meningitis cases and the nonmeningitis breakpoints for all of the other cases.

2.4. Statistical analysis

The impact of introducing PCV7 on the incidence of IPD was estimated based on percentage changes in incidence during the study period. The incidence of IPD was defined as the number of laboratory-confirmed IPD cases per 100,000 population. Population data were obtained from Statistics Japan (http://www.stat.go.jp/). Changes in serotype distribution and serotype-specific incidence were also analyzed. Serotype-specific IPD incidence was calculated by imputing the serotypes for missing isolates to serotypes assumed based on the distribution of known serotypes. Statistical analyses were performed using the χ^2 or Fisher's exact test. We calculated 95% confidence intervals (CIs), and two-sided P values of <0.05 were considered significant. The data were analyzed using the software GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA).

3. Results

3.1. Study populations and clinical features of IPD patients

From 2008 through 2013, a total of 1181 cases of IPD were reported from the 10 prefectures. The annual numbers of cases in 2008, 2009, 2010, 2011, 2012, and 2013 were 257, 249, 246, 206, 114, and 109, respectively.

Characteristics of the IPD cases before (2008–2010) and after (2011–2013) introduction of the subsidized PCV vaccination for children are summarized in Table 1. About 60% of patients were boys. The number of cases was highest in the 1-year-old age group. Among the 1181 IPD patients, 149 (12.6%) patients had meningitis, and 1032 (87.4%) had nonmeningitis IPD. The rates of vaccination by at least a single dose of PCV7 among the IPD patients before and after introduction of subsidizing PCV vaccination were 0.9% (7/752) and 40.8% (175/429), respectively. Of the 1181 patients, 104 (15.4%) had at least one comorbid disease, 232 (24.2%) had siblings, and 308 (45.6%) were associated with day care attendance. The case fatality rate was 0.9%.

The monthly number of IPD cases in children aged <5 years between 2008 and 2013 is shown in Fig. 1. The number of IPD cases peaked in the months April–June between 2008 and 2011. In the months July–September, the numbers of IPD cases remained low, except for October and November in 2010. This seasonality appeared to be markedly attenuated in 2012 and 2013, when PCV coverage had increased substantially.

3.2. Changes in the incidence of IPD

The yearly change in the incidence of IPD in children aged <5 years is shown in Fig. 2. Because the incidence was similar during the years 2008–2010 and the immunization rate by PCV7 remained low until 2010, we regarded the incidence during these 3 years as the baseline value before the introduction of PCV7. Compared with the average baseline rate of 25.0 per 100,000, the incidence of all IPD decreased significantly by 19% (95% CI, 3–29%, $P = 0.02$), 54% (95% CI, 44–62%, $P < 0.01$), and 57% (95% CI, 46–60%, $P < 0.01$) in 2011, 2012, and 2013, respectively. These results show that >50% of IPD cases were prevented by PCV7 within 2 years of PCV7 introduction.

The results were also analyzed according to types of disease: meningitis vs. nonmeningitis IPD (Fig. 2). Compared with the average incidence in 2008–2010, the incidence of meningitis declined by 25% (95% CI −15% to 52%, $P = 0.22$) in 2011, 71% (95% CI, 47–86%, $P < 0.01$) in 2012, and 61% (95% CI, 30–78%, $P < 0.01$) in 2013. Similarly, the incidence of nonmeningitis IPD declined by 18% (95% CI,

Table 1
Characteristics of children with invasive pneumococcal disease, Japan, 2008–2010 and 2011–2013.

Characteristics	Number (%) of patients		
	2008–2010[a], $n = 752$	2011–2013[b], $n = 429$	Total, $n = 1181$
Sex			
Boys	446 (59.3)	257 (59.9)	703 (59.5)
Girls	306 (40.7)	172 (40.1)	478 (40.5)
Age group			
<1 yr	178 (23.7)	104 (24.2)	282 (23.9)
1 yr	373 (49.6)	216 (50.3)	589 (49.9)
2 yr	113 (15.0)	54 (12.6)	167 (14.1)
3 yr	51 (6.8)	37 (8.6)	88 (7.5)
4 yr	37 (4.9)	18 (4.2)	55 (4.7)
Diagnosis			
Meningitis	102 (13.6)	47 (11.0)	149 (12.6)
Nonmeningitis	650 (86.4)	382 (89.0)	1032 (87.4)
PCV7 vaccination			
Yes[c]	7 (0.9)	175 (40.8)	182 (15.4)
No	745 (99.1)	248 (57.8)	993 (84.1)
Unknown	0 (0)	6 (1.4)	6 (0.5)
Comorbid diseases[d]			
Yes	37 (15.0)	67 (15.6)	104 (15.4)
No	209 (85.0)	358 (83.4)	567 (84.0)
Unknown	0 (0)	4 (0.9)	4 (0.6)
Presence of siblings[d]			
Yes	74 (30.1)	158 (36.8)	232 (34.4)
No	58 (23.6)	154 (35.9)	212 (31.4)
Unknown	114 (46.3)	117 (27.3)	231 (34.2)
Day care attendance[d]			
Yes	87 (35.4)	221 (51.5)	308 (45.6)
No	85 (34.6)	141 (32.9)	226 (33.5)
Unknown	74 (30.1)	67 (15.6)	141 (20.9)
Outcome			
Alive	697 (92.7)	383 (89.3)	1080 (91.4)
Dead	3 (0.4)	8 (1.9)	11 (0.9)
Unknown	52 (6.9)	38 (8.9)	90 (7.6)

[a] Seven-valent pneumococcal conjugate vaccine (PCV7) was introduced on a voluntary basis in February 2010.
[b] Financial support by the Japanese government started from November 2010. PCV7 has been included in the national immunization program since April 2013.
[c] At least one dose of PCV7.
[d] Data were not collected in 2008 and 2009.

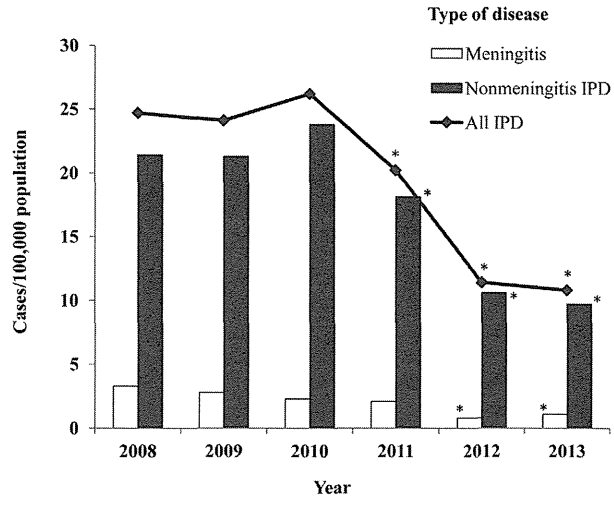

Fig. 2. Annual incidence of invasive pneumococcal disease among children aged <5 years during 2008–2013. The seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in Japan on a voluntary basis among children aged <5 years in February 2010. *$P < 0.05$ versus the average incidence in 2008–2010.

