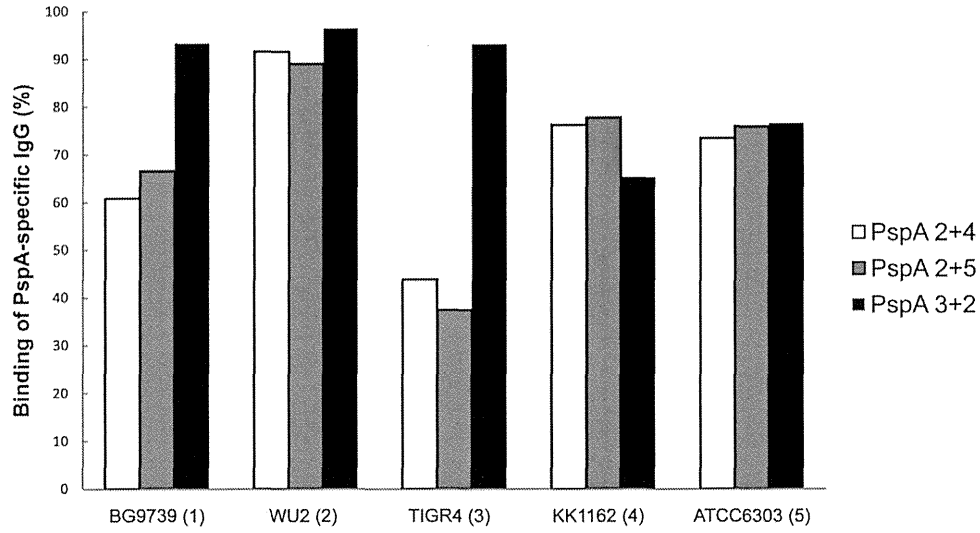

Fig. 3. The binding of PspA-specific IgG by antisera with PspA fusion proteins to the challenge strains with different clades. The mean percentages of fluorescent bacteria positive for IgG binding by antisera from mice immunized with PspA2+4, PspA2+5, or PspA3+2 in combination CpG ODNs plus AHG (double 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

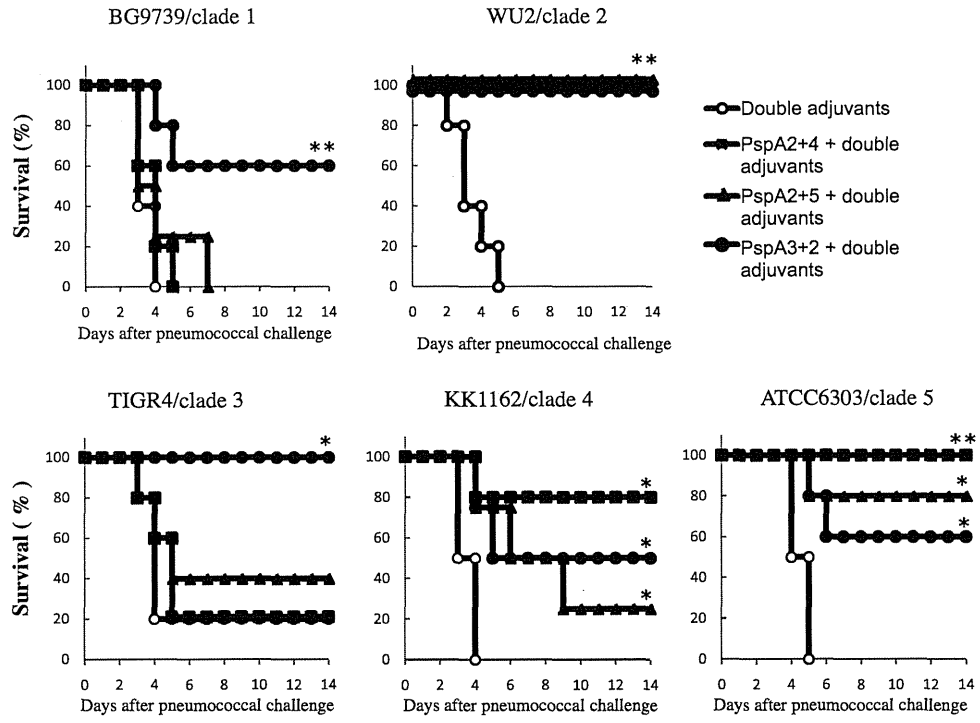

Fig. 4. Protective effects of immunization with fusion PspA proteins against pneumococcal challenge in mice. Mice were immunized subcutaneously with PspA2+4 (closed squares), PspA2+5 (closed triangles), or PspA3+2 (closed circles) in combination with CpG ODNs plus AHG (double adjuvants) or double adjuvants 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

