14 days after challenge. Similarly, 2 weeks after the final vaccination, mice were intranasally challenged with 1.0×10^2 CFU of *S. pneumoniae* strain EF3030. Nasal wash samples from 10 mice per group were collected on day 5 after challenge to determine the bacterial clearance from the nasopharynx. A quantitative bacterial culture of the nasal wash samples was performed.

All animal experiments were performed in accordance with the University of Tokyo's Regulations for Animal Care and Use, which were approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo (approval number PA 12-14). The committee acknowledged and accepted both the legal and ethical responsibility for the animals, as specified in the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science, and Technology.

Detection of pathogen-specific antibodies. Pathogen-specific antibodies in nasal wash samples, BALF, and sera were detected by means of an enzyme-linked immunosorbent assay (ELISA) (26). To detect virus-specific antibodies, we used 2-fold serially diluted serum, BALF, and nasal wash samples. In this assay, 96-well ELISA plate wells were coated with approximately 200 hemagglutination units (in 50 μl) of purified PR8 virus treated with disruption buffer (0.5 M Tris-HCl [pH 8.0], 0.6 M KCl, and 0.5% Triton X-100). After the diluted samples were incubated on the virus-coated plates for 1 h at room temperature, goat anti-mouse IgA or IgG antibody conjugated to horseradish peroxidase (Kirkegaard & Perry Laboratory Inc.) was added to detect bound antibody. The optical density at 405 nm (OD_{405}) was measured with a microplate reader. Endpoint titers are expressed as the reciprocal $\log_2$ of the last dilution whose OD value was more than the cutoff value. The cutoff value was determined by adding 3-fold standard deviations (SD) to the mean (i.e., mean + 3 SD) of the OD values of samples from naive mice. PspA-specific antibody titers in nasal wash samples, BALF, and sera were determined by use of an ELISA as previously described (15). Microtiter plates were coated overnight at 4°C with 100 μl of 1-μg/ml PspA. The plates were then washed with PBS with 0.05% Tween 20 (PBS-T). Serially diluted nasal wash samples, BALF, and sera (50 μl) were added to the plates, and the plates were then incubated for 30 min at 37°C. The plates were washed three times with PBS-T and incubated with alkaline phosphatase-conjugated goat anti-mouse IgG, IgG1, IgG2a, or IgA (Zymed) for 30 min at 37°C. After this incubation, the plates were washed three times with PBS-T, and then 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma-Aldrich) diluted with substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$) was added; the plates were then incubated for 30 min at room temperature in the dark. The OD at 405 nm was then measured with a microplate reader (Bio-Rad Laboratories). The endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution giving 0.1 OD_{405} unit above the OD_{405} of negative-control samples obtained from nonimmunized mice.

RESULTS

PspA and GFP expression in infected cells. To examine whether PspA was expressed in HA-KO/PspA virus-infected cells, we infected MDCK and HA-MDCK cells with HA-KO/PspA virus and attempted to detect PspA in virus-infected cells by use of an immunofluorescence assay. PR8 served as a control. PspA expression was detected in both cell types infected with HA-KO/PspA virus but not in cells infected with PR8 (Fig. 1). Although HA-KO/PspA virus could efficiently spread and express its viral proteins and PspA in HA-MDCK cells, the infection of HA-KO/PspA virus did not spread in MDCK cells (Fig. 1). Indeed, the virus titer of HA-KO/PspA reached $10^{7.6}$ PFU/ml in HA-MDCK cells. In both cell types infected with HA-KO/PspA virus, we found some cells that expressed the viral proteins, but not PspA (Fig. 1, white arrows). This may be because the HA gene segment encoding the PspA antigenic region was not incorporated into the virus particles that infected those cells. This is not surprising because not all virions contain authentic viral RNA segments (27). Taken together, these results indicate that HA-KO/PspA virus is replication incompetent but can express not only viral proteins but also PspA in virus-infected cells. We obtained similar results with HA-KO/GFP virus (data not shown).

Induction of antibodies against PspA and influenza virus by HA-KO/PspA virus. To assess the ability of HA-KO/PspA virus to induce antibodies against both PspA and PR8, mice were intranasally inoculated twice with 10^5 PFU of HA-KO/PspA virus. Mice inoculated with HA-KO/GFP virus or medium served as controls. Two weeks after the final vaccination, nasal wash samples, BALF, and serum samples were collected and subjected to ELISA to measure antigen-specific IgG and IgA in these samples. The induction of IgG against PR8 was detected in serum samples from mice inoculated with HA-KO/PspA or HA-KO/GFP virus (Fig. 2A). Moreover, both IgG and IgA against PR8 were detected in nasal wash samples and BALF from these mice, although IgA in the nasal wash samples of mice inoculated with HA-KO/PspA virus was not significantly induced compared with that in the nasal wash samples of mice inoculated with medium (Fig. 2B and C).

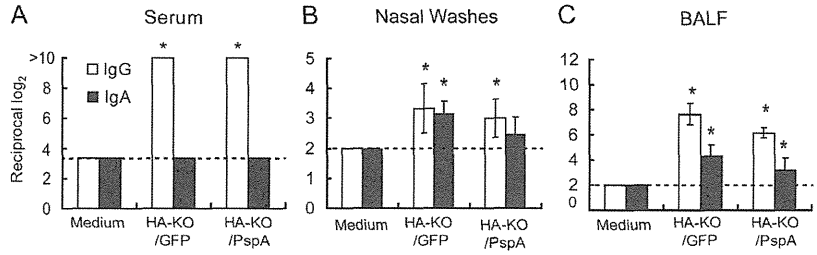

FIG 2 Induction of influenza virus-specific IgG and IgA in serum (A), nasal wash (B), and BALF (C) samples. Mice were intranasally inoculated with medium, HA-KO/GFP virus, or HA-KO/PspA virus with a 2-week interval between the inoculations. Samples from six mice from each group were collected 2 weeks after the final vaccination. Virus-specific antibodies were detected by using an ELISA. Results are expressed as the means of the reciprocal titer $\log_2$ (± standard deviations [SD]). Statistically significant differences between groups were determined by using the Dunnett method. The asterisk indicates a significant difference from samples taken from mice inoculated with medium (*, $P < 0.05$). The broken lines indicate the detection limits.