Table 2
Serotype distribution of *Streptococcus pneumoniae* Isolates from children with invasive pneumococcal disease, Japan, 2008–2010 and 2011–2013.

Serotype	Number (%) of isolates	
	2008–2010[a], $n=403$	2011–2013[b], $n=308$
6B	122(30.3)	34 (11.0)[c]
14	84(20.8)	31 (10.1)[c]
23F	48(11.9)	22 (7.1)[d]
19F	38(9.4)	10 (3.2)[c]
9V	9(2.2)	9(2.9)
4	12(3.0)	4(1.3)
18C	2(0.5)	2(0.6)
PCV7 type	315(78.2)	112 (36.4)[c]
1	3(0.7)	1(0.3)
5	0(0)	0(0)
7F	1(0.2)	1(0.3)
3	0(0)	4(1.3)
6A	16(4.0)	8(2.6)
19A	29(7.2)	86(27.9)[c]
PCV13 minus PCV7 type	49(12.2)	100 (32.5)[c]
6C	11(2.7)	8(2.6)
10A	3(0.7)	9 (2.9)[d]
11A/E	0(0)	2(0.6)
12F	1(0.2)	1(0.3)
15A	2(0.5)	18 (5.8)[c]
15B	4(1.0)	7(2.3)
15C	5(1.2)	14 (4.5)[c]
16F	0(0)	1(0.3)
22F	2(0.5)	7 (2.3)[d]
23A	4(1.0)	1(0.3)
24F	3(0.7)	15 (4.9)[c]
33F	0(0)	6 (1.9)[c]
35B	0(0)	2(0.6)
38	3(0.7)	4(1.3)
Non-PCV13 type	38(9.4)	95 (30.8)[c]
Nontypable	1(0.2)	1(0.3)

[a] Seven-valent pneumococcal conjugate vaccine (PCV7) was introduced on a voluntary basis in February 2010.
[b] Financial support by the Japanese government started from November 2010. PCV7 has been included in the national immunization program since April 2013.
[c] $P<0.01$ versus the proportion of isolates in 2008–2010.
[d] $P<0.05$ versus the proportion of isolates in 2008–2010.

1–28%, $P=0.04$) in 2011, 52% (95% CI, 39–60%, $P<0.01$) in 2012, and 56% (95% CI, 44–63%, $P<0.01$) in 2013. No difference was found in the trend of decline between the incidence of meningitis and nonmeningitis IPD.

3.3. Serotype change in pneumococcal isolates from before to after PCV7 introduction

A total of 711 pneumococcal isolates (60.3%) were subjected to serotyping during the study period. The percentages of IPD caused by PCV7 serotypes decreased markedly from 78.5% in 2010 to 3.3% in 2013 (Fig. 3). The percentages of IPD caused by PCV13 minus PCV7 serotypes increased from 11.6% in 2010 to 47.8% in 2013. Among the PCV13 minus PCV7 serotypes, 19A was the most frequently isolated serotype. The percentages of isolates containing serotype 19A increased gradually from 3.8% in 2008 to 44.6% in 2013. The percentages of IPD caused by non-PCV13 serotypes remained unchanged between 2008 and 2011 but then increased markedly in 2012 (41.5%) and 2013 (48.9%).

The distributions of serotypes causing IPD were compared before and after the introduction of PCV7 (Table 2). After the introduction of PCV7, the coverage rate of the vaccine for pneumococcal isolates from IPD cases decreased from 78.2% (315/403) in 2008–2010 to 36.4% (112/308) in 2011–2013 ($P<0.01$). By contrast, the percentages of IPD cases caused by serotype 19A and non-PCV13 serotypes such as 15A, 15C, and 24F increased markedly. Slight increases of the isolation rates for 10A, 22F, and 33F were also found. The differences in the percentages of IPD cases caused by these increasing serotypes were all significant.

3.4. Changes in the incidence of IPD according to different serotype groups

Compared with the baseline incidence of IPD in 2008–2010, the incidence of IPD caused by PCV7 serotypes decreased significantly by 32% (95% CI, 19–44%, $P<0.01$) in 2011, 85% (95% CI, 78–90%, $P<0.01$) in 2012, and 98% (95% CI, 95–99%, $P<0.01$) in 2013 (Fig. 4). These data confirmed the significant effect of PCV7 in preventing IPD caused by PCV7 serotypes. By contrast, compared with the baseline values, the incidence rates of IPD caused by PCV13 minus PCV7 serotypes and serotype 19A increased by 42% (95% CI, 3–97%, $P<0.01$) and 117% (95% CI, 44–246%, $P<0.01$) in 2011, 22% (95% CI, −14% to 72%, $P=0.3026$) and 91% (95% CI, 26–212%, $P<0.01$) in 2012, and 66% (95% CI, 22–127%, $P<0.01$) and 201% (95% CI, 105–358%, $P<0.01$) in 2013, respectively. Compared with the baseline value, the incidence of IPD caused by non-PCV13 serotypes increased by 96% (95% CI, 34–179%, $P<0.01$) in 2012 and 119% (95% CI, 53–210%, $P<0.01$) in 2013. The absolute increases in the incidence rates of IPD caused by PCV13 minus PCV7 serotypes, serotype 19A, and non-PCV13 serotypes between 2008 and 2013 were 2.1, 3.9, and 2.8 per 100,000, respectively.

3.5. Antimicrobial susceptibility

The MIC values of the 710 isolates (403 isolates in 2008–2010, 307 isolates in 2011–2013) are shown in Supplementary Table 1 and Supplementary Fig. 2. The percentages of penicillin G- and cefotaxime-nonsusceptible strains did not differ significantly from before to after PCV7 introduction. However, the percentages of the meropenem-intermediate strains increased significantly after PCV7 introduction ($P<0.01$). The serotypes of 15 meropenem-intermediate strains isolated in 2008–2010 were 6B (5 strains), 19A (5 strains), 23F (3 strains), 19F (1 strain), and 15A (1 strain). Of 15 meropenem-intermediate strains in 2008–2010, 9 (60%) were PCV7 type. By contrast, the serotypes of 38 meropenem-intermediate strains isolated in 2011–2013 were 19A (19 strains), 15A (8 strains), 6B (4 strains), 6A (3 strains), 35B (2 strains), 19F (1 strain), and 14 (1 strain). Thirty-two (84%) of 38 meropenem-intermediate strains isolated in 2011–2013 were the non-PCV7 type. All three meropenem-resistant strains isolated in 2011–2013 were serotype 19A. Collectively, our data demonstrate that the percentages of meropenem-nonsusceptible strains, which are predominantly serotypes 19A and 15A, increased after 2011. However, all of these nonsusceptible strains were sensitive to carbapenem antibiotic panipenem and vancomycin, which are commercially available in Japan [17].

