Table 1 Association of serotypes with multilocus sequence typing profiles and genetic *pbp* patterns among *S. pneumoniae* strains

Serotype	ST	CC	Genotype	No. of strains	Allele gene							First reported	
					aroE	*gdh*	*gki*	*recP*	*spi*	*xpt*	*ddl*	Year	Country (city)
1	**5239**	306	gPSSP	1	12	8	13	5	16	**336**	20		
3	**5234**	180	*2x*	1	7	15	2	10	6	1	**383**		
4	246	246	gPSSP	4	16	13	4	5	6	10	18	1997	UK
6A	3115	3115	gPRSP	2	7	32	6	1	6	14	14	1989	Korea
	2756	3787	gPRSP	1	8	8	19	16	77	1	68	2004	China
	5243	3787	gPRSP	1	8	8	4	16	77	1	26		
	3787	3787	*2x*	1	8	8	19	16	6	1	68	UN	Singapore
6B	90	156	gPRSP	7	5	6	1	2	6	3	4	1986	Spain
	3387	156	gPRSP	1	5	6	1	2	6	3	26	2002	Korea
	2983	156	*2x*, *1a+2x*	3	5	6	1	2	6	1	271	2003	Japan (Okayama)
	902	490	gPRSP	1	2	13	2	1	6	121	121	2000	Singapore
	2923	490	*2x*, *1a+2x*	2	2	13	2	5	6	121	29	2003	Japan (Kurume)
	5233	490	*2x+2b*	1	2	13	2	5	6	121	14		
	5232	Singleton	gPRSP	1	2	29	4	1	6	121	121		
	5238	Singleton	gPRSP	1	2	5	2	**149**	6	121	121		
	5244	Singleton	gPRSP	1	2	168	2	5	6	121	**385**		
	5245	Singleton	*2x*	1	2	13	19	5	6	124	29		
	2224	2224	gPRSP, *1a+2x*	2	7	12	7	1	116	14	29	1996	UK
	5235	2224	*1a+2x*	1	7	12	7	1	116	20	29		
	5230	180	gPRSP	1	7	15	2	10	6	1	**382**		
	2924	2924	*2x*	1	1	5	2	6	6	1	14	2003	Japan (Hyogo)
6C	2924	2924	*2x*	1	1	5	2	6	6	1	14	2003	Japan (Hyogo)
	5247	156	*2x*	1	1	29	8	6	6	6	14		
	2923	490	*2x*	1	2	13	2	5	6	121	29	2003	Japan (Kurume)
	5241	Singleton	*2x+2b*	3	7	9	8	6	1	6	**384**		
7C	2758	2758	gPSSP	1	10	5	1	1	9	220	8	2005	China
9V	280	280	*2x*, *2x+2b*	3	15	17	4	16	6	1	17	1998	Vietnam
	5231	280	*2x*	3	15	17	4	**148**	6	1	17		
10A	1263	280	*2x*	1	15	13	4	16	6	1	17	1998	USA
	5236	Singleton	gPSSP	1	7	12	1	1	10	1	11		
12F	4846	1527	*2b*	4	12	32	111	1	13	48	6	UN	Japan (Osaka)
14	343	554	gPRSP	2	8	8	4	15	39	12	14	1998	Norway
	236	320	gPRSP	1	15	16	19	15	6	20	26	1993	Taiwan
	5240	230	gPRSP, *1a+2x*	4	5	19	2	17	6	22	14		
	13	15	*1a+2x*	1	1	5	4	5	5	27	8	1997	Australia,USA
	2922	15	*1a+2x*	3	1	5	4	5	5	20	8	2003	Japan (Hyogo)
15A	63	63	*1a+2x*	1	2	5	36	12	17	21	14	1992	Sweden
15B	199	199	*1a+2x*	1	8	13	14	4	17	4	14	1987	Netherlands
15C	199	199	*2x*	1	8	13	14	4	17	4	14	1987	Netherlands
18C	3594	3594	gPSSP	1	10	13	34	16	6	1	31	2007	South Korea
19F	236	320	gPRSP	16	15	16	19	15	6	20	26	1993	Taiwan
	115	115	gPRSP	1	15	16	19	15	30	20	39	1994	Taiwan
19A	3111	3111	*2x*,*1a+2x*	2	61	60	67	16	10	104	14	1989	USA
	2331	2331	*2x*	1	10	16	150	1	17	1	29	1999	Czech
	5237	2331	*1a+2x*	1	10	16	150	1	30	1	29		
22F	433	433	*2x*	1	1	1	4	1	18	58	17	1997	Poland
23F	242	242	gPRSP	7	15	29	4	21	30	1	14	1996	Taiwan
	1437	1437	gPRSP	1	1	32	6	6	6	1	14	2000	Japan
	63	63	gPRSP	1	2	5	36	12	17	21	14	1992	Sweden
	338	156	gPRSP, *2x+2b*	3	7	13	8	6	1	6	8	1995	Colombia
	5246	156	*2x+2b*	1	7	13	8	6	1	138	8		
	5242	156	*2x+2b*	1	7	13	8	6	1	**337**	8		
23A	338	156	gPRSP, *2x+2b*	3	7	13	8	6	1	6	8	1995	Colombia
24	4982	4982	gPSSP	1	7	120	8	8	25	28	14	2001	USA
34	3116	Singleton	*2x*, gPSSP	3	10	8	6	1	9	1	279	2004	Japan (Okayama)
35	558	558	gPRSP	1	18	12	4	44	14	77	97	1998	South Korea
	2755	2755	*2x*	1	10	12	2	1	152	28	14	2004	China
38	393	393	gPSSP	1	10	43	41	18	13	49	6	1998	USA

New sequence types (STs) and alleles are shown in bold face and underlined. Country, first country of isolation for the same ST clone referred from the MLST database

CC clonal complex, *year*, first isolation year of the same ST clone referred from the MLST database

Table 2 Associations of isolation areas with serotypes and sequence types

Serotype	MLST type (n)	Area					
		Hokkaido Tohoku	Kanto	Chubu	Kinki	Chugoku Shikoku	Kyushu
4	246(4)		246(1)	246(2)			246(1)
6A	3115(2)		3115(1)			3115(1)	
6B	2923(2)				**2923(2)**		
	90(7)	90(3)	90(1)		90(2)		90(1)
	2983(3)		2983(3)				
	2224(2)				2224(1)		2224(1)
	2924(1)				**2924(1)**		
6C	2923(1)	**2923(1)**					
	2924(1)				**2924(1)**		
	5241(3)		5241(1)		5241(1)		5241(1)
9V	280(3)	280(1)		280(2)			
	5231(3)		5231(1)	5231(1)		5231(1)	
12F	4846(4)		4846(1)		4846(2)		4846(1)
14	343(2)			343(2)			
	5240(4)		5240(1)				5240(3)
	2922(3)		2922(1)		2922(1)		2922(1)
	236(1)		**236(1)**				
15A	63(1)		**63(1)**				
15B	199(1)						**199(1)**
15C	199(1)		**199(1)**				
19A	3111(2)		3111(2)				
19F	236(16)	**236(2)**	**236(4)**		**236(6)**	**236(1)**	**236(3)**
23A	338(3)		**338(2)**	**338(1)**			
23F	338(3)		**338(1)**		**338(2)**		
	63(1)				**63(1)**		
	242(7)	242(1)	242(2)	242(1)	242(1)	242(1)	242(1)
34	3116(4)	3116(2)		3116(1)	3116(1)		

Sequence types (STs) with two or more strains were selected. Novel STs are shown in bold face and underlined

The STs corresponding to the multiple serotypes are indicated in red bold face

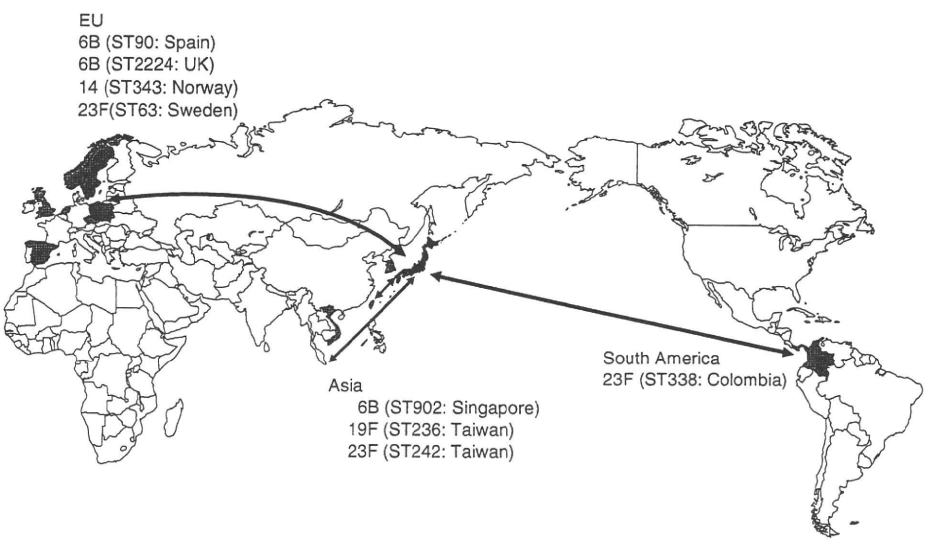

Fig. 3 Possible spread of prevalent serotypes, such as 6B, 19F, 23F, and 14, and corresponding sequence types between foreign countries and Japan

in serotype 19F, and ST63/ST242/ST338 in serotype 23F. As shown in the figure, pneumococcal strains now present in Japan and also other areas may have spread between Japan and European, Asian, or South American countries. In the future, clonal expansion is likely to result from human population drift.

Our manuscript represents the first report regarding associations of serotypes with MLST data and genotypic resistance classes based on PBP alterations in pneumococcal strains from children with meningitis in Japan. Pneumococcal MLST results have already been described in isolates from Japanese adults with community-acquired pneumonia [16]. Invasive pneumococcal disease is an important concern in Japan, and differences in serotype distributions between children and adults should be noted [21].

Continuous, accurate molecular epidemiologic surveillance regarding pneumococcal strains continues to be important in terms of global issues including vaccination and new antibiotic development.