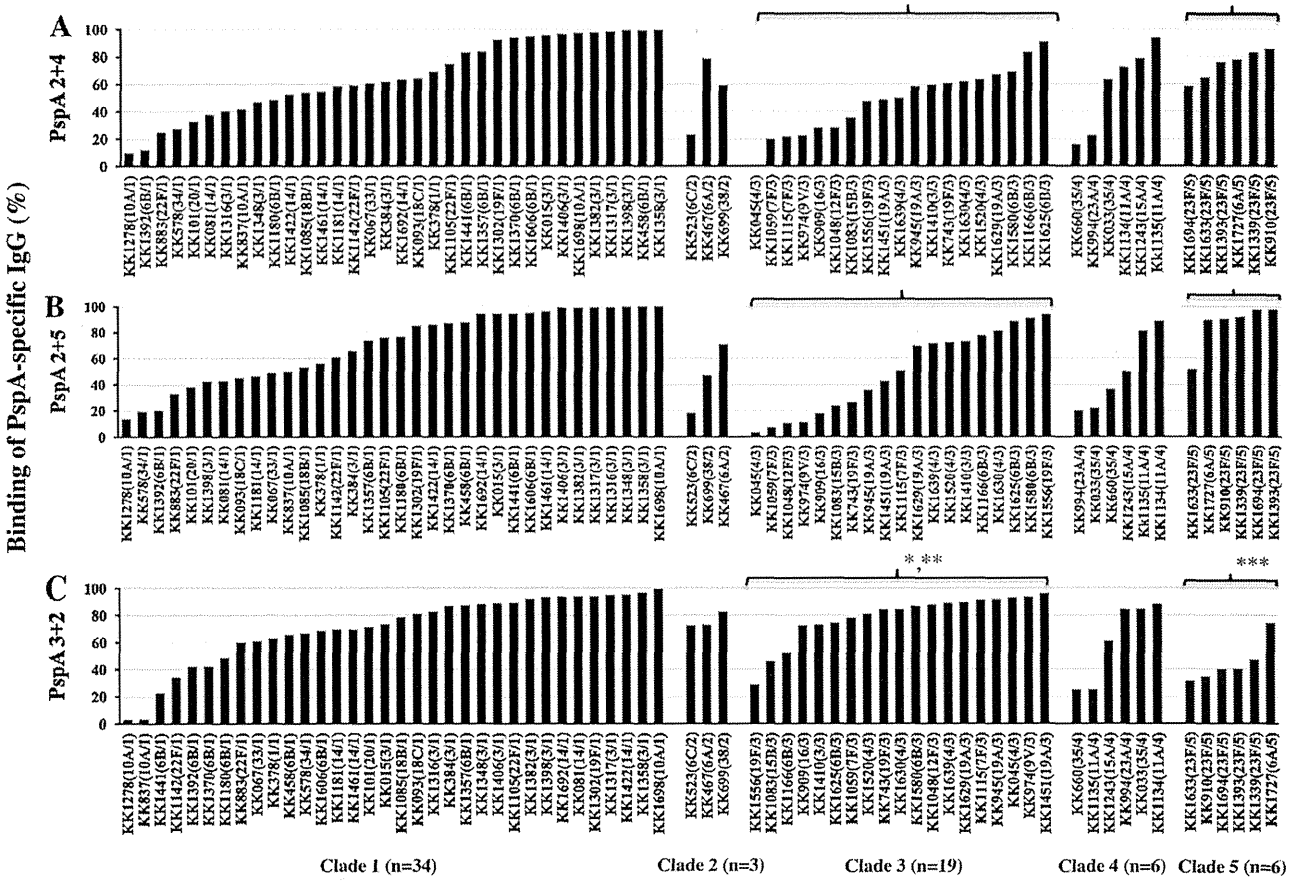

Fig. 5. Comparison of PspA-specific IgG binding by antisera with PspA2+4 (A), PspA2+5 (B), or PspA3+2 (C) in combination with CpG ODNs plus AHG (double adjuvants) to 68 pneumococcal isolates (34 for clade1, three for clade 2, 19 for clade 3, six for clade 4, and six for clade 5). The serotypes and PspA 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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Journal of Virology

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

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***J. Virol.* 2014, 88(22):13410. DOI: 10.1128/JVI.01205-14.**
Published Ahead of Print 10 September 2014.