4. Discussion

This nationwide population-based surveillance study clearly demonstrated significant declines of 98% in the incidence of PCV7-type IPD and 57% in the overall incidence of IPD in children <5 years of age after the introduction of PCV7 in Japan. The incidence of IPD peaked in April and May between 2008 and 2011, but this seasonal peak was not evident in 2012 or 2013. A rapid decrease in the incidence of PCV7-type isolates and a gradual increase of the incidence of 19A and non-PCV13-type isolates, such as 15A, 15C, and 24F, were found between 2010 and 2013; this is indicative of serotype replacement. The incidence rates of meropenem-nonsusceptible strains, such as 19A and 15A, also increased after 2011.

In Japan, a previous study reported that the incidence of pediatric pneumococcal meningitis per 1000 admissions at 159

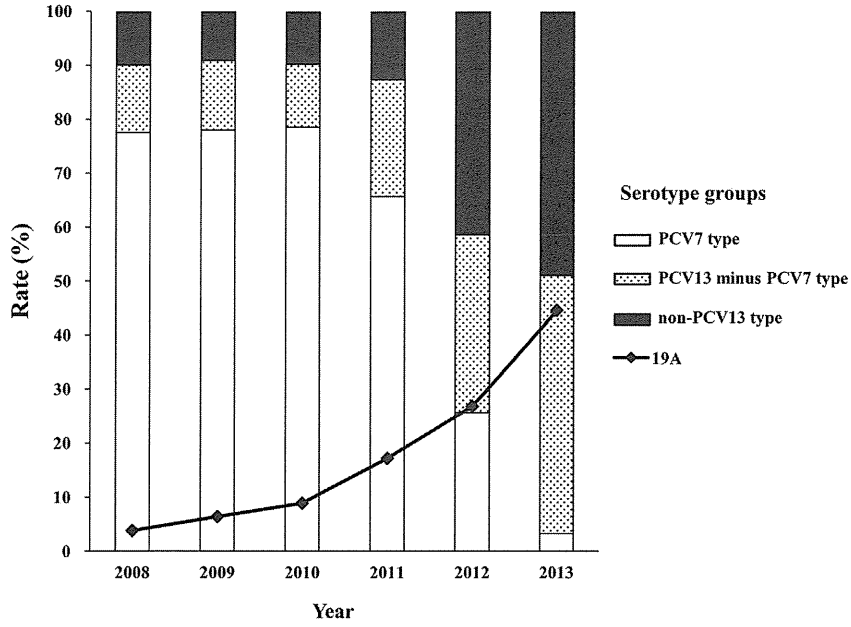

Fig. 3. Changes in serotype distribution of *S. pneumoniae* isolated from patients with invasive pneumococcal disease among children aged <5 years during 2008–2013. PCV13 minus PCV7 type denotes the six additional serotypes to PCV7.

hospitals was 80% lower in 2012 than in 2009 [18]. A rapid decrease in the prevalence of pediatric IPD cases by vaccine serotypes after the introduction of PCV7 was also seen in IPD surveillance involving 341 general hospitals [19]. In addition, a decrease in the population-based incidence of pediatric IPD in a single prefecture from before to after introduction of PCV7 was recently reported [20].

An observational cohort study demonstrated that the incidence of non-PCV7-type IPD increased by 32% in children <5 years of age in 2008–2010 after introduction of PCV7 compared with the baseline values in 2000–2006 in England and Wales [13]. In the USA and England and Wales, the non-PCV7 serotypes commonly found to be associated with serotype replacement were 10A, 15A/B/C, 19A, 21, 22F, 23B, and 33F [13,14,21]. Our study also demonstrated that serotype 24F, in addition to serotypes 15A, 15C, and 19A, was a replacement serotype. This finding is in agreement with a recent report from Japan [19]. Although an additional decrease in the incidence of pediatric IPD was expected after introduction of PCV13 on behalf of PCV7, our data suggest that about 40% of pediatric IPD would not be prevented by this vaccine. A further increase in the incidence of IPD caused by non-PCV13 serotypes is now of concern in Japan.

In our study, the absolute increase in the incidence of 19A serotype IPD between 2008 and 2013 was 3.9 per 100,000. The absolute increase in the incidence of PCV13 minus PCV7-type IPD was lower as a whole (2.1 per 100,000) than that of 19A serotype IPD, indicating an absolute decrease (−1.8 per 100,000) in the incidence of IPD caused by PCV13 minus PCV7 serotypes other than

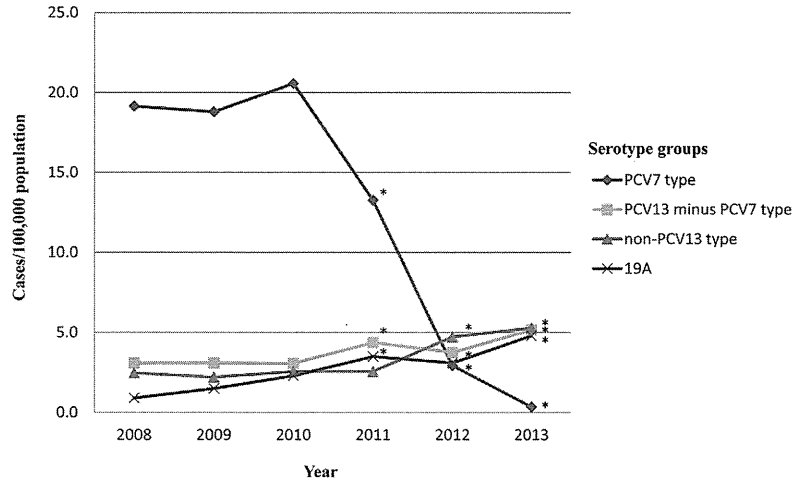

Fig. 4. Annual incidence of invasive pneumococcal disease by serotype groups among children aged <5 years during 2008–2013. The seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in Japan on a voluntary basis among children aged <5 years in February 2010. The incidence of every serotype group is estimated based on the results of the incidence of IPD and the percentages of pneumococcal isolates. PCV13 minus PCV7 type denotes the six additional serotypes to PCV7. *$P < 0.01$ versus the average incidence in 2008–2010.

19A (i.e., 1, 3, 5, 6A, and 7F). A recent study from the USA reported a similar pattern [14].

It has long been recognized that IPD epidemics demonstrate seasonal patterns, which generally show an increased incidence in winter in temperate areas of the world [22–24]. We found a unique seasonal pattern in pediatric IPD before the introduction of PCV7, with a peak prevalence in April and May (Fig. 1). A recent study from the USA reported an increased incidence of nonpneumonia IPD in August and September, which is at the start of the school year in the USA [25]. The authors suggested that the start of the school term may be related to the incidence of nonpneumonia IPD, which is driven by increased nasopharyngeal transmission of pneumococci. Because the new school term starts in April in Japan, the peak prevalence of pediatric IPD in the spring may also be explained by an increased nasopharyngeal transmission of the bacteria.

With any surveillance system, the possibility for bias exists if either case reporting or clinical practices change over time [26]. In the present study, all hospitals involved in pediatric care in each prefecture were actively contacted periodically to ensure identification of unreported IPD cases. This helped to minimize the possibility of reporting bias. Meningitis is a severe disease, for which the diagnostic criteria are unlikely to change significantly over time, whereas nonmeningitis IPD, especially occult bacteremia, may be more prone to changes in blood culture practices [26]. In our study, the declines in the incidence of meningitis (60%) and nonmeningitis IPD (56%) were similar after introduction of PCV7. Thus, it is unlikely that bias related to case reporting and blood culture practices could affect the epidemiological changes in the incidence of IPD after the introduction of PCV7 in Japan.