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ORIGINAL ARTICLE

Anti-polyribosylribitol phosphate antibody in pediatric patients with *Haemophilus influenzae* type b invasive disease

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Abstract *Haemophilus influenzae* type b conjugate vaccine was recently introduced to Japan for voluntary immunizations. *H. influenzae* type b remains a leading cause of pediatric invasive diseases in Japan. The purposes of this study were to verify the suitability of the *H. influenzae* type b conjugate vaccine for immunizing children with a history of invasive *H. influenzae* type b disease and to determine whether *H. influenzae* type b conjugate vaccine is immunogenic in these children. The subjects comprised 64 children with a history of invasive *H. influenzae* type b disease. Serum samples from 64 patients with *H. influenzae* type b systemic infection in the acute and convalescent phases were analyzed. Serum anti-polyribosylribitol phosphate antibody responses of patients <2 years old were poorer than those observed in patients ≥2 years old. Nineteen of the 64 patients received a single dose of *H. influenzae* serotype b conjugate vaccine, and then follow-up serum was taken and analyzed. Eighteen of 19 patients had ≥1 μg/mL of anti-polyribosylribitol phosphate antibody titer after the first dose of *H. influenzae* type b conjugate vaccine. *H. influenzae* type b conjugate vaccine is immunogenic in children with invasive *H. influenzae* type b disease. Children <4 years old, and particularly <2 years old, with invasive *H. influenzae* type b disease should receive subsequent immunization with a *H. influenzae* type b conjugate vaccine.

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Introduction

Haemophilus influenzae is one of the leading causes of pediatric infectious disease, and *H. influenzae* type b (Hib) strains are known to constitute a major cause of invasive infections such as meningitis, sepsis and epiglottitis in children. More than 100 countries have introduced Hib vaccines as a part of routine immunization programs. As a consequence, the prevalence of infectious diseases caused by Hib has decreased dramatically [1, 2]. Hib vaccine is regarded as highly safe, and is widely used [3]. Hib vaccine has only recently been introduced into the voluntary immunization schedule in Japan, and Hib remains a leading cause of pediatric invasive infections, particularly meningitis, in Japan [4]. Most invasive Hib disease occurs in children <5 years old, with a peak incidence between 7 and 23 months old [5]. Hib is an encapsulated bacteria, with the capsule composed of polyribosylribitol phosphate (PRP). PRP antibody is an important protective antibody against invasive Hib disease. Children <2 years old may not develop protective antibodies to PRP after episodes of invasive Hib disease [6]. Furthermore, a subpopulation of children who have recovered from invasive Hib disease may also be at risk of developing a second episode of invasive Hib disease [7]. Strategies aimed at preventing a second episode of Hib disease in children with a history of Hib disease have included immunization with Hib conjugate vaccine.

The purposes of this study were to verify the suitability of the Hib conjugate vaccine for immunizing children with a history of invasive Hib disease and to determine whether

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the Hib conjugate vaccine is immunogenic in these children.

Patients and methods

Subjects comprised 64 children with a history of invasive Hib disease. The children were admitted to either the Department of Pediatrics at Chiba University Hospital or to 25 other hospitals located in various areas throughout Japan between 1997 and 2009. Diagnoses included meningitis ($n = 38$), epiglottitis ($n = 13$), sepsis ($n = 4$), cellulitis ($n = 3$), arthritis ($n = 3$), pneumonia ($n = 1$), endocarditis ($n = 1$), and osteomyelitis ($n = 1$). Five of the 64 children had a history of recurrent invasive Hib disease. These 5 children did not suffer from congenital immunodeficiency or congenital anomalies, for example cerebrospinal fluid fistula and Mondini anomaly. Serum samples from 64 children with invasive Hib disease in the acute phase and convalescent phase (2–3 weeks after admission) were analyzed. The number of serum samples in the acute phase obtained <24 h, 1–2 days, and 3–5 days after onset of symptoms were 16, 38, and 10, respectively. Nineteen of the 64 children received a single dose of Hib conjugate vaccine. Serum for analysis was taken just before vaccination and at follow-up (4–8 weeks after vaccination). Serum samples were transported to our laboratory and stored at −20°C until needed. Informed consent was obtained from the parents and permission from the health care provider of each child was obtained. All study protocols were approved by the Chiba University Institutional Review Board for Clinical Investigations. Anti-PRP antibody titers were analyzed using a Bindazyme anti-haemophilus B enzyme immunoassay kit (The Binding Site, Birmingham, UK). This is the only commercially available EIA kit for measurement of anti-PRP antibody. Schauer et al. measured anti-PRP antibody in serum samples of 386 age-stratified subjects using this EIA kit. They reported that in all unimmunized infants below 1 year of age the concentration of anti-PRP antibodies was <1.0 μg/mL [8]. To date, there has been no comparative data between standard radioantigen-binding assay and this EIA kit. Statistical analyses were performed using SPSS software (SPSS, IL, USA). Fisher's exact test was used to compare the proportion of children in the convalescent phase of infection with ≥1.0 μg/mL of anti-PRP antibody. Geometric mean titers (GMTs) were calculated for pre and post-immunization titers. Titers <0.1 μg/mL (the low cutoff of assay sensitivity) were considered equal to 0.1 μg/mL for the purposes of data analysis. Pre and post-immunization GMTs were compared using a paired t test on log-transformed data.

Results

Anti-PRP antibody titers were <0.15 μg/mL for 40 of the 64 children with invasive Hib disease in the acute phase, and <1 μg/mL for 63 of the 64 children. Anti-PRP antibody titer for one 3-year-old child with endocarditis was ≥1 μg/mL in the acute phase (1.13 μg/mL). Table 1 shows immune responses after Hib invasive disease according to age. All 5 children ≥4 years old responded with ≥1 μg/mL of anti-PRP antibody titer after invasive Hib disease. Anti-PRP antibody titers were <0.15 μg/mL for 19 of the 59 children <4 years old with invasive Hib disease in the convalescent phase, and <1 μg/mL for 42 of the 59 children.

Three of 5 children with recurrent Hib invasive diseases did not respond with anti-PRP antibody titer ≥1 μg/mL after a second episode of invasive Hib disease. Anti-PRP antibody responses of children <2 years old were poorer than those of patients ≥2 years old. Anti-PRP antibody responses of children with meningitis were poorer than those of children with epiglottitis. Nineteen of the 64 children had been given one dose of Hib conjugate vaccine.

Table 1 Immune response after Hib invasive disease according to age group

Diagnosis	0 Year	1 Year	2 Years	3 Years	4 Years	≥5 Years	Total
Meningitis	14 (1)	13 (0)	4 (0)	3 (0)	1 (1)	3 (3)	38 (5)
Epiglottitis	0	1 (1)	6 (6)	5 (4)	1 (1)	0	13 (12)
Sepsis	3 (0)	1 (0)	0	0	0	0	4 (0)
Cellulitis	1 (0)	1 (1)	1 (1)	0	0	0	3 (2)
Arthritis	0	2 (0)	1 (1)	0	0	0	3 (1)
Endocarditis	0	0	0	1 (1)	0	0	1 (1)
Pneumonia	0	0	0	1 (0)	0	0	1 (0)
Osteomyelitis	0	0	1 (1)	0	0	0	1 (1)
Total	18 (1)	18 (2)	13 (9)	10 (5)	2 (2)	3 (3)	64 (22)

Numbers in parentheses are the number of children with anti-PRP antibody ≥1.0 μg/mL in the convalescent phase

Table 2 Characteristics and antibody responses of children with Hib invasive disease

Diagnosis	Age at diagnosis (months)	Age at vaccine (months)	Pre-GMT (μg/mL)	Post-GMT (μg/mL)
Meningitis	5	10	<0.1	1.31
Meningitis[a]	5	20	0.82	8.90
Meningitis	6	8	<0.1	9.14
Meningitis	6	33	<0.1	9.42
Meningitis	7	15	<0.1	3.20
Meningitis	7	15	0.35	4.83
Meningitis	8	34	<0.1	9.50
Sepsis	10	41	0.35	0.45
Meningitis	12	14	<0.1	1.68
Meningitis	12	29	<0.1	14.0
Meningitis	13	53	<0.1	9.22
Meningitis	14	19	<0.1	8.92
Meningitis	15	24	0.27	16.05
Meningitis[a]	16	29	<0.1	8.64
Sepsis[a]	17	39	0.47	15.90
Meningitis	19	23	0.86	8.80
Meningitis	24	36	0.1	10.18
Meningitis[a]	29	30	3.82	6.15
Pneumonia	41	43	<0.1	7.36
GMT			0.198	6.20[b]

[a] Second episode of Hib invasive disease

[b] $P < 0.001$, Pre-GMT versus Post-GMT

Eighteen of the 19 children had anti-PRP antibody titer ≥1 μg/mL after administration of Hib conjugate vaccine (Table 2). No serious adverse reactions to the vaccine occurred in any child who received Hib vaccine.

Discussion

The most important factor for susceptibility to Hib is young age. This is explained by the inability of children <24 months old to produce PRP antibodies in sufficiently large amounts to protect against the disease [9, 10]. Anti-PRP antibody titers of 0.15 and 1 μg/mL have been established as the minimum levels required to achieve protection and long-term protection, respectively [11]. In our study, 19 (29.7%) of the 64 children had antibody levels <0.15 μg/mL after invasive Hib disease and 42 (65.6%) of the 64 children had <1 μg/mL antibody. In particular, 15 (41.7%) of 36 children <2 years old had <0.15 μg/mL antibody after invasive Hib disease and 33 (91.7%) of these 36 children had <1 μg/mL antibody, confirming previous observations that young children typically do not develop protective levels of antibodies to invasive Hib disease. Similarly, Walter et al. [12] reported that only 1 of 10 children ≥12 months old and none of 13 children <12 months old had significant antibody responses after recovering from invasive Hib disease. Furthermore, 9 (39.1%) of 23 children 2–4 years old with invasive Hib disease in our study did not have ≥1 μg/mL antibody and 4 (80.0%) of 5 children with recurrent invasive Hib diseases likewise did not achieve ≥1 μg/mL after a second episode of invasive Hib disease. Interestingly, the proportion of children with ≥1 μg/mL anti-PRP antibody in the convalescent phase was significantly higher for the 13 children with epiglottitis than for the 38 children with meningitis. Johnson et al. compared levels of anti-PRP antibody in a larger group of children with either epiglottitis or meningitis. According to their results, children with epiglottitis respond more vigorously in convalescence than those with meningitis, a finding that cannot be explained by age alone. They suggested that the poor convalescent-phase response was not a general feature of children with Hib meningitis, but was instead attributable to a sub-group of poor responders [13]. Host factors related to lower antibody responses with invasive Hib disease have yet to be determined and further studies are warranted.