JVI
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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-*

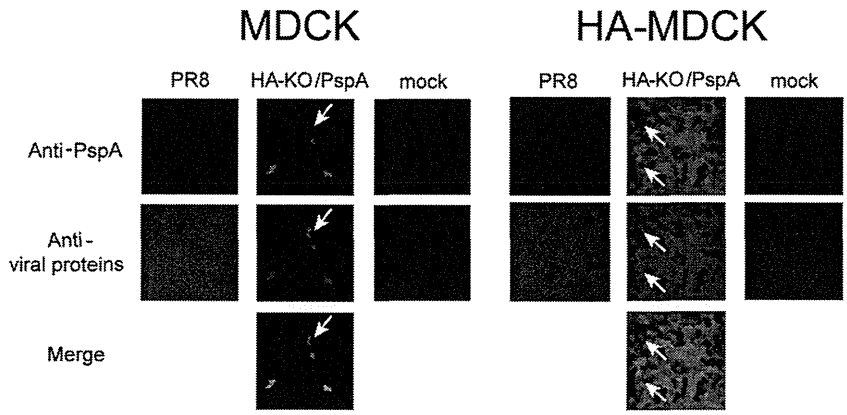

FIG 1 Expression of the PspA antigenic region and viral proteins in cells infected with PR8 or HA-KO/PspA virus. MDCK and HA-MDCK cells were infected with the indicated virus at an MOI of 0.0001, and an immunofluorescence assay was performed 36 h postinfection. PspA (green) and viral proteins (red) were 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).

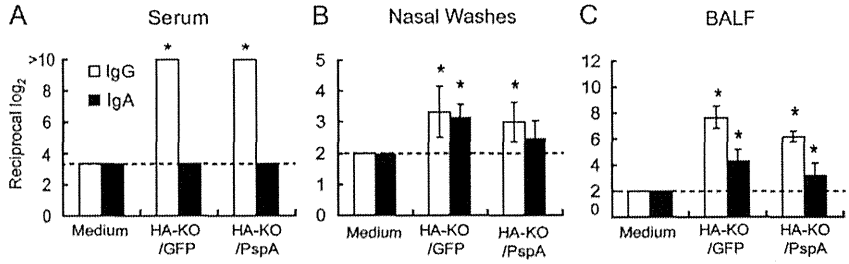

FIG 2 Induction of influenza virus-specific IgG and IgA in serum (A), nasal wash (B), and BALF (C) samples. Mice were intranasally inoculated with medium, HA-KO/GFP virus, or HA-KO/PspA virus with a 2-week interval between the inoculations. Samples from six mice from each group were collected 2 weeks 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.

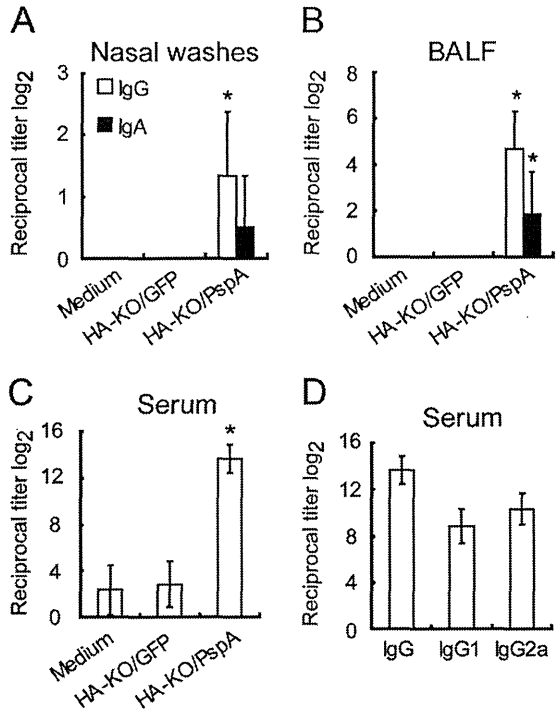

FIG 3 Induction of PspA-specific IgG and IgA levels in nasal wash fluids (A) and BALF (B) and IgG levels in sera (C), as well as IgG1 and IgG2a levels in sera (D). Mice were intranasally inoculated with medium, HA-KO/GFP virus, or HA-KO/PspA virus with a 2-week interval between the inoculations. Samples 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.