PCV is expected to be effective for preventing IPD caused by drug-resistant strains. Our study found unchanged percentages of penicillin G- and cefotaxime-nonsusceptible strains from before to after PCV7 introduction, suggesting that the vaccine has prevented IPD caused by both penicillin G- and cefotaxime-susceptible and -nonsusceptible strains. An increased percentage of meropenem-nonsusceptible strain was found after 2011. In Japan, cefotaxime and ceftriaxone are recommended as the first-line therapy against bacteremia in children >1 month of age, and meropenem, doripenem, or tazobactam/piperacillin are the second-line therapy [27]. It is noteworthy that the incidence rates of IPD caused by the meropenem-nonsusceptible strains, which are primarily non-PCV7 serotypes, are increasing. However, all of these meropenem-nonsusceptible strains were sensitive to vancomycin and panipenem/betamipron, as shown in Supplementary Table 1. Therefore, in Japan, vancomycin or panipenem/betamipron can be used for treatment of IPD, especially meningitis, caused by meropenem-nonsusceptible strains.

Mechanisms of resistance to meropenem in pneumococcal isolates have not been well understood. A previous study from Korea reported the rate of meropenem resistance (21.3%) was much higher than that of doripenem (1.2%) or imipenem resistance (3.2%) in 347 pneumococcal isolates [28]. The authors also described that a pneumococcal strain which was resistant to all of these carbapenem antibiotics, but sensitive to vancomycin, showed remarkably divergent sequences in penicillin-binding protein genes. In the present study, meropenem-nonsusceptible isolates showed high MIC values (1–4 μg/mL) against penicillin G (data not shown). Collectively, these data suggest the possibility that penicillin-binding proteins are involved in meropenem resistance of pneoumococcal isolates.

One limitation of our study is the relatively low percentage of isolates for serotyping. Only 54.3% of isolates were collected for serotyping in 2008–2010, although the percentage of isolates for serotyping increased to 71.8% in 2011–2013. Therefore, the incidence of serotype-specific IPD in these children might not be accurate for the interval 2008–2010. Another limitation is that our surveillance areas did not cover all prefectures in Japan. The incidence of IPD in children may differ between the 10 prefectures where the study was conducted and the other 37 prefectures.

In conclusion, this population-based surveillance demonstrated significant declines by 98% in the incidence of PCV7-type IPD and by 57% for all IPD in children <5 years of age within 3 years after the introduction of PCV7 in Japan. Serotype replacement and an increased percentage of meropenem-nonsusceptible strains among pneumococcal isolates from pediatric patients with IPD were also observed during this period. The continuation of nationwide population-based surveillance of IPD in children will enable the evaluation of the incidence, distribution of serotypes, and antimicrobial susceptibility, and will provide the data essential for assessing the effects of PCV13 introduction on pediatric IPD in Japan.

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Conflicts of interest

S.S. reports payments received from Pfizer Japan Inc. for lectures. H.A. reports payments received from Pfizer Japan Inc. and Japan Vaccine Co., Ltd. for lectures. J.N. reports payments received from Pfizer Japan Inc. for lectures. K. Okada reports payments received from Pfizer Japan Inc. for lectures. N.I. reports payments received from Pfizer Japan Inc. and Japan Vaccine Co., Ltd. for lectures. T.I. reports payments received from Mitsubishi Tanabe Pharma Co., Japan Vaccine Co., Ltd., MSD Japan, Takeda Pharmaceutical Co., Ltd., Astellas Pharma Inc., and Daiichi Sankyo Co., Ltd. for lectures. All other authors declare that they have no conflicts of interest.

Authors' contributions

T.I. contributed to the conception and design of the study. S.S., H.A., J.N., K. Okada, H.W., M.O., N.I., A.S., M.H., and T.T. established the surveillance system. S.S., B.C., K.A., H.A., J.N., K. Okada, H.W., A.M., M.O., N.I., A.S., T.O., M.H., and T.T. were responsible for data collection. B.C. performed the microbiological analysis. S.S., B.C., and K. Oishi were responsible for data management and analysis. S.S., B.C., and K. Oishi drafted the paper. All authors have revised the paper critically for important intellectual content. All authors approved the final version for submission.

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.2015.07.069

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Capsule Switching and Antimicrobial Resistance Acquired during Repeated *Streptococcus pneumoniae* Pneumonia Episodes

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***Streptococcus pneumoniae* colonizes the nasopharyngeal mucus in healthy people and causes otitis media, pneumonia, bacteremia, and meningitis. In this study, we analyzed an *S. pneumoniae* strain that caused 7 repeated pneumonia episodes in an 80-month-old patient with cerebral palsy during a period of 25 months. A total of 10 *S. pneumoniae* strains were obtained from sputum samples, and serotype 6B was isolated from samples from the first 5 episodes, whereas serotype 6A was isolated from samples from the last 2. Whole-genome sequencing showed clonality of the 10 isolates with 10 single nucleotide polymorphisms (SNPs) in the genomes. Among these SNPs, one single point mutation in the *wciP* gene was presumed to relate to the serotype switching from 6B to 6A, and the other mutations in *parC* and *gyrA* were related to fluoroquinolone resistance. These results suggested that an *S. pneumoniae* strain, which asymptomatically colonized the patient's nasopharynx or was horizontally transmitted from an asymptomatic carrier, caused the repeated pneumonia events. Phenotypic variations in the capsule type and antimicrobial susceptibility occurred during the carrier state. Hyporesponsiveness to serotypes 6B and 6A of *S. pneumoniae* was found even after vaccination with the 7-valent pneumococcal conjugate vaccine and the 23-valent pneumococcal polysaccharide vaccine. After an additional vaccination with the 13-valent pneumococcal conjugate vaccine, opsonic activities for both serotypes 6A and 6B significantly increased and are expected to prevent relapse by the same strain.**

Streptococcus pneumoniae is the etiologic agent of several bacterial diseases, such as otitis media, pneumonia, bacteremia, and meningitis (1–3), and it can asymptomatically colonize a child's nasopharynx for months (4). Colonization by *S. pneumoniae* is a prerequisite for various types of pneumococcal diseases and the source of transmission of the bacterium between people (5, 6). In Japan, approximately 80% of 3-year-old children were colonized by this bacterium, which may cause pneumococcal diseases, at least once (7).

Since 2000, the widespread use of 7- and 13-valent pneumococcal conjugate vaccines (PCV7 and PCV13, respectively) has reduced the incidence of pneumococcal infections (8–11) and the colonization rate of the vaccine serotypes but has not affected the overall colonization (12–15). In Japan, PCV7 was introduced in 2010 and was replaced by PCV13 in November 2013. After 2011, invasive pneumococcal disease (IPD) caused by *S. pneumoniae* isolates belonging to serotypes included in the PCV7 decreased (16). However, some unusual vaccine failures and/or breakthrough infection cases were reported together with the hyporesponsiveness to one or several serotypes (17).