The Hib conjugate vaccine is currently indicated for voluntary immunization of children at 2–59 months old in Japan. In this study we also measured the immunogenicity of the Hib conjugate vaccine (tetanus toxoid conjugate) in children with previous invasive Hib disease. Hib conjugate vaccine induced an immunogenic response in 18 of the 19 children tested. The mean age at vaccination was 27.1 months (range, 8–53 months). In a study similar to ours, Kaplan et al. [14] reported that 15 of 17 children responded with ≥1 μg/mL anti-PRP antibody after a single dose of Hib conjugate vaccine and all children responded

with ≥1 μg/mL anti-PRP antibody after two doses of Hib conjugate vaccine. Conversely, Walter et al. reported that only 9 of 19 children <15 months old responded with ≥1 μg/mL anti-PRP antibody after a single dose of vaccine. They suggested that a two-dose regimen should be considered for children <15 months old who are recovering from an episode of invasive Hib disease [12]. Hib conjugate vaccine is immunogenic in children with no anti-PRP response to invasive Hib disease, because children are most at risk of developing a second episode of Hib invasive disease within 6 months of the initial illness [7]. Indeed, our study included 5 children who experienced recurrent episodes of invasive Hib disease. Hib conjugate vaccine should optimally be used promptly after recovery from invasive Hib disease in any child <4 years old, particularly in those <2 years old, in Japan.

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Laboratory and Epidemiology Communications

Isolation of *Streptococcus pneumoniae* Serotypes 6C and 6D from the Nasopharyngeal Mucosa of Healthy Japanese Children

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Streptococcus pneumoniae, a primary causative agent of otitis media, pneumonia, bacteremia, and meningitis in children, results in substantial morbidity and mortality in many countries, including Japan (1–3). Of the 93 *S. pneumoniae* serotypes identified to date, serotypes 6C and 6D were recently differentiated from the classical serotypes 6A and 6B, respectively (4–6). Serotype 6C was subsequently reported to be isolated in several countries (5–9), especially as an important replacement serotype after introduction of the 7-valent pneumococcal conjugate vaccine (PCV7) (7,9,10). The naturally occurring *S. pneumoniae* serotype 6D was isolated from the Fiji Islands, Korea, and Poland (4,11,12). In this study, 32 6C and 1 6D *S. pneumoniae* isolates were identified from the nasopharyngeal mucosa of healthy children who had not received PCV7 residing on Sado Island, Niigata Prefecture, by using serological and genetic characterization.

S. pneumoniae, *Haemophilus influenzae*, and other pathogens among children residing on Sado Island, Niigata Prefecture, are monitored as part of the Sado Island, Antimicrobials, Day-care attendance, Older siblings (SADO) Study (13). In SADO study, which was conducted in 2008, pharyngeal swabs obtained from healthy children at check-up periods of 4, 7, 10, and 18 months old (mo) were cultured. Two of the children included had received PCV7. Fifty-two percent of the children at 18 mo had been attending day nursery. All *S. pneumoniae* isolates were serotyped using the conventional Quellung reaction using commercially available pneumococcal antisera (Statens Serum Institut [SSI], Copenhagen, Denmark) and home-made factor antiserum (designated factor 6dh [h indicates home-made]) for serotypes 6C and 6D. The factor 6b antiserum used in this study could react with both serotypes 6A and 6C; the new version of the factor 6b antiserum from SSI only reacts with serotype 6A (14,15). Factor 6dh antiserum was prepared by immunization of rabbits with formaldehyde-fixed serotype 6C whole cells and subsequent absorption of the antiserum with serotype 6A whole cells. In addition to the serological examination, serotypes 6C and 6D of the isolates were confirmed by genetic characterization involving comparison of the *wciN* region of 6A, 6B, 6C, and 6D isolates using PCR with primers 5106 and 3101 (5), and DNA sequencing of the *wciP* gene. The size of the *wciN* PCR products was determined by electrophoresis with 0.8% SeaKem GTG agarose gel (Takara Bio, Otsu, Japan). The DNA sequence of the *wciP* gene was determined using BigDye v1.1 (Applied Biosystems, Foster City, Calif., USA) and 3130*xl* Genetic Analyzer (Applied Biosystems). The antibiotic susceptibility of the isolates was analyzed by the microbroth dilution method according to the Clinical and Laboratory Standards Institute (CLSI M100-S18). Multi-locus sequence typing (MLST) was performed as described by Enright and Spratt (16).

A total of 337 *S. pneumoniae* isolates were obtained in this study. All isolates were initially serotyped using the Quellung reaction, and those that exhibited positive reactions with serogroup 6 antiserum were further tested using factor 6b, 6c, and 6dh antisera. Serotypes 6A and 6C exhibited positive reactions with factor 6b antiserum, whereas serotypes 6B and 6D exhibited positive reactions with factor 6c antiserum. Serotypes 6A and 6B exhibited negative reactions, and serotypes 6C and 6D exhibited positive reactions, with factor 6dh antiserum (Fig. 1). Thirty-two isolates (9.5%) exhibited positive reactions with both factor 6b and 6dh antisera, thus suggesting that they expressed the serotype 6C capsule. Furthermore, 1 isolate (0.3%) exhibited positive reactions with factor 6c and 6dh antisera, thus suggesting that it expressed serotype 6D capsule.

The *wciN* gene of the *S. pneumoniae* isolates was subsequently examined using PCR. The lengths of the PCR products for serotype 6A and 6B isolates found to be 2.0 (Fig. 2, lane 1) and 2.0/2.2 kb (Fig. 2, lanes 2 and 3), respectively. The length of each of the PCR products of the putative serotype 6C and 6D isolates was 1.8 kb (Fig. 2, lanes 4 and 5). The 2.0- and 2.2-kb *wciN* PCR products indicate the presence of capsular polysaccharide (PS) containing galactose, whereas the 1.8-kb PCR product indicates substitution of galactose by glucose (5). The DNA sequences of the *wciP* gene were determined for the isolates (4,5,11). The 138th amino acid residue in WciP for the 6A isolate is serine (AGT),

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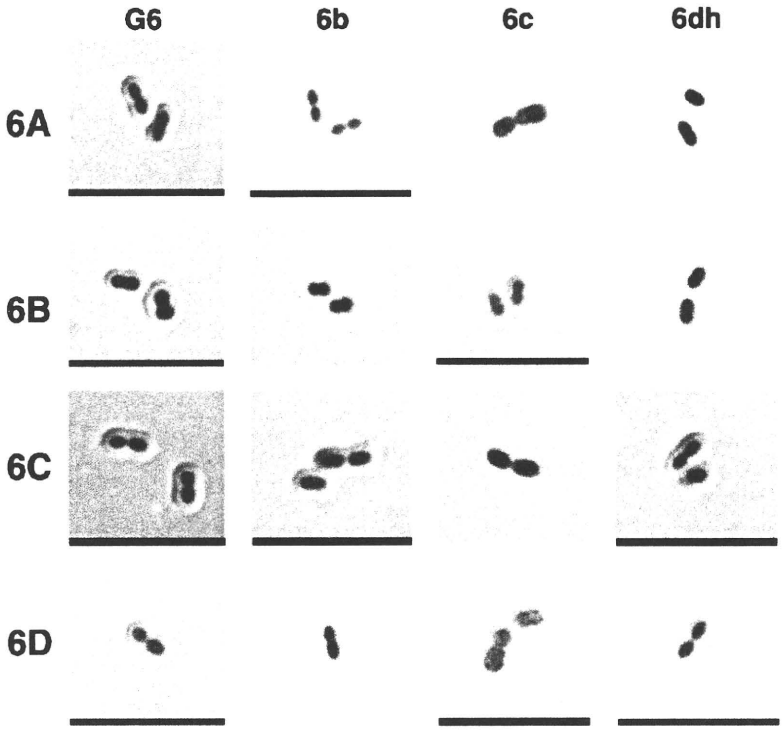

Fig. 1. Quellung reaction of *Streptococcus pneumoniae* serotypes 6A, 6B, 6C, and 6D. *S. pneumoniae* serotypes 6A (SP128) and 6B (KSP120) were isolated from cerebrospinal fluid. *S. pneumoniae* 6C (SP569) and 6D (SP687) were isolated from nasopharyngeal mucosa in this study. The antisera used are indicated on top of each column. G6, antiserum for serogroup 6; 6b, factor antiserum 6b; 6c, factor antiserum 6c; 6dh, home-made factor antiserum 6dh. Serotypes of *S. pneumoniae* are indicated on the left of the photographs. The underlined photographs illustrate positive results.

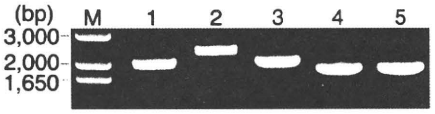

Fig. 2. PCR products of the *wciN* region of *Streptococcus pneumoniae* serogroup 6 isolates. M, 1 kb plus DNA ladder; lane 1, serotype 6A (SP128); lane 2, serotype 6B (KSP123); lane 3, serotype 6B (KSP120); lane 4, serotype 6C (SP569); lane 5, serotype 6D (SP687). The 2.0-kb or 2.2-kb fragments were obtained from serotype 6A (2.0-kb only) and 6B (2.0-kb or 2.2-kb) isolates, whereas the 1.8-kb fragments were obtained from serotype 6C and 6D isolates.

whereas that for the 6B isolate is asparagine (AAT) (17). The former amino acid is responsible for the rhamnose-(1→3)-ribitol linkage in the PS of serotype 6A, whereas the latter is responsible for the rhamnose-(1→4)-ribitol linkage in the PS of serotype 6B. The corresponding amino acids of the putative 6C and 6D isolates were serine and asparagine, respectively. The serological and genetic analyses yielded identical results in that both were consistent with the PS structure [→2)-glucose-(1→3)-glucose-(1→3)-rhamnose-(1→3)-ribitol-(5→phosphate] for 6C and [→2)-glucose-(1→3)-glucose-(1→3)-rhamnose-(1→4)-ribitol-(5→phosphate] for 6D, thus confirming the colonization of *S. pneumoniae* serotype 6C and 6D isolates in the nasopharynx of healthy Japanese children.