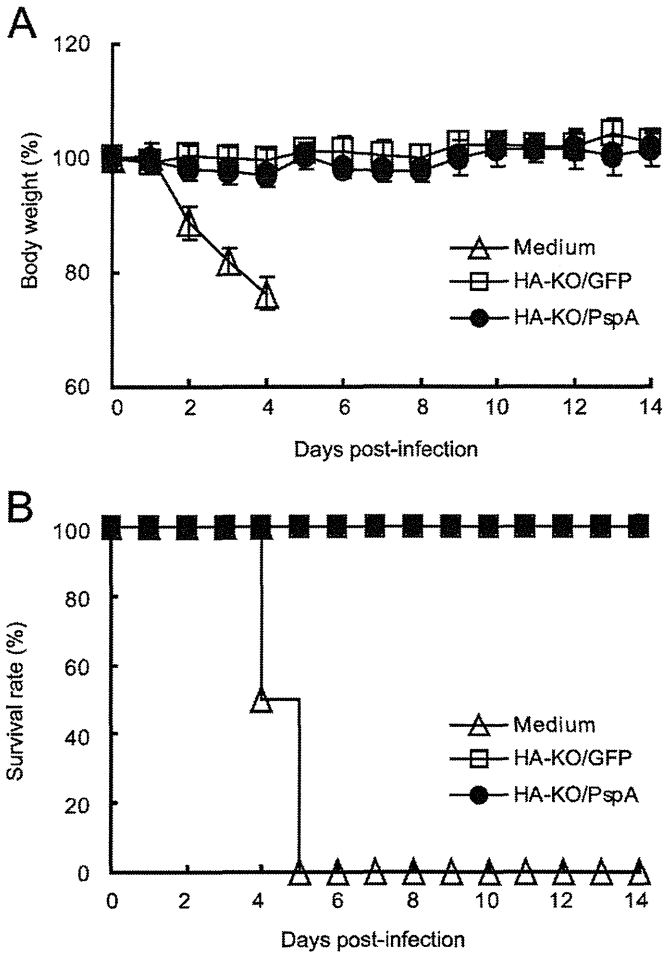

FIG 4 Body weight changes and survival curves for mice challenged with lethal doses of PR8. Eight mice per group were intranasally infected with 100 MLD_{50} of PR8 2 weeks after their final vaccination. Body weights were measured (A) and survival rate was monitored (B) for 14 days after infection. Open 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).

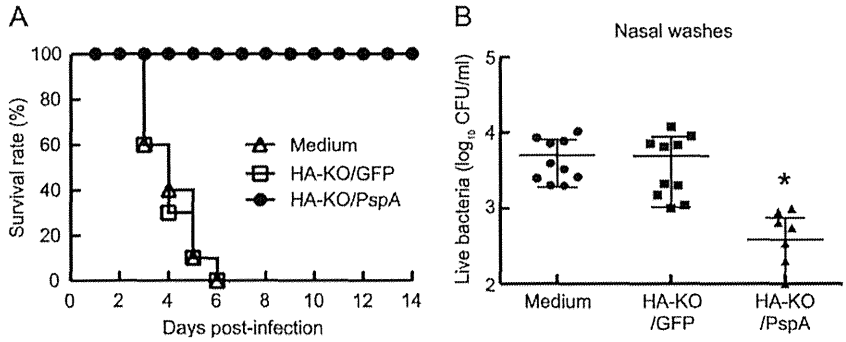

FIG 5 Survival curves for mice challenged with lethal doses of *S. pneumoniae* strain WU2 and bacterial densities in the nasopharynx 5 days after challenge with *S. pneumoniae* strain EF3030. (A) Ten mice per group were intranasally infected with 3 MLD_{50} of strain WU2 2 weeks after their final vaccination. 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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OUTBREAK OF *STREPTOCOCCUS PNEUMONIAE* SEROTYPE 3 PNEUMONIA IN EXTREMELY ELDERLY PEOPLE IN A NURSING HOME UNIT IN KANAGAWA, JAPAN, 2013

To the Editor: Streptococcus pneumoniae is the major cause of pneumonia in the elderly adults.[1] The vast majority of pneumococcal pneumonia cases are sporadic, although outbreaks of pneumococcal disease in crowded settings such as daycare centers,[2] nursing homes,[3] and military camps[4] have been reported.

In mid-April 2013, a local hospital in Kanagawa, Japan, identified a series of cases of pneumococcal pneumonia in nursing home unit residents aged 80 and older. The hospital physicians and local health center staff investigated the outbreak to confirm the etiological pathogen and to prevent disease transmission.

Demographic and clinical data of the residents were extracted from nursing home and hospital records using a standardized form. Confirmed pneumonia was defined as an episode of acute respiratory symptoms and evidence of a new infiltrate on chest radiography. Confirmed pneumonia was considered to be *S. pneumoniae* infection if *S. pneumoniae* was isolated from sputum or if a rapid urinary antigen test (Binax NOW *S. pneumoniae*, Binax, Portland, ME) was positive. Influenza-like illness (ILI) was defined as an episode of respiratory symptoms and sudden fever.