These results indicate that the HA-KO/PspA and HA-KO/GFP viruses elicited both virus-specific mucosal and systemic immunity. To assess whether these antibodies could neutralize wild-type PR8 virus, we performed neutralizing assays. However, we could not detect neutralizing antibodies in serum samples from mice inoculated with either HA-KO/GFP or HA-KO/PspA virus (data not shown). On the other hand, for the antibody response to PspA, both IgG and IgA titers in the BALF and IgG titers in the serum and nasal wash samples significantly increased only in mice inoculated with HA-KO/PspA virus (Fig. 3A, B, and C). Likewise, PspA-specific IgG1 and IgG2a titers were also elevated in the sera of these mice (Fig. 3D). While both isotypes inhibit the anticomplement effect of PspA, the complement-fixing ability of IgG2a is superior to that of other isotypes (28). Therefore, the increase in IgG2a titer in mice inoculated with HA-KO/PspA represents a significant response in terms of the efficient clearance of *S. pneumoniae* via opsophagocytic killing. A PspA-specific antibody response was not observed in samples from mice inoculated with HA-KO/GFP virus or medium. These results indicate that HA-KO/PspA virus can induce a significant antibody response against both influenza virus and PspA at the mucosal surface of the respiratory tract and in blood.

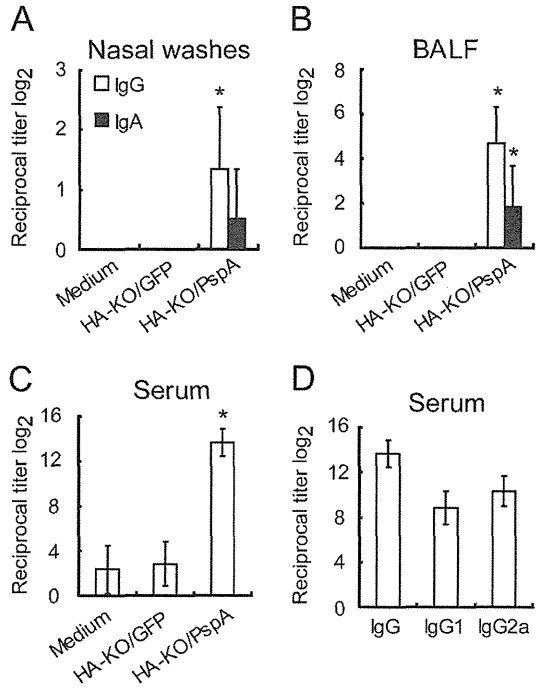

FIG 3 Induction of PspA-specific IgG and IgA levels in nasal wash fluids (A) and BALF (B) and IgG levels in sera (C), as well as IgG1 and IgG2a levels in sera (D). Mice were intranasally inoculated with medium, HA-KO/GFP virus, or HA-KO/PspA virus with a 2-week interval between the inoculations. Samples from six mice from each group were collected 2 weeks after the final vaccination. PspA-specific antibodies were detected by use of an ELISA. The value of IgG in panel D is identical to that of the IgG of HA-KO/PspA in panel C. Results are expressed as the means of the reciprocal titer $\log_2$ (± SD). Statistically significant differences between groups were determined by using the Dunnett method. The asterisk indicates a significant difference from samples taken from mice inoculated with medium (*, $P < 0.05$).

Protective efficacy of HA-KO/PspA virus against lethal doses of *S. pneumoniae* and influenza virus. To evaluate the protective efficacy of HA-KO/PspA virus against *S. pneumoniae* and influenza virus, we performed a challenge experiment. Mice were intranasally inoculated with medium, HA-KO/GFP, or HA-KO/PspA virus on the same schedule as the aforementioned experiment. Two weeks after the final vaccination, these mice were infected with lethal doses of either PR8 or *S. pneumoniae* serotype 3 strain WU2. Survival of mice challenged with either influenza virus or *S. pneumoniae* and body weight changes of mice challenged with influenza virus were monitored during the observation period.

In the case of influenza virus infection, the body weights of mice inoculated with medium rapidly decreased and all mice died by day 5 after infection (Fig. 4). On the other hand, mice inoculated with either HA-KO/PspA or HA-KO/GFP virus showed no reduction in body weight and all of these mice survived during the observation period (Fig. 4). We also determined virus titers in the lungs and nasal turbinates of each group of mice after challenge (Table 1). Although virus was recovered from the lungs of 2 out of 3 mice inoculated with HA-KO/PspA virus on day 3 after challenge, virus titers were appreciably lower than those in the lungs of mice inoculated with medium. Further, except for the lungs of these mice, virus in the nasal turbinates and lungs of mice inoculated with HA-KO/PspA or HA-KO/GFP virus was undetectable on days 3 and 6 after challenge. These results indicate that the HA-KO/PspA and HA-KO/GFP viruses confer protective immunity to mice against a lethal dose of influenza virus.

With regard to the *S. pneumoniae* infection, all mice mock immunized with medium died when challenged with *S. pneumoniae* strain WU2 of serotype 3. Moreover, in contrast to the PR8 infection, all mice immunized with HA-KO/GFP virus also died.