Since 2011, we have observed repeated pneumococcal pneumonia episodes in a child who has an underlying disease. Although vaccinations with PCV7 were performed, an additional 6 pneumococcal pneumonia infections occurred during the following 24 months. In this longitudinal study, we analyzed 10 *S. pneumoniae* isolates from the 7 pneumonia episodes by serotyping, drug susceptibility testing, and molecular typing (multilocus sequence typing [MLST]). We also compared the whole-genomic sequences to reveal the genetic relationship of the isolates and evaluated a host immune activity against *S. pneumoniae*.

MATERIALS AND METHODS

Diagnosis of pneumococcal pneumonia. Pneumonia was diagnosed based on a positive radiograph and one or more of the following clinical symptoms: fever, rapid or difficult breathing, cough, and crackle in lung fields on auscultation. Sputum samples were collected and pretreated as described by Tanaka et al. (18). Sputum and blood samples were cultured on Columbia agar with 5% sheep blood (Becton, Dickinson and Company Japan, Tokyo, Japan) at 37°C with 5% CO_2. Pathogens accounting for >50% of the colonies in culture or presenting $>1 \times 10^7$ CFU/ml of sputum were regarded as "dominant" (18).

This clinical study was reviewed and approved by the Ethics Committee of Yokohama Minami Kyosai Hospital and conducted according to the principles expressed in the Declaration of Helsinki.

Serotyping, molecular typing, and antimicrobial susceptibility testing. All *S. pneumoniae* isolates were plated on Columbia agar with 5% sheep blood at 37°C. The serotypes were determined using the Quellung reaction with pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark). MLST was performed as described by Enright and Spratt (19). Allelic numbers and sequence types (STs) of the strains were as-

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signed using the pneumococcal MLST website (http://spneumoniae.mlst.net/) for designations.

Susceptibilities to 10 antibiotics of the *S. pneumoniae* isolates were analyzed using the broth microdilution method according to a protocol of the Clinical and Laboratory Standards Institute (20). The antibiotics used were penicillin G, meropenem, panipenem, tebipenem, cefotaxime, tosufloxacin, levofloxacin, erythromycin, clindamycin, and vancomycin. The MIC breakpoints were defined according to the Clinical and Laboratory Standards Institute criteria (20). The MIC breakpoints for panipenem and tebipenem were defined as those for imipenem, whereas the MIC breakpoints for tosufloxacin were defined as those for levofloxacin.

Genome sequencing of the isolated *S. pneumoniae* strains. Whole-genome sequencing DNA libraries were constructed by a Nextera XT DNA sample prep kit (Illumina, San Diego, CA, USA) and then sequenced using a MiSeq instrument (Illumina) with a MiSeq reagent kit v.3 (Illumina). The trimmed and filtered short reads were assembled with the *de novo* assembler program in CLC Genomics Workbench v.6.5 (CLC Bio, Aarhus, Denmark). The phylogenetic analysis was performed with snpTree (21) using contigs of a clinical isolate and 24 available genomic sequences of *S. pneumoniae*. To extract single nucleotide variations and insertion-deletion (Indel) sites among the first 9 isolates, the short reads were aligned to 670-6B (GenBank accession no. CP002176) as a reference sequence using bwasw software (v.0.6.10) (22). All mutation sites were extracted by SAMtools (v.0.1.18) (23) and VarScan (v.2.3.4) (24) software with the default parameters. Single nucleotide polymorphisms (SNPs), revealed from the first 9 isolates, in the last isolate were determined by sequencing using an ABI 3700 analyzer. The PCR primer pairs flanking each SNP position were designed, and both DNA strands of the PCR products were sequenced on an ABI 3130xl sequencer (Applied Biosystems).

ELISA and MOPA. Serum samples from the patient were submitted to the Research Institute for Microbial Diseases (RIMD), Osaka University, Japan, for determination of the IgG levels by an enzyme-linked immunosorbent assay (ELISA) and of the opsonization indices (OIs) by a multiplex opsonophagocytic assay (MOPA). Concentrations of serotype-specific IgG antibodies and OIs were measured as described previously (17). The cutoff for IgG antibodies was 0.05 μg/ml, whereas that of the MOPA was a serum dilution of 1:4.

Nucleotide sequence accession number. The whole-genome sequencing reads are available from the DNA Data Bank of Japan (DDBJ) Sequence Read Archive under accession number DRA003687.

RESULTS

Repeated pneumococcal pneumonia episodes. Our patient was an 80-month-old child with severe multiple handicaps, who was diagnosed with congenital cerebral palsy of unknown cause when she was 5 months old and had repeated pneumococcal pneumonia episodes. We successfully followed the patient, identified the cause of the disease as *S. pneumoniae*, and tried to prevent another episode of pneumonia. The handicapped patient was bedridden and needed full tube feeding due to severe motor and intellectual disabilities. Based on the medical records, the patient had repeated episodes of aspiration pneumonia. We isolated *S. pneumoniae*, which was dominant in the sputum, in December 2011 when she was 54 months old. To prevent pneumococcal infection, she received the first PCV7 vaccination at 55 months old, the 26th day after the previous episode, and received a booster shot after 1 month. However, 6 other episodes of pneumonia occurred after the vaccinations, with the last episode in February 2014 (Table 1 and Fig. 1). To increase coverage for preventable serotypes, the patient received 23-valent pneumococcal polysaccharide vaccine (PPSV23) between the 2nd and 3rd episodes and PCV13 after the last (7th) episode (Table 1 and Fig. 1).

In Japan, when severe pneumonia is diagnosed in children,

TABLE 1 Information and characteristics of the *Streptococcus pneumoniae* strains isolated from the repeat pneumonia infections

No. of isolation	Episode[a]	Date of isolation (yr/mo)	Vaccination history[b]	Diagnosis	Specimens	Serotype	ST[c]	MICs (μg/ml) for[d]: PCG	MEPM	PAPM	TBPM	CTX	VCM	EM	CLDM	TFLX	LVFX
1	1	2011/12	—[e]	Pneumonia	Sputum	6B	4250	1	0.12	0.03	0.015	0.5	0.25	≥8	≥8	≤0.12	1
2	2	2012/6	PCV7 twice	Pneumonia	Sputum	6B	4250	1	0.25	0.03	0.015	1	0.5	≥8	≥8	≤0.12	1
3	3	2012/8	PCV7 twice, PPSV23 once	Pneumonia	Sputum	6B	4250	1	0.25	0.03	0.015	0.5	0.25	≥8	≥8	0.25	2
4		2012/9	PCV7 twice, PPSV23 once	Pneumonia	Sputum	6B	4250	1	0.12	0.03	0.015	0.5	0.25	≥8	≥8	0.25	2
5	4	2012/11	PCV7 twice, PPSV23 once	Pneumonia	Sputum	6B	4250	1	0.25	0.03	0.015	1	0.25	≥8	≥8	0.25	2
6		2012/11	PCV7 twice, PPSV23 once	Pneumonia	Sputum	6B	4250	1	0.12	0.03	0.015	0.5	0.25	≥8	≥8	0.25	2
7	5	2012/12	PCV7 twice, PPSV23 once	Pneumonia	Sputum	6B	4250	2	0.25	0.06	0.03	1	0.25	≥8	≥8	2	4
8		2012/12	PCV7 twice, PPSV23 once	Pneumonia	Sputum	6B	4250	1	0.25	0.06	0.03	1	0.25	≥8	≥8	2	4
9	6	2013/4	PCV7 twice, PPSV23 once	Pneumonia	Aspirate sputum	6A	4250	1	0.25	0.03	0.015	1	0.25	≥8	≥8	2	4
10	7	2014/2	PCV7 twice, PPSV23 once	Pneumonia	Aspirate sputum	6A	4250	1	0.25	0.06	0.03	1	0.25	≥8	≥8	4	4