Thirty-one residents with physical disabilities and cognitive impairment resided in the nursing home unit at the end of March. Most were female aged 80 and older and bedridden. Twenty-seven (87%) residents had been vaccinated for influenza in the 2012–13 season, but only two (7%) had been vaccinated with the 23-valent polysaccharide pneumococcal vaccine (PPV23).

The index case presented symptoms on March 28. From that date until late April, 10 confirmed cases of pneumonia and 16 ILI cases were identified in residents. The attack rates of pneumonia and ILI were 32.2% and 83.9%, respectively. All individuals with pneumonia were hospitalized, and one died from heart failure. The characteristics of the residents with confirmed pneumonia were nearly identical to those of other residents; the median age was 87.5, and none had been vaccinated with PPV23. In the same period, six of 28 (attack rate = 21%) staff members presented with ILI; none developed pneumonia. The last case was recorded on April 25.

Sputum samples were available for seven of 10 confirmed pneumonia cases, five of which revealed *S. pneumoniae*. The other two confirmed pneumonia cases were positive for the urinary antigen, for a total of seven cases of pneumococcal pneumonia (Table 1). Eight of the 10 residents with confirmed pneumonia underwent a rapid influenza diagnostic test, and none was positive. Nasopharyngeal swab samples were also obtained from three residents with ILI on April 19, and one was *S. pneumoniae* culture positive.

All six *S. pneumoniae* isolates (five from residents with pneumonia patients one from a resident with ILI) were identified as serotype 3, and multilocus sequence typing demonstrated that these isolates belonged to sequence type (ST) 180. All isolates showed identical

Table 1. Clinical and Microbiological Findings of Individuals with Confirmed Pneumonia (N = 10)

Patient	Sex	Age	Cognitively Impaired	Underlying Conditions[a]	Care-Need Level	Onset Date	Sputum Culture/ Serotype	Urine Antigen Test for SP	Influenza Rapid Test	Outcome
1	Female	96	No	Yes	Fully supported	28-Mar	NA	Positive	NA	Recovered
2	Female	82	Yes	Yes	Not supported	8-Apr	PSSP/3	Positive	Negative	Recovered
3	Female	95	Yes	Yes	Partially supported	10-Apr	PSSP/3	NA	Negative	Recovered
4	Female	91	Yes	Yes	Fully supported	10-Apr	Methicillin-resistant *Staphylococcus aureus*	NA	Negative	Deceased
5	Female	84	Yes	Yes	Partially supported	13-Apr	PSSP/3	Positive	Negative	Recovered
6	Female	99	Yes	No	Fully supported	14-Apr	Methicillin-sensitive *Staphylococcus aureus*	Positive	Negative	Recovered
7	Female	80	Yes	Yes	Partially supported	16-Apr	NA	Negative	Negative	Recovered
8	Female	91	Yes	Yes	Fully supported	18-Apr	PSSP/3	NA	Negative	Recovered
9	Male	84	Yes	Yes	Partially supported	18-Apr	PSSP/3	Negative	NA	Recovered
10	Male	76	Yes	Yes	Fully supported	25-Apr	NA	NA	Negative	Recovered

[a]Hypertension, hyperlipidemia, diabetes mellitus, cerebrovascular diseases, chronic respiratory diseases, and malignant neoplasms.
NA = not available; SP = *Streptococcus pneumoniae*; PSSP = penicillin-susceptible SP.

pulsed-field gel electrophoresis patterns. These isolates were susceptible to penicillins, cephalosporins, carbapenems, and vancomycin and resistant to erythromycin and clindamycin.

Previous studies have documented pneumococcal disease outbreaks caused by various serotypes,[2–6] but no serotype 3 outbreak has been reported. Although systematic data on *S. pneumoniae* serotype and ST distributions are lacking in Japan, several studies have suggested that serotype 3 is one of the major pneumococcal pneumonia serotypes in adults[7,8] and that the ST180 clone is dominant in this serotype.[9] We believe that this outbreak became obvious because it occurred in the most vulnerable group; nearly all of the residents with patients were extremely old people with underlying diseases. Swallowing disturbances and impaired immunity increase the risk of pneumonia in this age group.[1] Nursing home residents in crowded living conditions are at an especially high risk of *S. pneumoniae* infection.[10]

At the time of the study, no national recommendation for PPV23 vaccination existed in Japan. Although local governments partially or fully subsidize the cost of PPV23, vaccine coverage remained less than 20% for people aged 65 and older in 2012, and the figure was lower in very elderly people. In the current study, only 7% of residents had received PPV23, and none with pneumonia had been vaccinated. Japan has the world's most rapidly aging population, and the number of elderly people who require long-term care is rising. Given that the pneumonia burden is expected to rise substantially in the coming decades, effective vaccination programs for adults, and particularly for extremely elderly people, must be implemented in Japan.