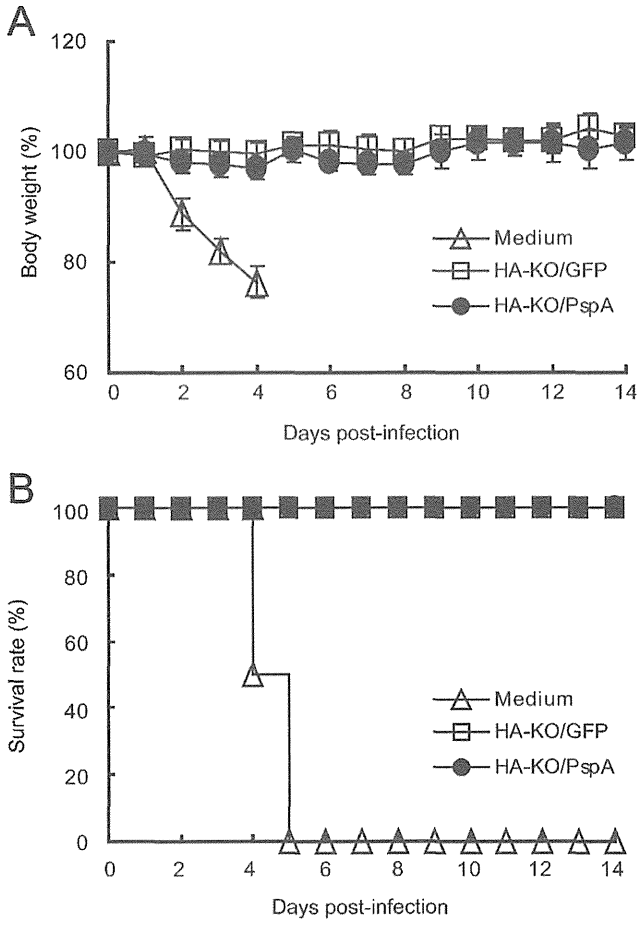

FIG 4 Body weight changes and survival curves for mice challenged with lethal doses of PR8. Eight mice per group were intranasally infected with 100 MLD_{50} of PR8 2 weeks after their final vaccination. Body weights were measured (A) and survival rate was monitored (B) for 14 days after infection. Open triangles, mice inoculated with medium; open squares, mice inoculated with HA-KO/GFP virus; closed circles, mice inoculated with HA-KO/PspA virus.

However, all mice immunized with HA-KO/PspA virus survived (Fig. 5A). To determine the effect of the vaccine on the level of bacterial colonization of the nasopharynx, we challenged immunized mice with *S. pneumoniae* serotype 19F strain EF3030; we did not use *S. pneumoniae* WU2 for this experiment because it causes bacteremia, which would make it problematic to differentiate true bacterial colonization from bacteria derived from blood. Although the bacterial densities in the nasopharynx of mice inoculated with HA-KO/GFP virus were comparable to those in the nasopharynx of mice inoculated with medium, the bacterial densities in the nasopharynx of mice inoculated with HA-KO/PspA virus were significantly lower than those in the nasopharynx of mice inoculated with medium or HA-KO/GFP virus (Fig. 5B). These results indicate that HA-KO/PspA virus confers immunity against *S. pneumoniae* of a heterologous serotype because the PspA gene in HA-KO/PspA virus was derived from serotype 2 (strain Rx1), which differs from the serotype of the challenge bacterium (i.e., serotypes 3 and 19F).

Overall, these results demonstrate that HA-KO/PspA virus provides protective immunity to mice against lethal infection with influenza virus and *S. pneumoniae*, suggesting that HA-KO virus can be used as a platform for a bivalent vaccine against respiratory infectious diseases.

DISCUSSION

Secondary bacterial infections after influenza virus infections complicate disease severity and increase mortality and morbidity. Indeed, most victims of the 1918-1919 influenza virus pandemic likely died from secondary bacterial pneumonia (29). In addition, autopsy samples from those who succumbed to infection with the 2009 pandemic H1N1 influenza virus exhibited signs of secondary bacterial infections, and the severity of the infections caused by this influenza virus was correlated with *S. pneumoniae* coinfection (30, 31). Damage to mucosal epithelial cells, exposure of receptors that facilitate bacterial adherence, and dysfunction of immune effectors by influenza virus infection are prominent features that allow bacteria access to the lower respiratory tract (4). It was, therefore, once thought that secondary pneumococcal infections could be prevented by administering influenza vaccine alone because if the influenza virus infection was prevented, the above-described features that contribute to bacterial invasion would be minimized (32, 33). However, protection from bacterial infection via influenza vaccination *per se* is not feasible because of the lack of specific immunity against bacteria. Therefore, the induction of antibodies against *S. pneumoniae* via vaccination is important to prevent such bacterial infections. Here, we generated a replication-incompetent HA-KO virus that encodes the PspA antigenic region in the coding region of its HA segment gene (HA-KO/PspA virus). This virus induced not only influenza virus but also PspA-specific antibodies on the respiratory mucosa and in the sera of mice. We also demonstrated that mice inoculated with HA-KO/PspA virus were completely protected from lethal challenge with *S. pneumoniae* or influenza virus. In addition, we also demonstrated that nasal immunization with HA-KO/PspA virus significantly decreased the levels of bacterial colonization in the nasopharynx of mice. Therefore, our findings suggest that nasal

TABLE 1 Protection against challenge with a lethal dose of PR8 in mice inoculated with HA-KO/GFP or HA-KO/PspA virus[a]

Inoculum	Days postinfection	Organ	Mean virus titer ± SD (log_{10} PFU/g)
Medium	3	NT	6.3 ± 0.4
	3	Lung	7.9 ± 0.2
	6	NT	NA
	6	Lung	NA
HA-KO/GFP	3	NT	ND
	3	Lung	ND
	6	NT	ND
	6	Lung	ND
HA-KO/PspA	3	NT	ND
	3	Lung	2.9, 4.3
	6	NT	ND
	6	Lung	ND

[a] Six mice from each group were intranasally infected with 100 MLD_{50} of PR8 (50 μl per mouse) 2 weeks after the final vaccination. Three mice per group were sacrificed on days 3 and 6 postinfection, and lungs and nasal turbinate were collected to determine virus titers. When virus was not recovered from all three mice, individual titers are given. Abbreviations: NT, nasal turbinate; NA, not applicable because the mice died; ND, not detected (detection limit, 10 PFU/lung or 5 PFU/NT).

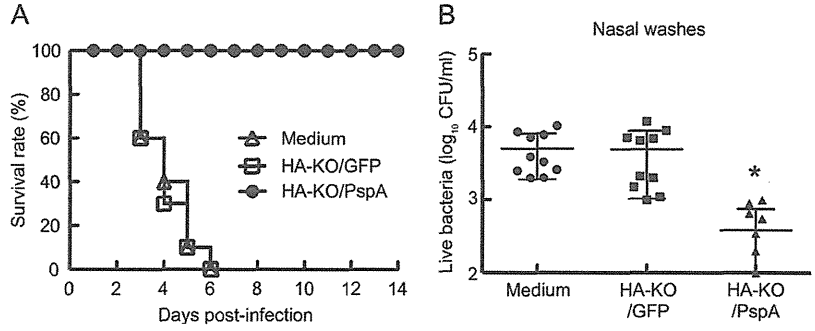

FIG 5 Survival curves for mice challenged with lethal doses of *S. pneumoniae* strain WU2 and bacterial densities in the nasopharynx 5 days after challenge with *S. pneumoniae* strain EF3030. (A) Ten mice per group were intranasally infected with 3 MLD_{50} of strain WU2 2 weeks after their final vaccination. The survival rate was monitored for 14 days after infection. Open triangles, mice inoculated with medium; open squares, mice inoculated with HA-KO/GFP virus; closed circles, mice inoculated with HA-KO/PspA virus. (B) Mice were intranasally infected with 1×10^2 CFU/mouse of strain EF3030 2 weeks after their final vaccination. Five days after challenge with strain EF3030, nasal washes were collected, and a quantitative bacterial culture of nasal washes was performed. Values represent the log_{10} CFU/ml (mean ± SD) for 10 mice per group. Closed circles, mice inoculated with medium; closed squares, mice inoculated with HA-KO/GFP virus; closed triangles, mice inoculated with HA-KO/PspA virus. Statistically significant differences between groups were determined by using the Kaplan-Meier log rank test for the survival analysis or the Mann-Whitney test for the bacterial clearance analysis. The asterisk indicates a significant difference (*, $P < 0.05$).