The 32 6C *S. pneumoniae* isolates were obtained from a total of 30 children (3 from 4-mo children, 5 from 7-mo children, 13 from 10-mo children, and 11 from 18-mo children); 2 of the isolates were obtained from the same child at 7- and 10-mo, and a furthre 2 isolates, which showed different colony morphologies and different antibiograms, were simultaneously obtained from a child at 18 mo. The *S. pneumoniae* serotype 6D was isolated from an 18-mo child. None of the children who carried the *S. pneumoniae* serotypes 6C or 6D had received PCV7. As for the children's residential area and day nursery attendance, there was no obvious association between the 30 children from whom the *S. pneumoniae* serotype 6C was isolated. The minimum inhibitory concentration (MIC) of penicillin G for the serotype 6C isolates ranged between ≤ 0.015 and 0.25 μg/ml, and that for 26 (81.3%) of the isolates being ≤ 0.06 μg/ml. All of the 6C isolates were susceptible to both cefotaxime (MIC ≤ 1 μg/ml) and meropenem (MIC ≤ 0.25 μg/ml), whereas 30 (93.8%) of them were resistant to erythromycin (MIC ≥ 1 μg/ml). The 6D isolate was susceptible to penicillin G (0.03 μg/ml), cefotaxime (0.25 μg/ml), and meropenem (≤ 0.008 μg/ml) but resistant to erythromycin (≥ 8 μg/ml). MLST analysis revealed that the frequent sequence types (STs) of the serotype 6C isolates were ST2923 (40.6%) and ST2924 (31.3%), whereas the ST of the 6D isolate was ST2924. The MLST analysis showed that the serotype 6C isolates from children on Sado Island comprised multiple clones.

The routine immunization of infants and toddlers in the United States with PCV7 has successfully reduced

the incidence of invasive pneumococcal disease (IPD) in children caused by the vaccine serotypes (18–20). Vaccination of children with PCV7 has also lowered the incidence of IPD among the elderly, a phenomenon known as the herd-immunity effect (18–20). The observed reduction in the incidence of IPD among the nonimmunized population is likely to be due to a change in the nasopharyngeal colonization of *S. pneumoniae* in immunized individuals. There has, however, been a rise in the incidence of IPD caused by non-PCV7 serotypes (known as replacement serotypes), including serotypes 19A, 6C, and others, in the United States (7,9,19, 21–24). As far as 6D is concerned, this serotype was isolated at a high rate (41%) from the nasopharyngeal mucosa of Fijian children, 86% of whom had received at least 1 dose of PCV7, thereby suggesting that serotype 6D may have a selective advantage after immunization with the vaccine (11). In addition, 5 IPD cases due to *S. pneumoniae* serotype 6D were reported in Poland (12). Because serotypes 6C and 6D were recognized after the introduction of PCV7, the surveillance data for infection with these serotypes in the United States and other countries are retrospective (12,18,19). PCV7 was released in Japan in February 2010 and widespread PCV7 vaccination is expected to lead to a similarly large reduction in pneumococcal infections, including IPD, pneumonia, and otitis media, in both the immunized and nonimmunized populations to that observed in other countries. We have initiated a population-based study to monitor the changes in IPD incidence and the serotype distribution among Japanese children, and we are also monitoring the colonized *S. pneumoniae* in the nasopharynx of healthy children. Initial results showed that *S. pneumoniae* serotype 6C was isolated from less than 2% of IPD cases without PCV7 vaccination (unpublished data) but could be isolated from the nasopharyngeal mucosa of 9.5% of the healthy children. PCV7, which includes only serotype-6B conjugate, would not affect the colonization or infection by *S. pneumoniae* serotypes 6C and/or 6D. A prospective surveillance on both colonization and infection by *S. pneumoniae* serotypes 6C, 6D, and others is therefore warranted to obtain an accurate evaluation of the effects of the 7- and 13-valent conjugate vaccines.

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Conflict of interest None to declare.

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肺炎球菌感染症

和田 昭仁

肺炎球菌について

肺炎球菌は，小児，成人の上咽頭にコロナイズすることがある一方，呼吸器感染の主要な起炎菌であり，また中耳炎，菌血症/敗血症，髄膜炎の起炎菌としても重要である．肺炎球菌が持つ莢膜は，最も重要な病原因子であり，莢膜を持つ肺炎球菌は，持たない菌に比べて著しく病原性が強いことが知られている[1]．これは，莢膜が主として白血球貪食抵抗因子として働くためである[2]．莢膜は多糖(ポリサッカライド)からなり，その抗原性の違いから91種類の型(遺伝子組み換えにより人工的に作り出した型を含めると92種類)に分類されている．それぞれの型に特異的な抗体を用いることにより，血清型別を行うことができる．**図**に，肺炎球菌とその型に特異的な血清を反応させた顕微鏡像を示す．黒く見えるのが菌体(実際には色素により青く染まっている)，菌体を取り囲むように見えているのが莢膜と抗体の複合体である．通常は菌体周囲の莢膜を観察することはできないが，莢膜と抗体の複合体は，抗体と反応していない莢膜より高い屈折率を持つため，陽性反応ではこのような像が得られる．**表**に肺炎球菌の血清型を示す．Typeのカラムに記載した数字にはそれぞれ1種類の型，Groupのカラムに記載した数字には複数の型が含まれ，例えば19F，19A，19Bといったように数字と大文字のアルファベットでそれぞれの型を表す．

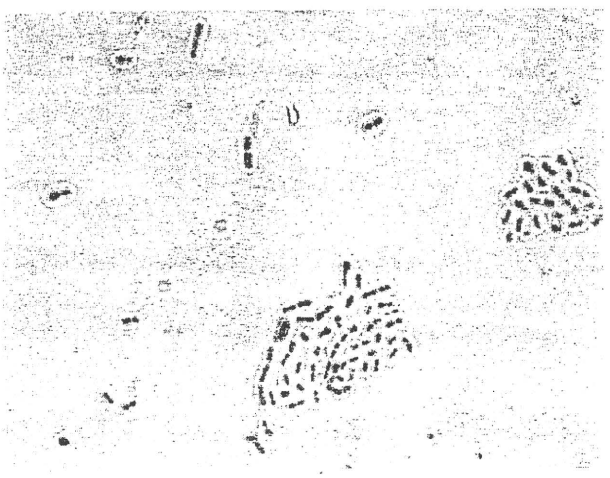

図　莢膜膨化反応

疫学

近年の推計によると，全世界で，肺炎球菌感染による5歳未満小児の死亡数は年間82万人とされている[3]．日本の小児の侵襲性感染症(血液，髄液など本来であれば無菌である部位から菌が検出される感染症)罹患率に関しては，厚生労働省研究班により，2007年から1道9県で調査が行われており，総合データの発表が待たれている．以前には，1999～2004年の北海道道北地域の5歳未満および2歳未満小児の肺炎球菌菌血症罹患率は，それぞれ30.9/10万/年，61.4/10万/年であったと報告されている[4]．また，1988～2002年の同地域の5歳未満小児肺炎球菌性髄膜炎罹患率は0.7～2.7/10万/年と報告されている[5]．小児の肺炎球菌性肺炎罹患率，中耳炎罹患率は不明であ

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るが，菌血症罹患率を大幅に上回るものになるであろう．一方，成人，特に高齢者における肺炎球菌感染症は重要な問題で，国内における罹患率の数字はないが，米国では23価肺炎球菌ワクチン罹患率の後ろ向きコホート研究で，ワクチン未接種群の肺炎球菌菌血症は，68/10万/年の割合で見られたとの報告がある[6]．

診断と治療

日本呼吸器学会から，『成人市中肺炎診療ガイドライン』，『成人気道感染症診療の基本的考え方』，『成人市中肺炎診療の基本的考え方』が出されている．米国からは，成人市中肺炎診療に対するInfectious Diseases Society of AmericaとAmerican Thoracic Societyからのコンセンサスガイドラインが発表されている[7]．いずれの出版物/文献にもリスク分類に基づく肺炎球菌性肺炎の治療に関する詳しい記載がある．小児感染症に関しては，日本小児呼吸器疾患学会と日本小児感染症学会から『小児呼吸器感染症診療ガイドライン〈2007〉』が出版されている[8]．

1. ペニシリン感受性，低感受性，耐性の分類

前述のコンセンサスガイドライン[7]では，ペニシリンのMICが2 μg/mL以上の肺炎球菌を耐性としているが，2008年に出されたClinical Laboratory Standards Institute M100-S18では，髄液以外の検体から分離された肺炎球菌は，ペニシリンGのMIC 4 μg/mL以上が耐性と分類されるようになった[9]．一方，髄液から分離された肺炎球菌は，ペニシリンGのMIC 0.12 μg/mL以上が耐性と分類されるようになった[9]．

2. 尿中抗原検出

診断用にイムノクロマト法を利用した尿中抗原検出キット(Binax NOW *Streptococcus pneumoniae*)が利用可能である．これは，肺炎球菌のC-ポリサッカライドを検出するキットであり，すべての血清型の肺炎球菌を検出することができる(血清型の区別はできない)．成人に対する特異性は高いが，小児に対する特異性は低く，高率に偽陽性が出ると報告されている[10]．しかし，小児では重篤な症状を伴わない肺炎球菌性菌血症が起こることがあり，キットに用いられている検出系の特異性の問題ではなく，小児特有の病態を反映した結果であると考えられる．小児髄膜炎の診断に用いた報告もあり[11]，小児肺炎球菌感染症診断キットとしての再評価が必要であると思われる．

表　肺炎球菌の血清型

Type	Group	Type	Group
1			24(24F, 24A, 24B)
2			25(25F, 25A)
3		27	
4			28(28F, 28A)
5		29	
	6(6A, 6B, 6C [6D])	31	
	7(7F, 7A, 7B, 7C)		32(32F, 32A)
8			33(33F, 33A, 33B, 33C, 33D)
	9(9A, 9L, 9N, 9V)	34	
	10(10F, 10A, 10B, 10C)		35(35F, 35A, 35 B, 35C)
	11 (11F, 11A, 11B, 11C, 11D)	36	
	12(12F, 12A, 12B)	37	
13		38	
14		39	
	15(15F, 15A, 15B, 15C)	40	
	16(16F, 16A)		41(41F, 41A)
	17(17F, 17A)	42	
	18(18F, 18A, 18B, 18C)	43	
	19(19F, 19A, 19B, 19C)	44	
20		45	
21		46	
	22(22F, 22A)		47(47F, 47A)
	23(23F, 23A, 23B)	48	

・2007年6Cが追加された．6Dは遺伝子組み換えにより作られた血清型である．
・23価ポリサッカライドワクチンに含まれる血清型は下線で示す．
・7価コンジュゲートワクチンに含まれる血清型はイタリックで示す．

予防

肺炎球菌に対するワクチンとして，莢膜由来のポリサッカライドを用いた23価肺炎球菌ワクチンと，ポリサッカライドとたん白質(CRM_{197})を共有結合させた7価コンジュゲートワクチンがわが国で承認されている．