[a] Episodes 1 to 7 occurred when the patient was 54, 60, 63, 65, 66, 71, and 80 months old, respectively.
[b] PCV7, 7-valent pneumococcal conjugate vaccine; PPSV23, 23-valent pneumococcal polysaccharide vaccine.
[c] ST, sequence type.
[d] PCG, penicillin G; MEPM, meropenem; PAPM, panipenem; TBPM, tebipenem; CTX, cefotaxime; VCM, vancomycin; EM, erythromycin; CLDM, clindamycin; TFLX, tosufloxacin; LVFX, levofloxacin.
[e] —, no immunization history of pneumococcal vaccines.

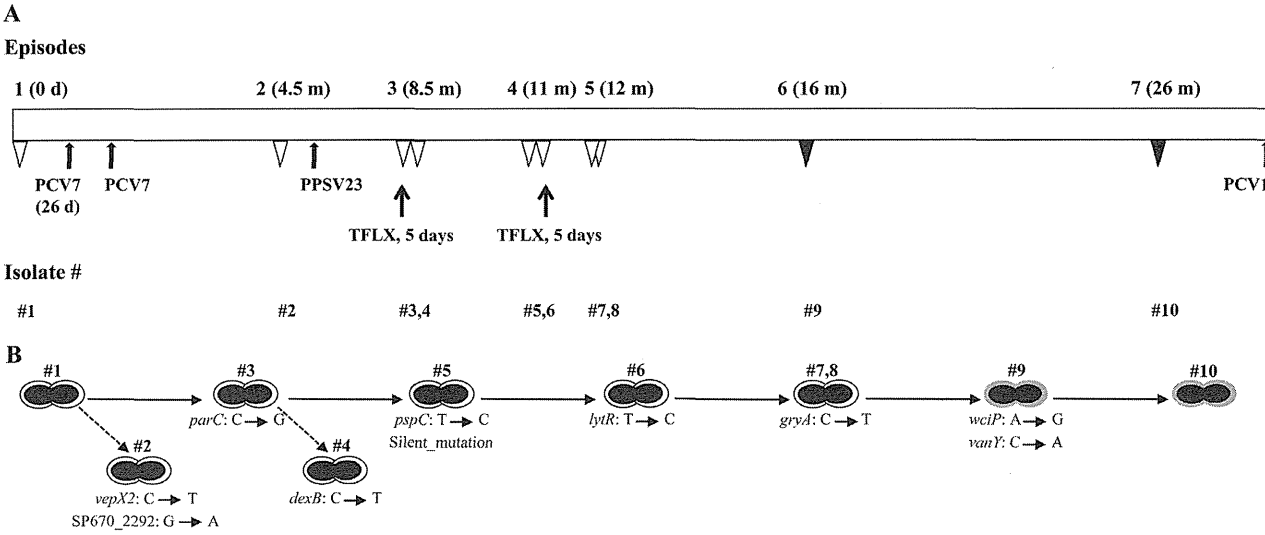

FIG 1 Schedule of the repeated pneumonia episodes and changes in the isolated *Streptococcus pneumoniae* strains. (A) Numbers in parentheses indicate the period from the first episode in December 2011, designated day 0. The vaccination dates for PCV7, PPSV23, and PCV13 are labeled. Tosufloxacin (TFLX) was administered at the 3rd and 4th episodes for 5 days. ▽, serotype 6B; ▼, serotype 6A. (B) Genetic basis of the phenotype changes of the *S. pneumoniae* strain during seven repeated pneumonia episodes.

meropenem, panipenem-betamipron, or tazobactam-piperacillin is recommended as the first-line therapy (25). Because the patient routinely received anticonvulsant medications, carbapenem antibiotics could not be used. Cefpirome was empirically selected as the first-line therapy when pneumonia occurred. Cefpirome was considered effective when the temperature decreased on the 2nd day after administration, the C-reactive protein level decreased to 1/3 on the 4th day, and no *S. pneumoniae* was further isolated from the sputum. The therapy was continued for a total of 5 days. At the 3rd and 4th episodes, because the clinical symptoms returned and *S. pneumoniae* was reisolated from the sputum, cefpirome was additionally administered for 5 days. Usually when someone, especially an adult, around the patient coughs, tosufloxacin has to be used for 5 days in order to prevent superinfection. In this case, tosufloxacin was used at the 3rd and 4th episodes, and the symptomatic therapy was continued until the symptoms improved.

Serotyping, MLST, and MIC of the strains. When pneumonia occurred in the patient, both sputum and blood cultures were performed before antimicrobial administration; no bacterium was found in the blood, whereas *S. pneumoniae* was dominantly detected from the sputum in each of the 7 episodes. Therefore, *S. pneumoniae* was determined to be the causative microorganism of the repeated pneumonia episodes. A total of 10 *S. pneumoniae* strains were obtained from the 7 episodes (Fig. 1 and Table 1). Serotyping, MLST, and antimicrobial susceptibility testing of the 10 *S. pneumoniae* strains were performed, and the results are summarized in Fig. 1 and Table 1. Serotyping revealed that the 8 strains from the first 5 episodes that occurred 1 year after the first episode belonged to serotype 6B, whereas the last 2 strains isolated from the last 2 episodes belonged to serotype 6A. MLST results showed that the 10 strains with the serotypes 6B and 6A were the same (ST4250).

The antimicrobial susceptibility tests showed that all the 10 *S. pneumoniae* strains were susceptible to penicillin G, meropenem, panipenem, tebipenem, cefotaxime, and vancomycin but were resistant to erythromycin and clindamycin (Table 1). The MICs for the 8 antibiotics did not obviously change during the following period. However, the MICs for tosufloxacin and levofloxacin increased during the series of repeated episodes. The strains (no. 3 to 6) isolated from the 3rd and 4th episodes showed higher MICs for these fluoroquinolones than those isolated from the 1st and 2nd episodes. Furthermore, the 4 strains (no. 7 to 10) from the last 4 episodes showed higher MICs than those from episodes 3 and 4 (Table 1). During the 26-month period, the patient received a fluoroquinolone (tosufloxacin) for 5 days just twice during episodes 3 and 4 (Fig. 1).