immunization with HA-KO/PspA virus can prevent pneumococcal colonization and protect mice infected with *S. pneumoniae* or with influenza virus. Therefore, the HA-KO/PspA virus is a promising bivalent vaccine against these important respiratory pathogens.

It has been reported that live-attenuated influenza vaccines may cause adverse effects, such as runny nose and sore throat, due to the replication of the vaccine virus (16). In contrast, replication-incompetent HA-KO/PspA virus can replicate only in HA-expressing MDCK cells and not in wild-type MDCK cells (Fig. 1). We previously found that no infectious virions were detected in mouse lungs infected with an HA-KO virus (unpublished data). Therefore, we believe that HA-KO virus-based vaccines are safer than live-attenuated influenza vaccines.

We did not test the stability of the foreign gene (i.e., the antigenic region of PspA) in HA-KO/PspA virus in this study, because we previously found that expression of a foreign gene in HA-KO virus gradually decreases during serial passages (unpublished data), and this is likely also the case for HA-KO/PspA virus. Therefore, the stability of the foreign gene in a replication-incompetent virus requires further evaluation and improvement.

In this study, neutralizing antibodies against influenza virus were not detected in mice immunized with HA-KO/PspA virus, although we did detect anti-influenza virus antibodies. It is possible that nonneutralizing antibodies might contribute to protection from a lethal dose of influenza virus infection as has been reported previously (34, 35). Furthermore, virus-specific cytotoxic T lymphocytes (CTLs) also play an important role in protection (36). Indeed, we have previously demonstrated that mice intranasally immunized with a replication-incompetent influenza virus elicit NP-specific CTLs in the lung (19). Thus, it is possible that CTLs were elicited in the lungs of mice immunized with HA-KO/PspA virus. In addition, these nonneutralizing antibodies and CTLs can mitigate infection of homologous and heterologous strains of influenza virus (35, 36). Therefore, HA-KO/PspA virus may confer heterosubtypic immunity as well as homosubtypic immunity. As to protection from *S. pneumoniae* infection, the induction of anti-PspA antibodies is considered a promising strategy. Anti-PspA antibodies disable PspA function, which inhibits the complement deposition on the bacterial surface (12, 13, 28) and can facilitate bacterial clearance by opsonization-mediated phagocytosis. Thus, HA-KO/PspA virus could provide mice with protective immunity against *S. pneumoniae* as well as influenza virus infection.

It has been previously demonstrated that intranasal administration of the PspA protein alone does not elicit an adequate antibody response and that administration of PspA with adjuvants, such as different types of TLR ligands, can confer sufficient immunity against *S. pneumoniae* in mice (37). Remarkably, however, we demonstrated that HA-KO/PspA virus induced efficient immunity against *S. pneumoniae* infection without any mucosal adjuvants. The possible mechanisms are as follows: first, infection with HA-KO/PspA virus triggers the innate immune response via recognition of vRNAs by pattern recognition receptors, such as TLR7 (38) and Retinoic acid-inducible gene I (*RIG-I*) (39, 40), in the infected cells since these vRNAs are amplified in HA-KO/PspA virus-infected cells even though infectious progeny virus cannot be generated; second, PspA is expressed in virus-infected cells as shown in Fig. 1; and third, antigen-presenting cells (APCs) phagocytose the infected cells that contain the ligands for the TLRs (vRNAs) and the antigens (PspA in addition to viral proteins), and the major histocompatibility complex classes I and II efficiently present these antigens on the cell surface of the APCs (41, 42). As such, it is possible for PspA-specific antibodies to be induced by HA-KO/PspA virus in the absence of any exogenous mucosal adjuvants.

There is a concern that HA-KO vaccines may not confer immunity to those who have been previously exposed to influenza viruses. However, because the antigenicity of seasonal influenza viruses changes (e.g., via antigenic drift), the HA used for HA-KO virus could be changed to match the antigenicity of the circulating strains. Therefore, HA-KO vaccines would not be neutralized by antibodies in vaccines and should be efficacious.

In conclusion, the replication-incompetent influenza virus-based approach presented here could be used as a platform to develop bivalent vaccine candidates against various pathogens that cause respiratory infectious diseases.

ACKNOWLEDGMENTS

We thank Takeo Gorai, Ryuta Uraki, and Eiryo Kawakami for helpful discussions and Susan Watson for editing the manuscript. We are also grateful to David E. Briles from the University of Alabama at Birmingham for providing the pneumococcal strains.

This work was supported by the Advanced Research for Medical Products Mining Programme of the National Institute of Biomedical Innovation (NIBIO), by grants-in-aid from the Ministry of Health, Labor and Welfare, Japan, by ERATO (Japan Science and Technology Agency), by the Strategic Basic Research Programs of the Japan Science and Technology Agency, and by National Institute of Allergy and Infectious Diseases Public Health Service research grants. H.K. is supported by a Grant-in-Aid from the Japan Society for the Promotion of Science.