1. 成人に対するワクチン

23価肺炎球菌ワクチンは，当初14価ワクチン

として1980年代に開発され，その後，23価ワクチンとしてわが国で承認されたワクチンである．2006年には新製法によるワクチン(ニューモバックスNP)が承認されたが，ワクチンに含まれるポリサッカライドの血清型(**表**)，含量は旧製法によるワクチンと変更はない．成分であるポリサッカライドは，抗原提示細胞によりT細胞に提示されることはなく，T細胞非依存性の抗体産生を惹起する．したがってメモリー効果はなく，接種後数年で抗体価の漸減が見られる．2歳未満の小児に対する適応はない．2009年10月に添付文書の改訂があり，重要な基本的注意として「過去5年以内に，多価肺炎球菌莢膜ポリサッカライドワクチンを接種されたことのある者では，本剤の接種により注射部位の疼痛，紅斑，硬結等の副反応が，初回接種よりも頻度が高く，程度が強く発現すると報告されている．本剤の再接種を行う場合には，再接種の必要性を慎重に考慮した上で，前回接種から十分な間隔を確保して行うこと」という記載が追加され，条件付きながら再接種が認められるようになった．

臨床効果として添付文書には6つの表が載っている．うち南アフリカでの2つの研究とマラウィ・モザンビークでの研究を示した3つの表にはワクチンに含まれる莢膜血清型による肺炎球菌性肺炎に対する予防効果があることが示されているが，米国，スウェーデン，ウガンダでの研究結果を示した残り3つの表には，肺炎予防効果が見られなかったことが示されている．前述した米国での23価ワクチンの後ろ向きコホート研究でも，ワクチンに含まれる血清型肺炎球菌の菌血症を44%減少させる効果は示されたが，肺炎予防効果は見られなかった[6]．

2. 小児に対するワクチン

表に斜体で示した7種類の血清型肺炎球菌から抽出，精製したポリサッカライドの各々に，ジフテリア毒素のアミノ酸配列に改変を加えて毒性を除いたCRM_{197}を結合させた7価コンジュゲートワクチン(プレベナー)が，わが国で2009年に承認された．海外で行われた二重盲験試験により，ワクチンに含まれる血清型の肺炎球菌による小児侵襲性感染症の予防効果が示された[12]．肺炎[13]，中耳炎[14]に対する予防効果も見られているが，わが国で承認された効能・効果には肺炎，中耳炎に対する予防効果は含まれていない．米国では2000年にこのワクチンが承認された後，ワクチンに含まれる7つの血清型肺炎球菌(4，6B，9V，14，18C，19F，23F)による侵襲性感染症だけではなく，6Bと交叉免疫性のある6Aによる侵襲性感染症の減少も見られている[15]．また，実際にはワクチンを接種していない高齢者の肺炎球菌感染症の減少も見られている[15]．これは，小児のワクチン接種率が90%に達したことによる集団免疫効果(小児から小児へ，小児から高齢者への感染が減少)として考えられている．興味深いことに，小児では6Aの侵襲性感染は減少しているが，高齢者では6Aの減少は見られていない[15]．咽頭にコロナイズする血清型6Aの菌を減少させるには，コンジュゲートワクチンに含まれる6Bによる免疫では不十分であるからと考えられている．

一方，ワクチンに含まれている血清型肺炎球菌による感染は減少しているものの，含まれていない血清型肺炎球菌(特に19A)による感染の増加(replacement)が見られている[15]．わが国で7価コンジュゲートワクチンが使われることにより，侵襲性感染，呼吸器感染，中耳炎がどのように減少し，分離される肺炎球菌の血清型にどのように変化が起こるか，継続したサーベイが必要である．

7価コンジュゲートワクチンに新しく6種類の血清型(1，3，5，6A，7F，19A)を加えた13価コンジュゲートワクチンの承認申請が2009年に予定されている．このワクチンでは，二重盲験による治験を行わないで，抗体価の上昇，白血球貪食アッセイのデータに基づき，申請がなされる予定である．

2008年に，ポリサッカライドワクチンとコンジュゲートワクチンを異なる順で成人に接種した場合，抗体価の推移にどのような差が見られるか報告された[16]．この文献には，最初にポリサッカ

ライドワクチンを接種し6か月後にコンジュゲートワクチンを接種した場合に比べ，最初にコンジュゲートワクチンを接種し6か月後にポリサッカライドワクチンを接種した場合のほうが，抗体価がより高くなるというデータが示されている．いずれの場合でも，菌血症の防止に必要な抗体価(0.35 μg/mL 以上)を達成することはできているが，呼吸器感染防止を目的とする場合，成人に対してどのようなワクチンをどのような順で接種するべきであるか，今後，様々な検討が必要である．わが国においては，成人用コンジュゲートワクチンの早期承認が待たれる．

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お知らせ

第1回　日本臨床試験研究会学術集会 2010 in 東京

一般社団法人日本臨床試験研究会の創設にあたり，第1回学術集会を開催します．本研究会の活動目的は，臨床試験や臨床研究に携わる幅広い専門家の方々の知識と技術の向上をはかり，日本の臨床試験・臨床研究の推進と質の向上に寄与することにあります．そのためには職種の枠を超えた情報交換と生涯教育の場が必要であり，研修会の開催，定期刊行物の発行・インターネットによる情報提供，将来的な専門職認定制度の設立などを行っていく予定です．多くの皆さまの参加をお待ちしております．

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東京都港区虎ノ門2丁目9番16号
会長：大橋靖雄(東京大学大学院医学系研究科)
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Intranasal vaccination with pneumococcal surface protein A plus poly(I:C) protects against secondary pneumococcal pneumonia in mice

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ABSTRACT

Effective pneumococcal vaccines are required for preventing secondary bacterial pneumonia, a life-threatening condition, during epidemics of influenza. We examined whether nasal administration of a low dose of pneumococcal surface protein A (PspA) plus polyinosinic–polycytidylic acid (poly(I:C)) could protect against a fatal secondary pneumococcal pneumonia after influenza A virus infection in mice. PspA-specific IgG but not IgA level was higher in the airways and blood of mice nasally administered a low dose of PspA plus poly(I:C) than in mice nasally administered PspA alone or poly(I:C) alone. Binding of PspA-specific IgG increased C3 deposition on the bacterial surface. The survival rate during secondary infection was higher in mice immunized with PspA plus poly(I:C) than in mice immunized with poly(I:C) alone. The significant reduction in bacterial density in the lung and blood was associated with increased survival of immunized mice with secondary pneumonia. Passive transfer of sera from mice immunized with PspA plus poly(I:C) increased the survival of mice infected with secondary pneumonia. Our data suggest that an intranasal PspA vaccine has promising protective effects against secondary pneumonia after influenza and that PspA-specific IgG plays a critical role in this protection.

1. Introduction

Although influenza is a seasonal viral infection associated with significant morbidity and mortality [1], most of the deaths during the 1918 influenza pandemic were caused by secondary bacterial pneumonia, primarily *Streptococcus pneumoniae* [2,3]. Although recent studies on the 2009 pandemic of H1N1 virus infection showed that the most frequent cause of death was viral pneumonia [4–6], bacterial coinfection by *S. pneumoniae* was found in fatal cases or cases with severe respiratory failure associated with confirmed pandemic H1N1 virus infection [7,8].

Although pneumococcal conjugate vaccine provides protective immunity against pneumonia and invasive disease in infants [9,10], polysaccharide-based vaccines are not ideal because they must include multiple polysaccharide serotypes and do not protect against strains with nonvaccine serotypes [11]. Previous investigators have examined several pneumococcal proteins as potential vaccine candidates with promising results [12–15]. One of these candidates is pneumococcal surface protein A (PspA), which is a choline-binding protein exposed on the cell surface [16–18]. PspA is present on all pneumococcal strains, and anti-PspA antibody increases bacterial clearance and induces cross-protection against infection from strains with different serotypes [19]. Anti-PspA antibodies neutralize the anticomplementary effect of PspA, increasing C3 deposition on PspA-bearing bacteria [20,21]. Human antibody to PspA could protect mice from fatal pneumococcal infection, suggesting that PspA can be an effective human vaccine [22].

We reported previously on increased bacterial clearance in the lung of mice given intranasal immunization of PspA with a Toll-like receptor (TLR) ligand [23]. Intranasal immunization of each TLR agonist in combination with PspA works as a potent mucosal adjuvant in the induction of PspA-specific antibodies in the airway and blood. In the present study, we developed a murine model of secondary pneumococcal pneumonia and studied the effect of intranasal immunization with PspA with polyinosinic–polycytidylic acid (poly(I:C)), a double-stranded RNA (dsRNA) ligand for both TLR3 and melanoma-associated 5 [24] in this model.

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2. Materials and methods

2.1. Viral and bacterial strains

Influenza virus (H1N1) A/New Caledonia strain was obtained from The Research Foundation for Microbial Diseases, Osaka University. *S. pneumoniae* WU2 strain (serotype 3) was grown in Todd-Hewitt Broth (BD, Franklin Lakes, NJ) supplemented with 0.1% 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, St. Louis, MO). Bacteria were suspended in THY, and the aliquots were snap frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Immunogen and adjuvant

The recombinant plasmid pUAB055 [25] containing the 0.9 kb *pspA* Rx1 gene encoding $PspA_{32-333}$ was transformed into *Escherichia coli* strain BL21 (DE3) (Invitrogen, Carlsbad, CA) for protein production. The recombinant PspA (rPspA) was purified with Ni-NTA agarose (Qiagen, Valencia, CA). The purified His-tag PspA was purified further with an ion exchange column Mono Q 5/50 GL (GE Healthcare Bio-Sciences, Piscataway, NJ), followed by gel filtration chromatography with Superdex 75 10/300 GL (GE Healthcare Bio-Sciences). We used poly(I:C) (InvivoGen, San Diego, CA), which is a synthetic analog of dsRNA, as the adjuvant for PspA [23].

2.3. Immunization of mice

6–8-Week-old C57BL/6 mice were purchased from CLEA Japan, Inc. (Tokyo, Japan). Mice were anesthetized by subcutaneous administration of ketamine (2.46 mg/mouse) and xylazine (0.216 mg/mouse). The anesthetized mice were immunized intranasally with 0.5 μg of PspA together with 10 μg of poly(I:C) or 10 μg of poly(I:C) alone in 12 μl of PBS once a week for 3 weeks. Mice were sacrificed 1 week after the last immunization, and serum and bronchoalveolar lavage (BAL) fluid were sampled for PspA-specific antibody assays. All animal experiments were performed in accordance with institutional guidelines for the Osaka University animal facility.