Comparison of whole-genome sequences of the strains. These strains belonged to the same ST but presented phenotypic variations in capsule types and fluoroquinolone susceptibilities, indicating that the modification occurred while they resided in the patient. In order to clarify the genetic relatedness between the 10 *S. pneumoniae* strains, whole-genome sequencing was performed. Genomic sequences of 24 *S. pneumoniae* reference strains obtained from the NCBI database were used for comparison, and 1 strain isolated in this study was closely related to a 6B strain, 670-6B (see Fig. S1 in the supplemental material). Therefore, the genomic sequence of 670-6B was used as a reference, and mutation sites were detected by mapping analysis among the first 9 strains isolated in this study (Table 2). A total of 2,254 SNPs were found between the sequences of 670-6B and those of the 9 strains isolated in this study. On the other hand, only 10 SNPs were detected among the 9 strains. Among them, 6 SNPs were permanent, whereas the remaining 4 were transient (Table 2; see also Fig. S2 in the supplemental material). The 10 SNPs were also confirmed in the genomic DNA of strain no. 10 by sequencing of the corresponding region. All of the results suggested that the 10 strains were related to *S. pneumoniae* serotype 6B and acquired some minor genetic variations during the asymptomatic state.

The presumed function of each gene described below is based on the genomic sequence of 670-6B. Among the 6 retained SNPs,

TABLE 2 SNPs between the 10 isolated *Streptococcus pneumoniae* strains

Reference strain 670-6B				Base									
Locus[a]	Product	Position from the start base of the ORF[b]	Sequence[c]	1	2	3	4	5	6	7	8	9	10
SP670_1470	DNA topoisomerase IV subunit A, ParC	307	C	C	C	G	G	G	G	G	G	G	G
SP670_2336	Surface protein, PspC	2080	T	T	T	T	T	C	C	C	C	C	C
SP670_2028	Transcriptional regulator	235	T	T	T	T	T	T	C	C	C	C	C
SP670_1060	DNA gyrase subunit A, GryA	242	C	C	C	C	C	C	C	T	T	T	T
SP670_0425	Rhamnosyl transferase Cps6bS, WciP	584	A	A	A	A	A	A	A	A	A	G	G
SP670_0687	D-Alanyl-D-alanine carboxypeptidase	382	C	C	C	C	C	C	C	C	C	A	A
SP670_0411	Trehalose-6-phosphate hydrolase	1202	C	C	C	C	T	C	C	C	C	C	C
SP670_0660	ABC transporter ATP-binding protein, Vexp2	34	C	C	T	C	C	C	C	C	C	C	C
1203448[d]	Silent_mutation		C	C	C	C	C	A	C	C	C	C	C
SP670_2292	Putative alpha-L-fucosidase 1	1213	G	G	A	G	G	G	G	G	G	G	G

[a] Position of the nucleotide acid on the genomic DNA of reference strain 670-6B.
[b] ORF, open reading frame.
[c] Sequence of the nucleotide acid of reference strain 670-6B.
[d] Position of the nucleotide acid of reference strain 670-6B.

the first mutation occurred in strain 3 from episode 3. A mutation from C to G was found at position 2130 in the *parC* gene, leading to an amino acid substitution in ParC (position 103, from Ala to Pro). This mutation was found in 8 strains (no. 3 to 10) that showed higher MICs for tosufloxacin and levofloxacin than strains 1 and 2, suggesting that the *parC* mutation increased the MICs for fluoroquinolones. Another related mutation, a C to T mutation at position 242 that resulted in a substitution in the 81st amino acid (from Ser to Phe), leading to higher MICs for tosufloxacin and levofloxacin, was found in the *gyrA* gene of strains 7 to 10. These results indicated that ParC and GyrA were involved in the resistance of *S. pneumoniae* to quinolone antibiotics.

Although the first 8 strains (no. 1 to 8) and the last two strains (no. 9 and 10) possessed different capsule types, 6B and 6A, respectively, strains 9 and 10 were genetically related to the first 8 strains. Two SNPs found in strains 9 and 10 may be related to the differentiation of the capsule type. One was the mutation in the *wciP* gene encoding rhamnosyl transferase, and the other was the mutation in the *vanY* gene encoding D-alanyl-D-alanine carboxypeptidase. The A to G mutation at position 584 in the *wciP* gene, which resulted in the substitution at the 195th amino acid (from Asn to Ser), may be involved in the serotype change from 6B to 6A (26). Although D-alanyl-D-alanine carboxypeptidase is presumed to play a role in the outer membrane biogenesis, the relation of this mutation to the serotype change is unknown. The other mutations on the *pspC* and *lytR* genes were introduced in strains 5 and 6 from episode 5 and were maintained in the following strains. The changes corresponding to the 2 SNPs are still unknown.

Among the 4 SNPs that were transient and detected only once (Table 2; see also Fig. S2 in the supplemental material), 3 did not result in amino acid substitutions. Only the C to T mutation at position 34 in the *vexp2* gene resulted in a substitution in the 12th amino acid (from Arg to Cys). This gene encodes an ABC transporter ATP-binding protein, but the change corresponding to this SNP is unknown.

Vaccine booster. Although vaccination with PCV7 was performed twice after the 1st episode, and vaccination with PPSV23 was performed once after the 2nd episode, 5 further pneumococcal pneumonia episodes occurred. Serotyping revealed that the isolated *S. pneumoniae* strains belonged to serotypes 6B and 6A (a 6B-related serotype). Serotype 6B is contained in both PCV7 and PPSV23. In theory, pneumococcal infections caused by serotype 6B and by the 6B-related 6A *S. pneumoniae* can be prevented by the vaccines. To understand the hyporesponsiveness to the infecting serotypes 6B and 6A of *S. pneumoniae*, concentrations of serotype-specific IgG antibodies and opsonic activities for PCV7-containing and -related serotype *S. pneumoniae* were measured. Serum samples obtained before vaccination with PCV13 and after the booster with PCV13 were used, and the results are shown in Table 3. The concentrations of IgGs specific for all of the tested serotypes, including 6B and 6A, were >0.05 μg/ml. On the other hand, the OIs for 6B and 6A serotypes of *S. pneumoniae* were lower than 1:8 before the booster. After vaccination with PCV13, the OIs increased for 6B (3,136) and 6A (2,957), and other PCV13-containing serotypes of *S. pneumoniae* were detected. Taken together, the results suggested that the patient received effective anti-6B-

TABLE 3 Serotype-specific IgG levels and OIs in sera of the patient[a]

Date of sampling (yr/mo/day)	Results for serotype: 4		6B		9V		14		18C		19F		23F		19A		6A	
	IgG[b]	OIs[c]	IgG	OIs	IgG	OIs	IgG	OIs	IgG	OIs	IgG	OIs	IgG	OIs	IgG	OIs	IgG	OIs
2014/2/19	1.3	3,095	2.1	7	2.8	17,460	2.8	17,496	2	2,589	7.1	17,496	1.4	17,496	3.3	3,440	2.2	2
2014/5/29	2.6	1,875	4.3	3,136	2.4	1,098	11.6	17,496	3.4	1,861	17.1	6,271	4.7	17,496	-	4,097	5.8	2,957

[a] The patient was vaccinated with PCV7 on 11 January 2012 and 13 February 2012, with PPSV23 on 12 July 2012, and with PCV13 on 1 May 2014, respectively.
[b] Concentration of serotype-specific IgG (mg/ml).
[c] OIs, opsonization indices.

and anti-6A-specific IgG antibodies for the prevention of pneumococcal infection caused by the two *S. pneumoniae* serotypes.