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Vaccine 32 (2014) 1181–1186

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Sustained functional serotype-specific antibody after primary and secondary vaccinations with a pneumococcal polysaccharide vaccine in elderly patients with chronic lung disease

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ARTICLE INFO

Article history:
Received 13 July 2013
Received in revised form
18 September 2013
Accepted 24 September 2013
Available online 9 October 2013

Keywords:
Serotype-specific IgG
Opsonophagocytic activity
Pneumococcal polysaccharide vaccine
Revaccination

ABSTRACT

An observational study was conducted to determine immunogenicity before and after primary and secondary vaccinations with 23-valent pneumococcal polysaccharide vaccine in a cohort of 40 elderly patients with chronic lung diseases. Safety of this vaccine was also compared between primary and secondary vaccination. We analyzed serotype-specific immunoglobulin G (IgG) and the opsonization index (OI) for serotypes 6B, 14, 19F, and 23F and compared adverse local and systemic reactions. The levels of serotype-specific IgG and the OIs significantly increased 1 month after primary and secondary vaccinations. Peak levels of IgG after secondary vaccination were 5–20% lower than those after primary vaccination, while serotype-specific OIs after secondary vaccination were comparable with those after primary vaccination. The levels of serotype-specific IgG required for 50% killing significantly decreased 1 month after vaccination. These values for serotypes 14, 19F, and 23F were slightly elevated immediately before secondary vaccination, but those for serotype 6B did not change. After secondary vaccination, these values declined slightly for serotypes 14, 19F, and 23F and remained low for serotype 6B. Although self-limited local and systemic reactions were more frequent after secondary vaccination compared with primary vaccination, no serious systemic reaction was found after either vaccination. Our data suggest a sustained functional serotype-specific IgG after primary and secondary vaccination and confirmed the safety of secondary vaccination among elderly individuals with chronic lung disease.

1. Introduction

Streptococcus pneumoniae is a leading human pathogen that causes a variety of diseases, such as invasive pneumococcal disease (IPD) and non-bacteremic pneumonia, in children and adults. The rates of IPD are highest among children under 5 years of age and among adults who are older than 65 years of age [1–3]. Community-acquired pneumonia (CAP), which is most likely to be caused by *S. pneumoniae*, and the incidence of pneumococcal CAP is also high among the elderly [3–5].

The efficacy, immunogenicity, and safety of the 23-valent pneumococcal polysaccharide vaccine (PPV23; Pneumovax®, Merck Sharp & Dohme) has been extensively studied in adults [6,7]. Although *S. pneumoniae* is commonly responsible for 8–25% of exacerbation in patients with chronic lung diseases such as chronic obstructive pulmonary disease, the immunogenicity studies of PPV23 in this population are scarce [8–12]. Consistent results from observational studies have demonstrated that PPV23 reduces the risk of IPD in immunocompetent older adults. Recent studies from Japan reported that PPV23 prevented pneumococcal pneumonia and reduced the death rate due to pneumococcal pneumonia among nursing home inhabitants in Japan and that PPV23 was effective for all-cause pneumonia for study subjects older than 75 years of age after routine immunization with the influenza vaccine [13,14]. As the percentage of the elderly population (aged 65 years and over) is 23.1% in Japan [15], the demand for receiving PPV23 revaccination 5 years or more after primary vaccination is increasing. The US Advisory Committee on Immunization Practices recommends a single revaccination for persons at increased risk, including adults ≥65-years old who had received their first vaccination ≥5 years previously and were less than 65 years old at the time of their first vaccination in 1997 [16]. Revaccination with

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0264-410X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.vaccine.2013.09.060

PPV23 was contraindicated in Japan until October 2009 [17], but revaccination has been approved for adults who had received their first dose of PPV23 more than 5 years previously.

Jackson et al. reported that secondary vaccination with PPV23 induced local reactions more frequently than primary vaccination, but that these reactions were not serious and resolved within 3 days [18]. In addition, two recent studies from the United States demonstrated comparable functional antibody responses between primary and secondary vaccination in adults older than 65 years of age [19,20]. Hammitt et al. also demonstrated that repeat revaccination with PPV23, administered 6 years or more after the prior dose, was immunogenic and generally tolerated [21]. Furthermore, IgG concentrations were found to exceed vaccine-naïve levels for seven of eight serotypes tested 10 years after the first or second doses of PPV23 [22]. In this study, we examined the immunogenicity and safety of PPV23 in a cohort of patients with chronic lung disease (CLD) who were followed up through the time of primary to secondary vaccination at a single institution and report on the sustained and functional serotype-specific antibodies raised by primary and secondary vaccinations with PPV23.

2. Materials and methods

2.1. Study subjects

Between October 2001 and November 2002, 101 patients with CLD who were 65 years of age or older received primary vaccination with PPV23 at our outpatient clinic. Serum samples from these study subjects had been acquired before and 1 month after primary vaccination and had been preserved for antibody titer analyses [23]. Of 101 patients, 30 patients died and 31 patients were lost for follow-up at our outpatient clinic until September 2009. All patients provided written informed consent.

This study was reviewed and approved by the ethics committee of the National Hospital Organization, Tokyo National Hospital, and was conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Samples

Blood samples were drawn from 40 study subjects before and 1 month after secondary vaccination with PPV23. Sera were separated by centrifugation and stored at −80 °C until used.

The levels of serotype-specific immunoglobulin G (IgG) and the opsonization index (OI) were measured in the serum samples obtained before and 1 month after primary vaccination and before and 1 month after secondary vaccination.

2.3. ELISA

Antipneumococcal IgG antibodies were measured with the World Health Organization (WHO)-approved ELISA methodology, using standard reference serum (89-SF or 007sp) and C-polysaccharide and 22F polysaccharide absorptions, as previously described [24,25]. The levels of serotype-specific IgG for four serotypes, 6B, 14, 19F, and 23F, were determined according to the WHO protocol (a detailed protocol is available at www.vaccine.uab.edu/ELISAProtocol [89SF]). These four serotypes are commonly found in adult patients with CAP in Japan [5].

2.4. Multiplexed opsonophagocytic killing assay

A multiplexed opsonophagocytic killing assay (MOPA) for the four serotypes, based on antibiotic-resistant target bacteria, was performed at the Research Institute for Microbial Diseases, Osaka University, as previously described [26]. The quality control serum was prepared from the pooled sera of adults vaccinated with PPV23 and was used in each assay. The OI was defined as the serum dilution that killed 50% of bacteria, and the OI was determined using opsotiter3 software according to the WHO protocol (a detailed protocol is available at www.vaccine.uab.edu/UAB-MOPA). Functional activity of serotype-specific IgG was expressed as the concentration of IgG required for 50% killing of the pneumococcal strain by dividing the IgG concentration of a test sample by the OI [27].

Table 1
Baseline characteristics of 40 patients with chronic pulmonary diseases.