2.4. PspA-specific antibody assays

Microtiter plates (Thermo Fisher Scientific, Waltham, MA) were coated overnight at 4 °C with 100 μl of 1 μg/ml of PspA. The plates were washed with PBS containing 0.05% Tween 20 (PBS-T). Serially diluted serum and BAL fluid were added to the plates, and the plates were 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 (Zymed, San Francisco, CA) for 30 min at 37 °C. After the incubation, the plates were washed three times with PBS-T, 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich) diluted with substrate buffer (1 M diethanolamine, 0.5 mM $MgCl_2$) was added, and the plates were incubated for 30 min at room temperature in the dark. The optical density was read at 405 nm with a microplate reader (Bio-Rad Laboratories, Hercules, CA).

2.5. Secondary pneumonia model

Anesthetized mice were infected intranasally with 1×10^3 plaque-forming units (PFU) of influenza virus (H1N1) A/New Caledonia strain in 30 μl of PBS and infected intranasally with 6×10^3 colony-forming units (CFU) of *S. pneumoniae* WU2 strain in 30 μl of PBS 5 days after the viral infection. Two weeks after the last immunization, immunized mice were similarly infected intranasally with influenza virus, followed 5 days later by nasal infection with *S. pneumoniae*. Mice were sacrificed and lung and blood samples were obtained 2, 6, 16, 24, 48, and 72 h after pneumococcal infection. The lungs were homogenized in 2 ml of PBS, and quantitative culture of the lung homogenates and blood samples were performed on sheep blood agar.

2.6. Lung histopathology

Lungs were excised from mice and fixed in 10% formalin. The fixed lungs were embedded in paraffin, and 5 μm-sliced sections were stained with hematoxylin–eosin. Four types of histopathological changes (peribronchiolitis, perivasculitis, interstitial pneumonitis, and alveolitis) were scored independently by a pathologist who was unaware of the animal status and were given histopathological scores using a 0–4 scale as described previously [26,27]. BAL fluid was obtained from the nasally immunized mice after pneumococcal infection at the indicated times. Cells were stained with Diff-Quik (Sysmex, Kobe, Japan), and the cell morphology was determined in cell monolayers prepared using a Cytospin 2 (Shandon Southern Products, UK).

2.7. Immunoblotting

S. pneumoniae WU2 strain and rPspA protein were lysed in sample buffer (60 mM Tris–HCl pH 6.8 containing 5% glycerol, 1.6% SDS, 0.1 M DTT, and 0.002% bromophenol blue), and the lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After the electrophoresis, the gel was transferred to a polyvinyl difluoride membrane (Bio-Rad Laboratories), the membrane was blocked with 5% skim milk for 1 h at room temperature and then incubated with sera from PspA-immunized mice at a dilution of 1:5000 for 1 h at room temperature. After the incubation, the membrane was washed three times and then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (SouthernBiotech, Birmingham, AL) at a dilution of 1:10,000 for 1 h at room temperature. The separated bands were detected with an ECL kit (GE Healthcare Bio-Sciences).

2.8. Antibody binding and C3 deposition assay

S. pneumoniae WU2 strain was grown in THY to mid-log phase. The bacteria were harvested by centrifugation and washed once with PBS. The pellet was suspended with an appropriate amount of PBS to prepare a bacterial suspension at a concentration of 1×10^8 CFU/ml, and 100 μl of Alexa-Fluor 488-conjugated goat anti-mouse IgG antibody (Invitrogen, Eugene, OR) or 90 μl of the bacterial suspension was incubated with 10 μl of heat-inactivated mouse serum for 30 min at 37 °C. After the incubation, the suspension was washed once with PBS, suspended in 90 μl of gelatin-veronal buffer with Mg^{2+} and Ca^{2+} with 10 μl of naive mouse serum, and then incubated for 30 min at 37 °C. Following the incubation, the bacterial suspension was washed once with PBS, suspended in 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse C3 antibody (MP Biomedicals, Solon, OH), 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.

2.9. Passive immunization

Mice were infected intranasally with 1×10^3 PFU of influenza virus and infected intranasally with 6×10^3 CFU of *S. pneumo-*

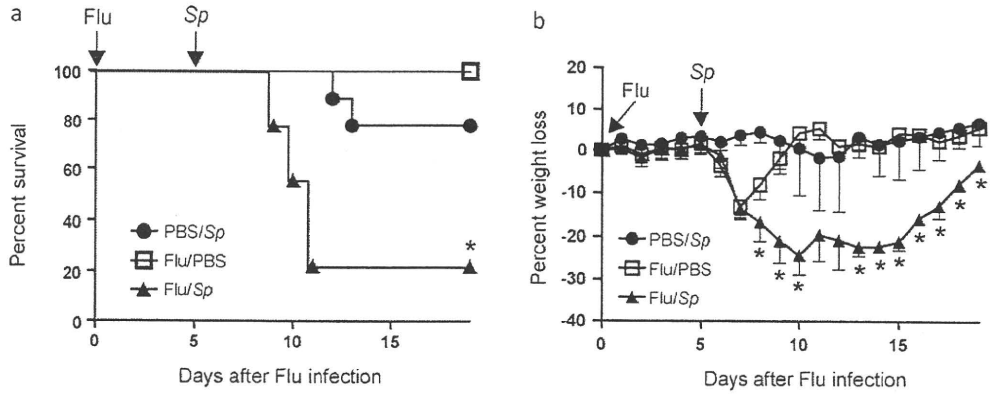

Fig. 1. Survival (a) and change in body weight (b) of mice after influenza virus infection. The data for the mice ($n=9$) administered PBS nasally, followed 5 days later by administration of *S. pneumoniae* are shown as PBS/*Sp* (closed circles). The data for mice ($n=7$–9) that received influenza virus, followed by administration of PBS or *S. pneumoniae* are shown as Flu/PBS (open squares) or Flu/*Sp* (closed triangles). *$P<0.05$ (versus Flu/PBS and PBS/*Sp*).

niae WU2 strain 5 days after the viral infection. Immediately after pneumococcal infection, 100 μl of serum obtained from mice immunized intranasally with PBS alone, with 10 μg of poly(I:C) alone, or with 0.5 μg of PspA plus 10 μg of poly(I:C) was administered intraperitoneally to mice with secondary pneumococcal pneumonia. The survival of the infected mice was monitored.

2.10. Statistical analysis

All data are expressed as the mean ± SD. Student's *t*-test, the Mann–Whitney test or one-way ANOVA and Turkeys' multiple comparison methods were used to analyze the data. We used the Kaplan–Meier log-rank test for the survival analysis. *P* values <0.05 were considered significant.

3. Results

3.1. Secondary bacterial pneumonia

All mice that received the nasal challenge of influenza virus followed by PBS (Flu/PBS) and 80% of mice that received the nasal challenge of PBS followed by *S. pneumoniae* (PBS/*Sp*) survived. By contrast, only 20% of mice that received the nasal challenge of influenza virus followed by *S. pneumoniae* (Flu/*Sp*) survived during the observation period (Fig. 1a). Body weight declined transiently by 15% after the PBS challenge in mice that received the Flu/PBS challenge but did not change in mice that received the PBS/*Sp* challenge (Fig. 1b). By contrast, body weight decreased by 25% during the observation period in surviving mice that received the Flu/*Sp* challenge.

To characterize further the model of secondary pneumonia, we next examined the bacterial density in the lung and blood of mice. The bacterial densities of *S. pneumoniae* were approximately 10^3 CFU in the lung tissue of mice that received the PBS/*Sp* challenge 2 h after the pneumococcal challenge and 10^3 to 10^4 CFU/lung 16–72 h after the challenge (Fig. 2b). None of these mice was bacteremic between 2 and 72 h after the pneumococcal challenge, except for one of five mice at 48 h after the challenge (Fig. 2a). By contrast, the bacterial density in the lung tissue increased from 10^3 CFU/lung at 2 h to 10^6 CFU/lung 16 h after the pneumococcal challenge in mice receiving the Flu/*Sp* challenge (Fig. 2b). At 16, 24, 48, and 72 h after the pneumococcal challenge, the bacterial density in the lung tissue was significantly higher in mice that received Flu/*Sp* than in those that received PBS/*Sp* at the same time points ($P<0.05$). In these mice that received Flu/*Sp*, the bacterial density was higher than 10^7 CFU in the lung tissue, and three of four mice had bacteremia 72 h after the pneumococcal challenge (Fig. 2a).

A histopathology study showed minimal inflammatory changes in the lungs of mice that received PBS/PBS and in mice that received PBS/*Sp* (Fig. 3a). Marked inflammatory changes were evident in mice that received Flu/PBS or Flu/*Sp* compared with mice that received PBS/PBS or PBS/*Sp*. Interstitial pneumonitis was prominent only in mice that received Flu/*Sp* and not in mice that received Flu/PBS. These data suggest that advanced pulmonary inflammation was caused by secondary pneumonia.

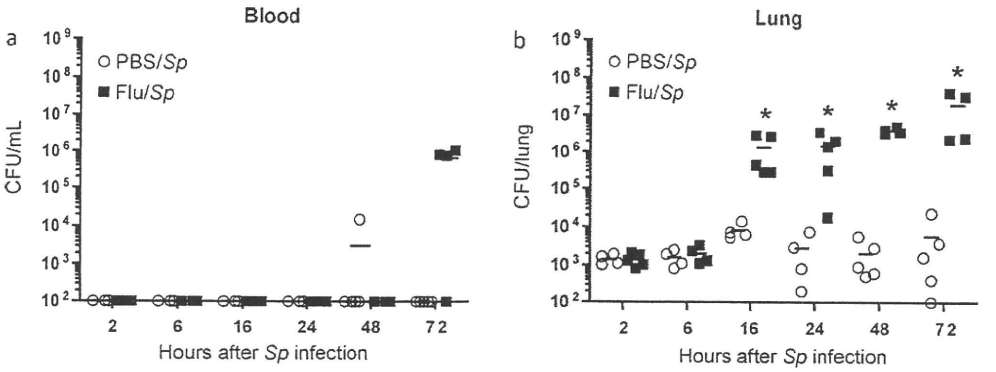

Fig. 2. Bacterial densities in blood (a) and lung (b) of mice given PBS (open circles) or influenza virus (closed squares) nasally followed by administration of *S. pneumoniae*. Lung tissue and blood were harvested 2, 6, 16, 24, 48, and 72 h after pneumococcal challenge, and bacterial density was counted. Values represent the $\log_{10}$ CFU/mL or CFU/lung (mean ± SD) for 4–5 mice per group. *$P<0.05$ versus PBS/*Sp* at the same time points.