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ACKNOWLEDGMENTS

We thank the nursing home staff for assistance with data collection.

Conflict of Interest: None.

This work was supported by the Institute of Tropical Medicine, Nagasaki University, Japan.

Author Contributions: Study concept and design: Kuroki, Ishida, Suzuki, Furukawa, Ohya, Watanabe, Konnai, Aiharam, Morimoto. Acquisition of participants and data: Kuroki, Ishida, Suzuki, Furukawa, Ohya, Watanabe, Konnai, Aiharam, Chang, Ohnishi. Analysis and interpretation of data: Kuroki, Ishida, Suzuki, Ariyoshi, Morimoto. Preparation of manuscript: Kuroki, Ishida, Suzuki, Ariyoshi, Oishi, Ohnishi, Morimoto.

Sponsor's Role: None.

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小児科

Q ロタウイルスワクチン服用患者嘔吐時の対応法

2種類のロタウイルスワクチンである経口弱毒生ヒトロタウイルスワクチン（ロタリックス®）と5価経口弱毒生ロタウイルスワクチン（ロタテック®）を服用した患者が嘔吐した場合，再投与は必要か。また，そのほかの対応法があれば併せて。

（佐賀県　S）

A 原則として再投与は必要ないが，ワクチンの大半を嘔吐したと判断すれば考慮することもある。吐物の処理に際しては，弱毒生ワクチンであることに配慮する

ロタウイルスワクチンを嘔吐した場合の対応

現在は国内外で2種類のロタウイルスワクチンが使用されているが，いずれも経口投与する弱毒生ワクチンである。本剤を服用した児が嘔吐した場合の対応としては，2つの点に注意する必要がある。1つは有効性に関する事項で，再投与が必要か否かである。もう1つは，本剤は弱毒生ワクチンであり，感染性への配慮を含めた吐物の適切な処理方法である。

再投与の考え

①ロタリックス

「接種直後にワクチンの大半を吐き出した場合は，改めて本剤1.5mLを接種させることができる」と添付文書に記載がある。

②ロタテック

「接種直後に本剤を吐き出した場合は，その回の追加接種は行わないこと。（臨床試験において検討が行われていない。）」と添付文書に記載がある。

③再投与の必要性に関する考え方

2剤に関する我が国での添付文書を参照すると，前者は大半を嘔吐した場合は再投与，後者は嘔吐しても再投与は行わない，と薬によって対応に差異があるように思われる。しかし，これらの記載は，米国疾病予防管理センター（Centers for Disease Control and Prevention；CDC）が発信した予防接種諮問委員会（Advisory Committee on Immunization Practices；ACIP）による推奨事項を，それぞれ異なる出典から引用したものである（**表1**）。

前者は，1994年の経口ワクチンに関する推奨事項[1]が参考にされている。そこでは，「乳児ではしばしば経口薬［たとえば，経口生ポリオワクチン（oral poliovirus vaccine；OPV）など］を飲み込むことに失敗することがある。もし，ワクチン接種者の判断により，ワクチンの大半を吐き戻した，あるいは接種直後（たとえば，5～10分以内）に嘔吐したと考えられた場合には，改めてその来院時にワクチンを接種することができる」と記載されている。

後者は，2009年のロタウイルスワクチンに関する推奨事項[2]が参考にされている。国内および外国の臨床試験において，追加接種に関する検討が行われていないことから，「接種直後に本剤を吐き出した場合は，その回の追加接種は行わず，接種スケジュールに従い，接種を行うこと」と記載されている。

表1　経口ワクチンやロタウイルスワクチンに関する米国ACIPによる推奨事項

a. 1994年の経口ワクチンに関する推奨事項

Regurgitated Oral Vaccine Infants may sometimes fail to swallow oral preparations(e.g., oral poliovirus vaccine[OPV])after administration. If, in the judgment of the person administering the vaccine, a substantial amount of vaccine is spit out, regurgitated, or vomited shortly after administration(i.e., within 5～10 minutes), another dose can be administered at the same visit.	乳児は経口生ポリオワクチンなどの液剤の接種を失敗することがある。 接種者の判断において，ワクチン接種後すぐに（例：5～10分以内）ワクチンの大半を吐き出した場合，追加接種も可能である。

b. 2009年のロタウイルスワクチンに関する推奨事項

Regurgitation of Vaccine The practitioner should not readminister a dose of rotavirus vaccine to an infant who regurgitates, spits out, or vomits during or after administration of vaccine. No data exist on the benefits or risks associated with readministering a dose. The infant should receive the remaining recommended doses of rotavirus vaccine following the routine schedule (with a 4-week minimum interval between doses).	ロタウイルスワクチン接種中あるいは接種後にワクチンを吐き出した場合，再接種の必要はない。その理由は，再接種に関する有効性や安全性について検討したデータが存在しないためである。 被接種児はワクチンスケジュールに従って，最低でも4週間の間隔をおいて，残りの必要な接種回数を完了すること。