Characteristics	Values
Male sex: No. (%)	18(45)
Mean age: years of age (SD)	77(6.1)
65–69 years of age: No. (%)	4(0.1)
70–79 years of age: No. (%)	22(55)
≧80 years of age: No. (%)	14(35)
Comorbid illness: No. (%)	
Sequela of pulmonary tuberculosis	13(33)
Bronchiectasis	7(18)
Asthma	7(18)
Nontuberculous mycobacterial infection	6(15)
Aspergillosis	3(8)
Chronic obstructive pulmonary disease	3(8)
Interstitial pneumonia	1(3)
Home oxygen therapy: No. (%)	15(38)
Mean time to revaccination: months (SD)	91(3.7)

SD, standard deviation.

2.5. Adverse reactions

Subjects were provided a diary to record their body temperature and any local or systemic reactions that occurred from the day of secondary vaccination to day 14. They were instructed to assess the maximal diameter of any redness or swelling at the site of injection; this was expressed as mild for a maximum diameter of 1–5 cm, as moderate for a maximum diameter ≧5 cm, and as severe for a maximum diameter ≧10 cm. A systemic symptom was considered mild when the subjects felt a certain symptom but had no difficulty in daily life. A physical examination with an interview was conducted to record the condition of the study subject on the day of secondary vaccination and 14 days after. Data of adverse reactions after primary vaccination were used for comparison with those after secondary vaccination [23].

2.6. Statistical analysis

Average antibody concentrations and increases were expressed as geometric means. Differences in the geometric mean concentrations (GMCs) of serotype-specific IgG, the OIs, or the IgG required for 50% killing were assessed by the Wilcoxon matched-pairs signed-ranks test. The frequencies of adverse reactions were compared between primary and secondary vaccinations by the Student *t*-test. Differences with a P value <0.05 were considered to be statistically significant.

3. Results

The subject patient group comprised 18 males and 22 females, and all of them were Japanese (Table 1). Four subjects were in their 60s, 22 in their 70s, and 14 in their 80s; the mean age was 77 years. The mean interval between primary and secondary vaccinations was 7 years and 7 months. Their comorbid illnesses included sequelae of pulmonary tuberculosis (33%), bronchiectasis (18%), bronchial asthma (18%), nontuberculous mycobacterial infection

Table 2
Comparison of geometric mean concentrations (GMCs) and geometric increases (*n*-fold) in levels of serotype-specific IgG in sera and opsonization index (OI) of sera from 40 study subjects before and after primary and secondary vaccination.

Serotype	Time point	GMC of IgG (mg/ml) (95% CI)	Geometric mean increase (*n*-fold; post/pre)	GMT of OI (95% CI)	Geometric mean increase (*n*-fold; post/pre)
6B	Pre dose 1	1.29 (1.02–1.64)		75 (44–129)	
	Post dose 1	2.99 (2.18–4.10)[a]	2.32	557 (327–949)[a]	7.43
	Pre dose 2	1.65 (1.20–2.28)		267 (142–500)	
	Post dose 2	2.73 (1.97–3.79)[b]	1.65	768 (489–1206)[b]	2.88
14	Pre dose 1	3.11 (2.36–4.12)		120 (67–216)	
	Post dose 1	8.75 (7.01–10.91)[a]	2.81	1028 (539–1958)[a]	8.55
	Pre dose 2	4.31 (2.82–6.58)[c]		344 (182–648)	
	Post dose 2	6.96 (4.70–10.32)[b,e]	1.61	699 (384–1273)[b]	2.03
19F	Pre dose 1	2.46 (2.05–2.94)		34 (19–62)	
	Post dose 1	4.61 (3.81–5.59)[a]	1.87	538 (325–892)[a]	15.77
	Pre dose 2	2.81 (2.20–3.58)[d]		158 (91–275)	
	Post dose 2	4.38 (3.53–5.44)[b]	1.56	556 (349–887)[b]	3.51
23F	Pre dose 1	1.31 (1.00–1.70)		17 (10–27)	
	Post dose 1	3.10 (2.34–4.11)[a]	2.37	142 (74–271)[a]	8.57
	Pre dose 2	1.57 (1.12–2.20)		47 (27–80)	
	Post dose 2	2.56 (1.75–3.75)[b]	1.63	179 (100–322)[b]	3.84

GMC, geometric mean concentration, GMT, geometric mean titer; CI, confidence interval; dose 1, primary vaccination; dose 2, secondary vaccination. Pre dose 1: before primary vaccination; post dose 1: after primary vaccination; pre dose 2: before secondary vaccination; post dose 2: after secondary vaccination. Sera were collected 1 month before and after primary and secondary vaccinations.

[a] $P<0.01$ (vs. pre dose 1).
[b] $P<0.01$ (vs. pre dose 2).
[c] $P<0.01$ (vs. pre dose 1).
[d] $P<0.05$ (vs. pre dose 1).
[e] $P<0.01$ (vs. post dose 1)

(15%), and others. Fifteen patients (38%) were on home oxygen therapy.

The GMCs of serotype-specific IgG for serotypes 14, 19F, 6B, or 23F of the study subjects were significantly elevated 1 month after primary vaccination ($P<0.01$) and remained slightly higher than that before primary vaccination, even at the time of prevaccination. The GMCs of serotype-specific IgG for serotypes 14 and 19F were significantly higher immediately before secondary vaccination compared with those before primary vaccination, whereas the GMCs of serotype-specific IgG for serotypes 6B and 23F before secondary vaccination did not significantly differ from those before primary vaccination. The GMCs of serotype-specific IgG for all serotypes significantly increased by secondary vaccination, but the GMCs of serotype-specific IgG for all serotypes 1 month after secondary vaccination were lower than those 1 month after primary vaccination. The proportions of the peak IgG levels 1 month after secondary vaccination compared with those 1 month after primary vaccination were 79.5–95.0% for all serotypes. The GMCs of serotype-specific OIs for all serotypes significantly increased 1 month after primary vaccination (Table 2). The GMTs of serotype-specific OIs 1 month after secondary vaccination were higher or comparable with those 1 month after primary vaccination for serotypes 6B, 19F, and 23F, but not for serotype 14. The GMTs of serotype-specific OIs for all serotypes decreased thereafter, but secondary vaccination significantly increased the GMTs of serotype-specific OIs at 1 month postvaccination. The GMCs of serotype-specific IgG required for 50% killing for all serotypes significantly decreased after primary vaccination ($P<0.01$; Fig. 1). The GMCs of serotype-specific IgG required for 50% killing was significantly elevated for serotype 19F but did not change for serotypes 6B, 14, and 23F during the interval between primary and secondary vaccinations. The GMCs of serotype-specific IgG required for 50% killing significantly decreased for serotypes 14, 19F, and 23F between before and after secondary vaccinations ($P<0.01$) but remained unchanged for serotype 6B.