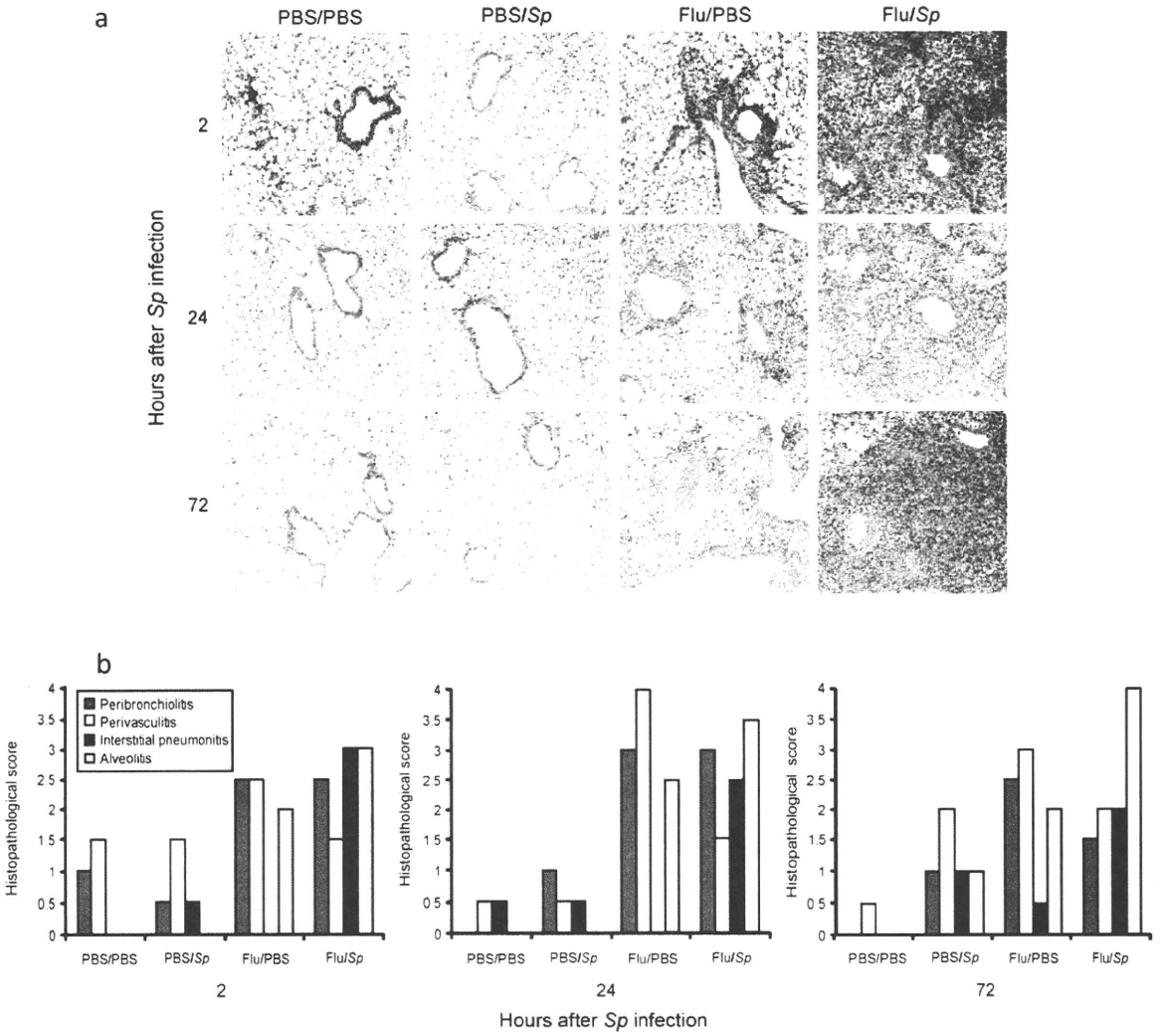

Fig. 3. Histopathological findings in lung tissues from mice with secondary pneumonia (a) and the histopathological scores of lung tissues (b). Mice were given PBS nasally followed by administration of PBS (PBS/PBS), PBS followed by administration of *S. pneumoniae* (PBS/*Sp*), influenza virus followed by administration of PBS (Flu/PBS), or influenza virus followed by administration of *S. pneumoniae* (Flu/*Sp*). Mice administered PBS or influenza virus nasally were sacrificed at the times indicated after intranasal administration of *S. pneumoniae* or PBS challenge, and their lungs were excised, fixed, and stained with hematoxylin–eosin. The histopathology was scored from microscopic analysis. Two mice per group were examined.

3.2. PspA-specific IgG and its function

A negligible level of PspA-specific IgG, IgG1 or IgG2a was found in serum from mice immunized nasally with poly(I:C) alone (PBS/poly(I:C)) or PspA alone (Fig. 4a and b). A higher level of PspA-specific IgG was found in serum from mice immunized nasally with PspA plus poly(I:C) (PspA/poly(I:C)). PspA-specific IgG in serum comprised both PspA-specific IgG1 and IgG2a, and a low level of PspA-specific IgG was detected in BAL fluid of these mice (Fig. 4b and c). No PspA-specific IgA was detected in BAL fluid in mice immunized nasally with PspA alone or with PspA/poly(I:C) (data not shown).

The serum from mice immunized nasally with PBS/poly(I:C) exhibited no binding to the cell lysates of the WU2 strain or rRx1 PspA protein. Faint binding was detected in the serum from mice immunized nasally with PspA/poly(I:C) irrespective of whether they received the nasal challenge of influenza virus followed by *S. pneumoniae* (Fig. 4d). Binding of mouse IgG was observed to bacteria treated with serum from mice immunized nasally with PspA/poly(I:C) regardless of whether they had received the nasal challenge of influenza virus followed by *S. pneumoniae* (Fig. 4e). No binding was observed to bacteria treated with serum from mice immunized nasally with PBS/poly(I:C), suggesting the binding of PspA-specific IgG to the surface of the WU2 strain. Similarly, C3 binding was found on the bacteria treated with serum from mice immunized nasally with PspA/poly(I:C) regardless of whether they received the nasal challenge of influenza virus followed by *S. pneumoniae* (Fig. 4f). No binding was observed to bacteria treated with serum from mice immunized nasally with PBS/poly(I:C), suggesting that the increased C3 deposition was caused by PspA-specific IgG.

3.3. Protective effect of intranasal PspA vaccine

The survival rates did not differ between mice immunized nasally with PspA alone or with PBS alone (Fig. 5a). By contrast, the survival rate was significantly higher in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) ($P < 0.0001$, Fig. 5b). Body weight was significantly

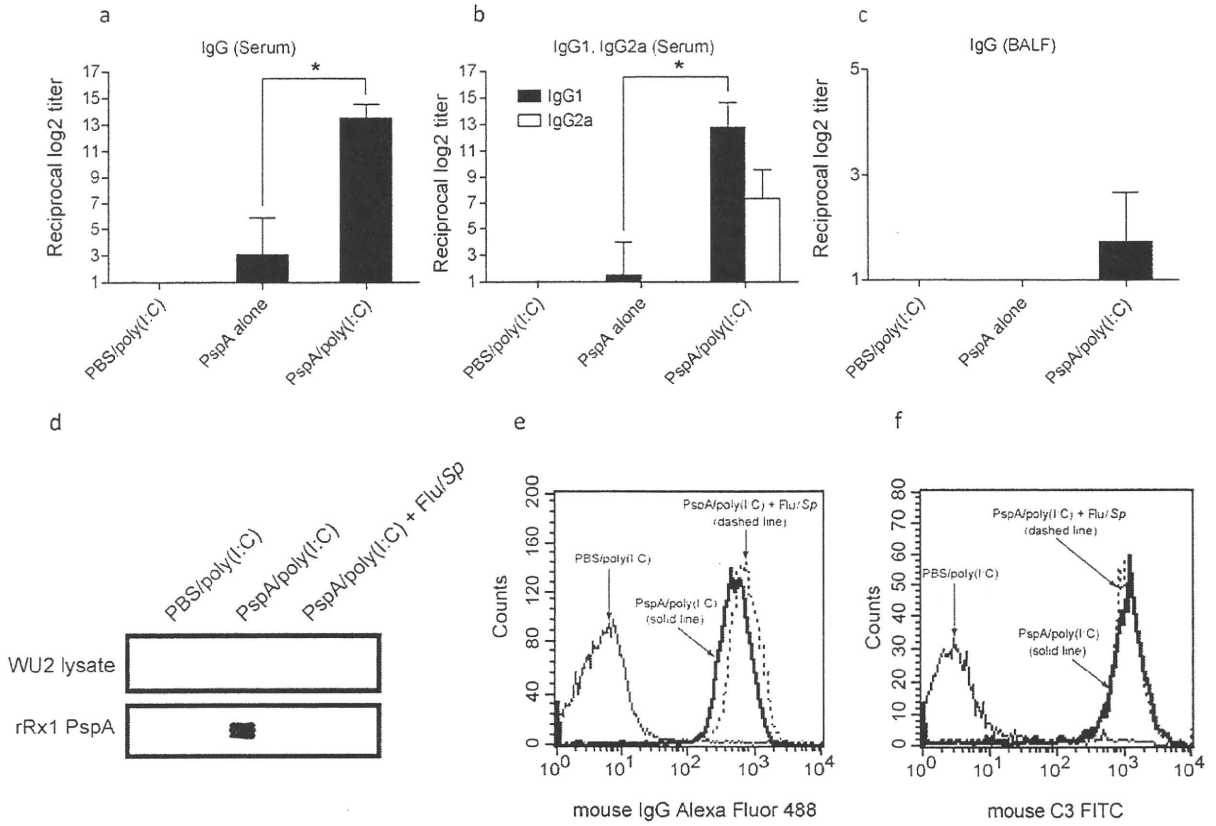

Fig. 4. Induction of PspA-specific IgG levels in serum (a), IgG1 and IgG2a levels in serum (b), and IgG levels in BAL fluid (c), immunoblot analysis of lysates of *S. pneumoniae* WU2 strain and recombinant Rx1 PspA with sera from mice immunized with PBS/poly(I:C) and PspA/poly(I:C) (d), the binding of PspA-specific antibodies (e), and C3 deposition on the bacterial surface (f). Sera from mice immunized with PBS/poly(I:C), PspA alone or PspA/poly(I:C) were used for the determination of PspA-specific antibodies. Sera from mice immunized with PBS/poly(I:C), PspA/poly(I:C), or PspA/poly(I:C) and followed by pneumococcal infection (PspA/poly(I:C) + Flu/*Sp*) were used for the immunoblot analysis. Sera from mice immunized with PspA/poly(I:C) (solid line) or sera from mice immunized with PspA/poly(I:C) + Flu/*Sp* (dashed lines) were tested for the ability to bind to the pneumococcal surface and for the ability to bind C3 on the pneumococcal surface by flow cytometry. Sera from mice immunized with PBS/poly(I:C) (grey areas) were used as the control. The results are expressed as mean ± SD for 9–10 mice per group for the measurement of PspA-specific antibodies. *$P < 0.05$ versus PspA alone.

higher in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) at 2–6 days after the pneumococcal challenge ($P < 0.005$, Fig. 5c).