ACIP recommendation（文献1，2より作成）

実際の対応

現状での実際の対応としては，次のように考える。服用して一定の時間が経過している，嘔吐物が少量である，などの場合は，再投与の必要はない。一方，注意して投与すれば起こる頻度は高くないであろうが，もし投与直後にワクチンの大半を嘔吐した際には，再投与を考慮してもよい。

吐物の処理

身体に付着した吐物については，拭き取る，洗い流すなど標準予防策に基づいた対処を行う。衣類や物品については，ウイルス性胃腸炎を来す野生株への対応を準用して，次亜塩素酸や家庭用漂白剤などの塩素系消毒薬を用いて洗濯や拭き取りを行えば，より確実な感染制御策となる。

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◈回 答

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中野貴司

特集②

ワクチンの定期接種化
今後のあゆみ

中野貴司（川崎医科大学小児科教授）

Point
- ▶「ワクチン・ギャップ」解消をめざして，定期接種ワクチンの種類は増加しつつある
- ▶2014年10月から水痘ワクチンと成人用肺炎球菌ワクチンが定期接種に加わる
- ▶接種対象者や接種スケジュールを確認の上，ワクチンの普及に努めたい
- ▶ムンプス，B型肝炎，ロタウイルスワクチンについて，定期接種化に向けての検討が継続されている

1．定期接種の充実によりワクチン・ギャップの解消をめざす

2013年4月1日，改正予防接種法が施行された。本改正における大きな目的の1つは，海外先進諸国と比較して公的に接種するワクチン（定期接種）が少ないというわが国の現状，いわゆる「ワクチン・ギャップ」の問題を解消することであった。

本改正により，定期接種のA類疾病にインフルエンザ菌b型（*Haemophilus influenzae* type b：Hib）感染症，小児の肺炎球菌感染症，ヒトパピローマウイルス（human papillomavirus：HPV）感染症が追加された。HPVワクチンについては定期接種導入直後に国内で安全性についての懸念事項に関する議論が湧き上がり，いまだ普及には程遠い状況である。一方，Hib感染症と小児の肺炎球菌感染症については，結合型ワクチンの小児への接種普及に伴い，細菌性髄膜炎をはじめとするこれらの菌による小児の侵襲性感染症（invasive infection）の発症報告が大幅に減少し，既にワクチンによる素晴らしい疾病予防効果が認められている。

改正された予防接種法では，予防接種の総合的な推進を図るための計画策定が謳われ，予防接種施策の立案にあたって評価・検討組織への付議が定められ，厚生科学審議会に新しく設置された予防接種・ワクチン分科会で議論が重ねられてきた。その過程で，水痘と成人（高齢者）用肺炎球菌ワクチンは2014年10月からの定期接種が決定され，ムンプス，B型肝炎，ロタウイルスワクチンは今後の定期接種化に向けて検討が継続されている。

2．2014年10月に定期接種化――水痘，成人用肺炎球菌ワクチン

2013年4月以降，予防接種・ワクチン分科会および予防接種基本方針部会において，現行では任意接種の各種ワクチンについて，定期接種化を念頭に置いた接種対象者や接種方

表1　水痘ワクチン定期接種の接種対象者と接種方法

【対象者】
○生後12月から生後36月に至るまでの間にある者

【接種方法】
○乾燥弱毒生水痘ワクチンを使用し，合計2回皮下に注射する。3月以上の間隔をおくものとして，接種量は毎回0.5mLとする。

【標準的な接種期間】
○生後12月から生後15月に至るまでに初回接種を行い，追加接種は初回接種終了後6月から12月に至るまでの間隔をおいて1回行う。

【経過措置】
○生後36月から生後60月に至るまでの間にある者を対象とし，1回注射する。
ただし，平成26（2014）年度限りとする。

【その他】
○既に水痘に罹患したことがある者は接種対象外とする。
○任意接種として既に水痘ワクチンの接種を受けたことがある者は，既に接種した回数分の接種を受けたものとみなす（経過措置対象者も含む）。
○当該疾病はA類疾病として規定される。