No serious adverse reaction, such as anaphylactic shock, was found after secondary vaccination. The frequencies of local reactions, including local swelling, reddening, and pain, peaked 1 day after secondary vaccination and then gradually disappeared within 1 week (Table 3). Only one subject developed fever over 38 °C on the day of revaccination, while none of the other subjects developed fever. Several subjects developed systemic symptoms, including nausea, headache, muscle pain, joint pain, or malaise. These symptoms were found more frequently during the first 2 days after secondary vaccination and improved slowly thereafter. All adverse reactions disappeared within 2 weeks after secondary vaccination.

The frequency of local reactions, including pain and swelling or redness, was significantly higher in subjects after secondary vaccination than after primary vaccination (Fig. 2A). The frequency of systemic reactions, including headache, malaise, muscle pain, and joint pain, was also significantly higher in subjects after secondary vaccination than after primary vaccination (Fig. 2B).

4. Discussion

In this study, we demonstrated immunogenicity during the period from before primary vaccination to 1 month after secondary vaccination in a cohort of elderly patients with CLD. Significant increases in the GMCs of serotype-specific IgG or serotype-specific OIs occurred after primary and secondary vaccinations. Peak levels of serotype-specific IgG after PPV23 secondary vaccination were only 5–20% lower than peak levels after primary vaccination, which is in agreement with previous studies [19,20,28]. This phenomenon of hyporesponsiveness induced by PPV23 was found to be associated with a depletion of the memory B-cell population [29]. In contrast, we found serotype-specific OIs after secondary vaccination to be comparable with those after primary vaccination. Therefore, hyporesponsiveness was not found in opsonic activity after secondary vaccination. The GMCs of serotype-specific IgG or the OIs after primary vaccination declined within several years, but these levels before secondary vaccination were still higher than those before primary vaccination. The values of OIs

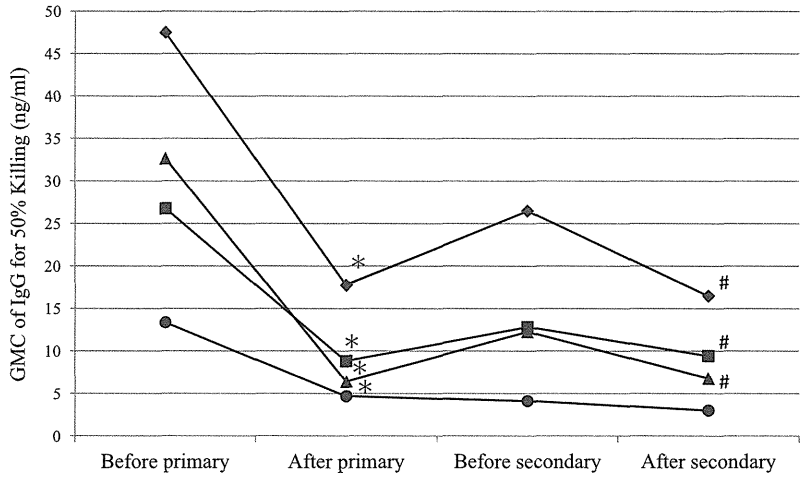

Fig. 1. The GMCs of serotype-specific IgG required for 50% killing of bacteria for serotypes 6B (closed circle), 14 (closed square), 19F (closed triangle), and 23F (closed diamond) before and after primary and secondary vaccinations with 23-valent pneumococcal polysaccharide vaccine. The number of subjects examined for serotypes 6B, 14, 19F, and 23F were 30, 33, 23, and 21, respectively. $^{*}P < 0.05$ versus before primary vaccination; $^{\#}P > 0.05$ versus 1 month before secondary vaccination.

for serotypes 14 and 23F before and after primary and secondary vaccination were comparable to those reported in a previous paper [20].

A previous study reported that serotype-specific IgG 30 days after secondary vaccination was modestly lower than that after primary vaccination, but the difference was transient and was resolved within several years after secondary vaccination [19]. Levels of OI were similar after primary or secondary vaccination, although levels 30 days after secondary vaccination were insignificantly lower than those 30 days after primary vaccination [20]. A recent study also demonstrated that serotype-specific IgG levels for seven of eight serotypes tested during 10 years after secondary vaccination were higher than those after primary vaccination [22].

Although we determined the levels of serotype-specific IgG, but not IgM or IgA, the serum opsonic activity correlated best with levels of serotype-specific IgG in healthy adults after vaccination with PPV23 [30,31]. Significant decreases in IgG levels required for 50% killing for serotypes of 6B, 14, 19F, and 23F after primary vaccination indicated an improved efficiency of opsonic activity in sera. These values of IgG required for 50% killing slightly increased during the mean interval more than 7 years, except for serotype 6B. However, these values for all serotypes were still lower than those before primary vaccination, suggesting a sustained functional serotype-specific IgG after primary vaccination. The values of IgG required for 50% killing in serotype 14, 19F, and 23F further declined after secondary vaccination. These findings suggest an improved efficiency of opsonic activity of serotype-specific IgG for these three serotypes after secondary vaccination. Our results may provide new insights for protective immunity more than five years after primary vaccination and after secondary vaccinations in the elderly population.

Among the previous studies of immunogenicity by PPV23 in patients with chronic lung diseases [8–12], we could directly compare the levels of serotype-specific IgG in the present study with those of our previous study in 13 hospitals, Japan [12], because the WHO approved ELISA [24,25] was used to determine the IgG levels for serotypes 6B, 14, 19F, and 23F in our previous study [12] and in the present study. The IgG levels in our previous study before primary vaccination with PPV23 were slightly higher for all serotypes than those after primary vaccination in the present study. Because of the higher prevaccination IgG level, the fold increases for all serotypes 1 month after primary vaccination in previous our study were lower than those in the present study.

It remains uncertain whether the immune response in adult patients with chronic lung diseases is inferior to that in healthy adults. We previously reported that 31% of 84 patients with chronic lung diseases were low responders to PPV23 [12]. Rubins et al. also reported that 20% of elderly patients with chronic illnesses

Table 3
Proportion of local and systemic adverse reactions after secondary vaccination among 40 subjects.