Bacterial density in the lung was 500-times lower at 24 h, 6000-times lower at 48 h, and 13,000-times lower at 72 h after the pneumococcal challenge in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) (Fig. 6b). The bacterial density in blood was also lower in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) at 48 h and 72 h after the pneumococcal challenge (Fig. 6a).

A histopathology study of nasally immunized mice showed similar inflammatory cell infiltration in the lung tissue in mice immunized nasally with PspA/poly(I:C) and in mice immunized with PBS/poly(I:C) (Fig. 7a). The observed alveolitis was slightly milder in mice immunized nasally with PspA/poly(I:C) than in mice immunized nasally with PBS/poly(I:C) at 24 h and 72 h after the pneumococcal challenge. The total number and the differen-

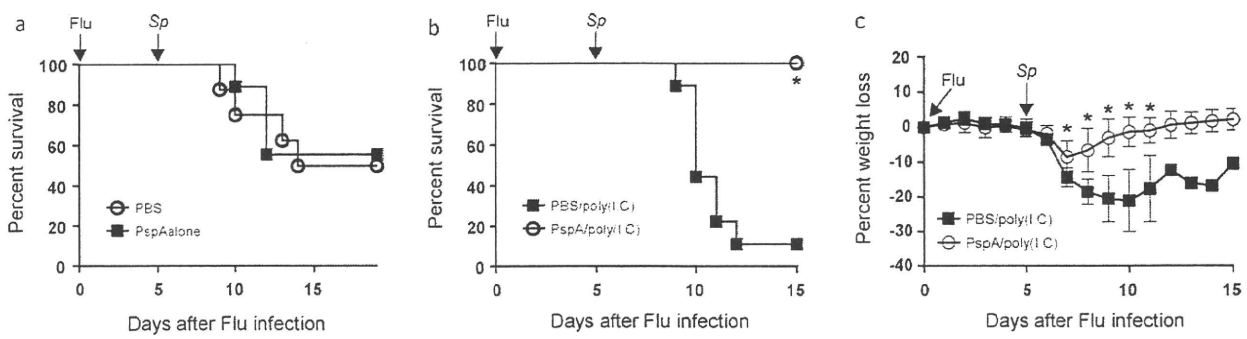

Fig. 5. Survival of C57BL/6 mice immunized nasally with PspA alone or with PBS after secondary pneumonia (a), nasally immunized with PBS/poly(I:C) or with PspA/poly(I:C) after secondary pneumonia (b), and the change in body weight of C57BL/6 mice immunized nasally with PspA/poly(I:C) or PBS/poly(I:C) alone after secondary pneumonia (c). In (a), the groups of mice ($n = 8–9$) immunized nasally with PspA alone or with PBS are shown as PBS (open circles) or PspA alone (closed squares). In (b) and (c), the group of mice ($n = 9–10$) were nasally immunized with PBS/poly(I:C) (closed squares) or with PspA/poly (I:C) (open circles).

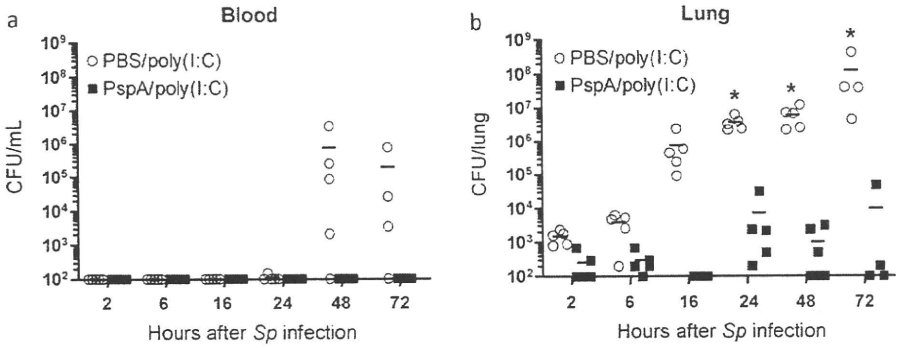

Fig. 6. Bacterial densities in blood (a) and lung (b) of mice immunized nasally with PBS/poly(I:C) (open circles) or PspA/poly(I:C) (closed squares) after secondary pneumonia. Lungs and blood were harvested 2, 6, 16, 24, 48, and 72 h after pneumococcal infection, and bacterial density was counted. Values represent the $\log_{10}$ CFU/mL or CFU/lung (mean ± SD) for 4–6 mice per group. *$P < 0.05$ versus PBS/poly(I:C).

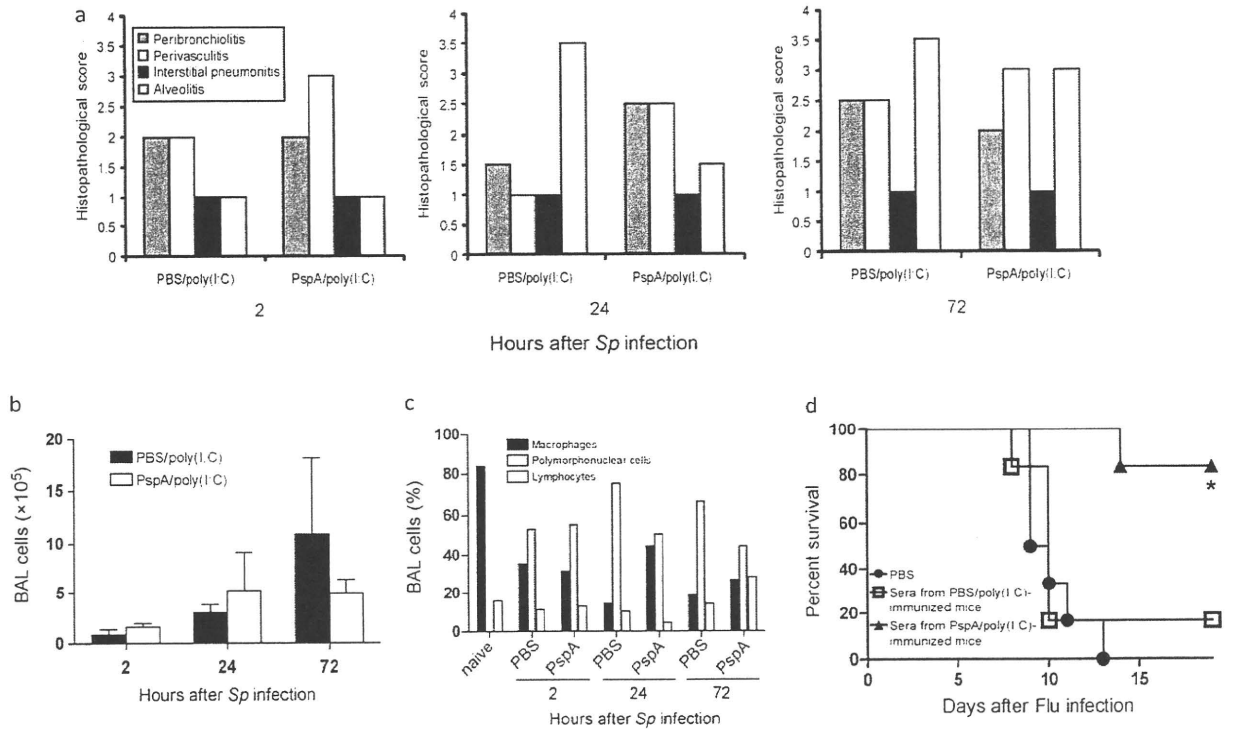

Fig. 7. Histopathological findings of lung tissues (a), the total cell count (b), and differential cell count (c) of BAL cells from nasally immunized mice after secondary pneumonia, and the effect of passive immunization in mice infected with secondary pneumonia (d). Nasally immunized mice were sacrificed at the times indicated after pneumococcal infection, and their lungs were removed, fixed, and stained with hematoxylin–eosin. The histopathology was scored using microscopic analysis. BAL was performed in the nasally immunized mice after pneumococcal infection at the times indicated, and the total number of BAL cells and cell morphology were determined. Three mice per group were examined for analysis of BAL cells. Immediately after pneumococcal infection, 100 μl of PBS (closed circles), 100 μl of serum from mice nasally immunized with PBS/poly(I:C) (open squares) or with PspA/poly(I:C) (closed triangles) was administered intraperitoneally to mice with secondary pneumonia. Six mice per group were examined. *$P < 0.05$ versus PBS/poly(I:C) and PBS.

tial count of BAL cells did not differ significantly between mice nasally immunized with PspA/poly(I:C) and those immunized with PBS/poly(I:C) (Fig. 7b and c). These data suggest that the protective effect of nasal immunization with PspA/poly(I:C) occurred through the suppression of bacterial growth but not through the suppression of pulmonary inflammation.

3.4. Protection by passive immunization

The survival rate was higher in mice that received intraperitoneal administration of 100 μl of serum from mice immunized nasally with PspA/poly(I:C) than in mice that received intraperitoneal administration of 100 μl of PBS or serum from mice immunized nasally with PBS/poly(I:C) ($P < 0.05$, Fig. 7d).

4. Discussion

In this study, we demonstrated the protective effects of a low dose of PspA plus poly(I:C) against a fatal secondary pneumococcal pneumonia after influenza virus infection in mice. Since nasal challenge with the WU2 strain at 2×10^7 CFU caused fatal pneumonia in untreated, healthy C57BL/6 mice (Piao et al., unpublished data), the challenge dose of WU2 strain required for a fatal pneumonia in mice nasally infected with 1×10^3 PFU of influenza virus (H1N1) A/New Caledonia was about 3300-times lower than in untreated