（文献1より改変）

法などに関する技術的な検討が行われてきた。その結果，水痘，成人用肺炎球菌の2ワクチンについては，おおむね技術的な課題の整理が完了した。定期接種ワクチン追加のためには必要となる財源の捻出も必要であるが，地方財政措置に関して調整が図られた。

これらの経過を経て，水痘，成人用肺炎球菌の2ワクチンの定期接種化が決定した。水痘は主に集団予防を図ることが目的であるA類疾病，成人用肺炎球菌は個人予防目的に比重のあるB類疾病に位置づけられた。定期接種化の実施時期については，ワクチンの供給状況，自治体の準備期間，国民への周知期間などを勘案して，2ワクチンとも2014年10月からとなった。

1 水痘ワクチン

定期接種のスケジュールは，生後12カ月（1歳）以上，生後36カ月（3歳）未満の者に対して，乾燥弱毒生水痘ワクチンを合計2回接種する。2回の接種間隔は3カ月以上あける。標準的には，生後12カ月から生後15カ月の間に初回接種を行い，追加接種は初回接種終了後6カ月から12カ月間隔をあけて行う（**表1**）[1]。

なお，平成26（2014）年度に限り経過措置が定められており，生後36カ月（3歳）以上，生後60カ月（5歳）未満の者に対する1回の接種が定期接種として認められる。

接種不適当者については，発熱や急性疾患などワクチン全般に共通するもの以外に特記事項はない。また，水痘の既往歴がある者は定期接種の対象外である。

任意接種として過去に水痘ワクチンの接種を受けたことがある者は，既に接種した回数

表2　成人用肺炎球菌ワクチン定期接種の接種対象者と接種方法

【接種対象者】
①65歳の者〔経過措置終了後の平成31（2019）年度より実施〕
②60歳以上65歳未満の者であって，心臓，腎臓もしくは呼吸器の機能またはヒト免疫不全ウイルスにより免疫の機能に障害を有する者（インフルエンザの定期接種対象者と同様）

【接種方法】
○肺炎球菌ワクチン（ポリサッカライド）を使用し，1回筋肉内または皮下に注射する。接種量は0.5mLとする。

【経過措置】
○平成26（2014）年度から平成30（2018）年度までの間は，前年度の末日に各64歳，69歳，74歳，79歳，84歳，89歳，94歳，99歳の者（各当該年度に65歳，70歳，75歳，80歳，85歳，90歳，95歳，100歳となる者）を対象とする。
例：平成26年度における65歳への接種については，平成25（2013）年度末日に64歳の者〔生年月日が昭和24（1949）年4月2日～昭和25（1950）年4月1日の者〕が対象となる。
○平成26年度は，平成25年度の末日に100歳以上の者（平成26年度101歳以上となる者）を定期接種の対象とする。

【その他】
○既に肺炎球菌ワクチン（ポリサッカライド）の接種を受けたことがある者は対象外とする。
○平成31年度以降の接種対象者については，経過措置対象者の接種状況や，接種記録の保管体制の状況等を踏まえ，改めて検討する。
○当該疾病はB類疾病として規定する。

（文献1より改変）

分の接種を受けたものとみなす。これは経過措置対象者にも適用される。すなわち，3歳以上5歳未満の者が過去に1回の接種を受けていれば，2回目の接種を行う際は定期接種とみなされず任意接種の扱いとなる。1歳以上3歳未満の者で過去に1回の接種歴がある場合は，2回目の接種は定期接種の扱いとなる。

2 成人用肺炎球菌ワクチン

65歳以上の高齢者に対して肺炎球菌ワクチンを定期接種として行うわけであるが，平成26（2014）年度から平成30（2018）年度までの間は，前年度の末日に各64歳，69歳，74歳，79歳，84歳，89歳，94歳，99歳の者（各当該年度に65歳，70歳，75歳，80歳，85歳，90歳，95歳，100歳となる者）を対象に接種するという経過措置が実施される。5年間かけて，65歳以上の人口集団を肺炎球菌ワクチンでカバーすることを意図したスケジュールである。経過措置終了後の平成31（2019）年度からは，65歳の者が接種対象となる。また，同じくB類定期接種であるインフルエンザと同様に，60歳以上65歳未満の者で，心臓，腎臓もしくは呼吸器の機能またはヒト免疫不全ウイルスにより免疫の機能に障害を有する者は定期接種の対象者である（**表2**）[1]。

さらに経過措置として，平成26（2014）年度は，平成25（2013）年度の末日に100歳以上