	Adverse reactions	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
	Maximal diameter of redness or swelling at the injection site	No. of subject (%)						
	<5 cm	9(23)	11(8)	6(15)	6(15)	8(20)	5(13)	3(7.5)
Local	≧5 cm	7(17.5)	11(27.5)	8(20)	6(15)	3(8)	1(3)	2(5)
	≧10 cm	0(0)	1(2.5)	2(5)	0(0)	0(0)	0(0)	0(0)
	Mild soreness in arm	13(33)	23(58)	15(37.5)	10(25)	6(15)	1(2.5)	2(5)
	Moderate soreness in arm	4(10)	4(10)	0(0)	0(0)	0(0)	0(0)	0(0)
	Severe soreness in arm	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Individual symptom							
	Nausea	1(3)	1(3)	1(3)	1(3)	1(3)	1(3)	3(8)
	Headache	3(8)	5(12.5)	1(2.5)	3(7.5)	6(15)	4(10)	2(5)
Systemic	Myalgia	4(10)	5(12.5)	2(5)	1(2.5)	1(2.5)	2(5)	3(8
	Arthralgia	0(0)	0(0)	0(0)	2(5)	2(5)	1(3)	1(3)
	Fatigue	6(15)	9(23)	5(13)	7(18)	7(17.5)	3(8)	4(10)
	Body temperature ≧38 °C	1(2.5)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)

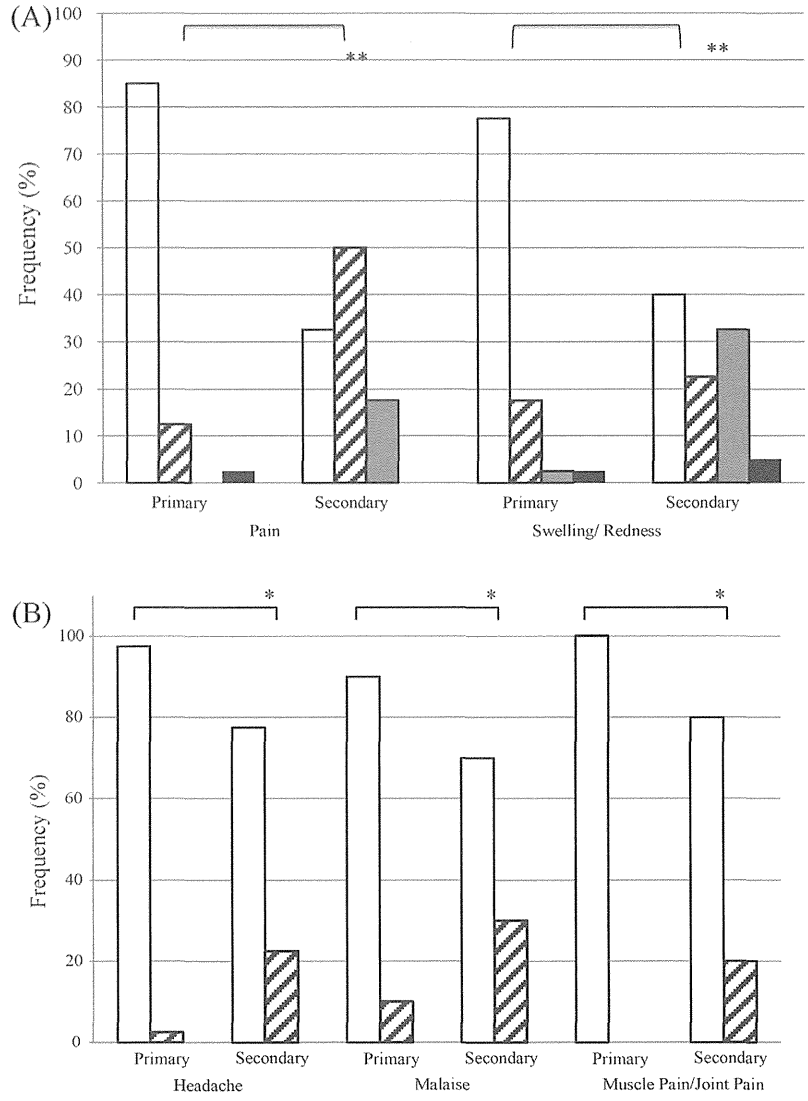

Fig. 2. Comparison of adverse reactions between primary and secondary vaccinations among the study subjects. Local adverse reactions (A) and systemic symptoms (B) were compared between primary and secondary vaccinations. Data are shown as the frequencies of subjects who received primary and secondary vaccinations ($n = 40$). The grade of finding or symptom was expressed as none (open bar), mild (slashed bar), moderate (gray bar), or severe (closed bar). $^{**}P < 0.01$, $^{*}P < 0.05$ versus primary vaccination.

including chronic lung diseases were poor responders, while none of the healthy young adults were poor responder [32]. Therefore, the great heterogenicity of the study subjects as shown in Table 1 might influence on the results of immunogenicity in this study.

Although we found self-limited local and systemic reactions to be more frequent after secondary vaccination than after primary vaccination, no serious systemic reaction was found after secondary vaccination. These findings are in agreement with two previously published reports [18,22].

The limitations of this study include (1) a small number of study subjects for comparative investigation of safety and immunogenicity between primary and secondary vaccination; (2) only four serotypes examined for the serotype-specific IgG and OIs; and (3) the antibody levels were examined only 1 month after secondary vaccination. In addition, it should be noted that the increased levels of opsonic activity after primary or secondary vaccination found in this study are not directly associated with protection of patients with chronic lung disease from IPD or non-bacteremic pneumococcal pneumonia, since the protective levels of OIs against such pneumococcal infections in adults remain unknown.

In conclusion, we demonstrated significant increases of GMCs of serotype-specific IgG and OIs after primary and secondary vaccinations with PPV23. The GMCs of serotype-specific IgG and OIs several years after primary vaccination were still higher than those before primary vaccination. Either primary or secondary vaccination conferred a long-term sustained and functional serotype-specific IgG. Our study also confirmed the safety of secondary vaccination with PPV23.

Acknowledgements

The authors are grateful to Yu T, Hayakawa M, and Hattori Y and for technical assistance in the measurement of the serotype-specific IgG and the opsonizing index, and to Uemura Y for analyzing the clinical and laboratory data. This work was supported

by research grants from the Ministry of Health, Labour, and Welfare of Japan.

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