DISCUSSION

In this report, we describe a patient with repeated episodes of pneumonia caused by an *S. pneumoniae* strain. The repeated episodes are due to the condition of the patient and to the characteristics of serotype 6B of *S. pneumoniae*. Since the patient had congenital cerebral palsy and needed tube feeding, she was susceptible to aspiration pneumonia. To prevent infections caused by bacteria, immunizations containing pneumococcal vaccines were recommended. However, hyporesponsiveness to serotype 6B of *S. pneumoniae* after vaccination has been frequently reported (27–30).

The same ST4250 *S. pneumoniae* strain, or one that was at least genetically closely related, was identified as the responsible bacterium in these infectious cases. From 2007 to 2014, a total of 311 6B and 84 6A strains isolated from pneumococcal infections in children who lived in Japan were analyzed in our laboratory. The major STs were ST90 in 6B and ST5833 in 6A (B. Chang, unpublished data). With the exception of the strains analyzed in this study, only 1 ST4250 strain belonging to serotype 6B was isolated from another patient. No epidemiological association between the patients was found. These results suggested that the ST4250 was rare, was not prevalent among Japanese children, and sustainably existed around the patient.

Since nasopharyngeal colonization of *S. pneumoniae* is thought to be a major risk for pneumococcal infections, sustained normal colonization of the ST4250 strain in the patient's nasopharyngeal mucosa was considered. However, no *S. pneumoniae* and only normal bacterial flora were detected in the nasopharyngeal mucosa when the patient recovered from each pneumonia episode and was healthy (data not shown). False-negative results due to the time of and techniques for sampling and isolation cannot be excluded. Another cause of the repeated infections might be the horizontal transmission of the bacterium from an asymptomatic carrier in close contact with the patient. Indeed, the patient has a brother who is 5 years older, and at the time of the infections, the patient attended a day care facility; both factors are considered to be major risks for asymptomatic colonization of *S. pneumoniae* in Japanese children (7). However, examination of the nasopharyngeal mucosa of her sibling and of the toddlers in the day care were not permitted, because the patient had pneumonia only, not a systemic infection. Therefore, the hypothesis that the strain was transmitted from others was not confirmed in this study. Although the exact route of infection is still unclear, both possibilities should be considered.

A follow-up study on chronic pediatric otitis media caused by *S. pneumoniae* was previously reported by Hiller et al. (31). In this pediatric patient, two STs of *S. pneumoniae* strains existed and were isolated during symptomatic episodes. By using whole-genome sequencing technologies, Hiller et al. determined that approximately 156 kb of the genomic content of the *S. pneumoniae* strain was replaced during a 7-month investigation period, results that are totally different from those obtained in this study. The pneumococcal dynamic evolution was due to the simultaneous presence of polyclonal populations of *S. pneumoniae*, chronic biofilm infection, or hyperrecombination of *S. pneumoniae* strains (31). In this study, only one ST of *S. pneumoniae* strain was detected during the investigation period, and pneumonia was acute and completely cured every time, and no chronic biofilm was formed. Furthermore, the ST4250 *S. pneumoniae* strain is unlikely to recombine at a high frequency, because only 10 SNPs were detected, and no long recombinant fragments were found during the following 25 months.

From the genomic sequencing of the 10 *S. pneumoniae* strains, two types of SNPs were detected. One type occurred in an open reading frame and was persistent. A total of 6 SNPs belonged to this type, and 3 of them had clear connections with phenotype variations: changes in serotype and MICs for fluoroquinolone antibiotics. Amino acid substitution resulted from a single point mutation from A to G on the *wciP* gene at position 584, which has been related by Mavroidi et al. (26) to a switch from serotype 6B to 6A in this study. Furthermore, the genomic analyses also confirmed the relation between ParC/GyrA and resistance to quinolone antibiotics. On the other hand, 4 SNPs were detected only once. The reason for the identification of transient SNPs might be the analysis of only one colony from each episode. Therefore, we might have observed just part of genome diversification and might have missed other transient SNPs.

Although receiving vaccinations with PCV7 twice and PPSV23 once, the patient had pneumococcal pneumonia caused by PCV7- and PPSV23-containing serotypes 6B and the 6B-related 6A of *S. pneumoniae*. The exact reasons for the nonresponsiveness to PPSV23 are still unclear, whereas several studies reported that hyporesponsiveness to specific serotypes after vaccination of children with PCV7 was detected in IPD cases (27–30). Although many causes might lead to this phenomenon, three factors are thought to be significant. First, no marked increase in IgG for a specific serotype was detected after vaccination, probably because of immune paralysis (27). Second, no sufficient opsonization activity was found, probably because of the lower avidity of serotype-specific IgG against pneumococcal infection (17, 30). Third, pneumococcal carriage in the nasopharynx was detected, which may also be caused by immune paralysis (32, 33). In this study, the level of anti-6B IgG was higher than 0.35 μg/ml (2.1 μg/ml), whereas the OI was <8. Only normal bacterial flora and no *S. pneumoniae* were found in the patient's nasopharyngeal mucosa when she was healthy. These results suggest that insufficient opsonization activity against serotype 6B of *S. pneumoniae* may be due to the repeated infections. Although the exact cause of insufficient opsonization activity has not been identified, the low avidity of anti-6B IgG and compromised immune function are the most probable causes. Fortunately, effective 6B- and 6A-specific IgG antibodies were detected in the patient after vaccination with PCV13.

After the introduction of PCV vaccines, incidence of IPD caused by the vaccines containing *S. pneumoniae* serotypes decreased (8–11). However, sometimes the vaccines are not effective against nasopharyngeal colonization (12, 13). Some vaccine serotypes, such as 6B and 19F, also colonized the nasopharyngeal mucosa. Furthermore, nonvaccine serotypes of *S. pneumoniae* were detected at a higher rate than before the use of vaccines. Thus, although IgG and opsonophagocytic activity specific for the serotypes of *S. pneumoniae* contained in the vaccines were confirmed in the patient, *S. pneumoniae* nasopharyngeal colonization has not been excluded. Therefore, routine hygiene management for the prevention of infections, including pneumococcal infection, is indispensable in the patient.

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We declare no conflicts of interest.

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The Journal of Infectious Diseases
MAJOR ARTICLE

Memory B-Cell Pools Predict the Immune Response to Pneumococcal Conjugate Vaccine in Immunocompromised Children

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Background. The immune responses to pneumococcal conjugate vaccine (PCV) are low in immunocompromised hosts. The effect of memory B cells on the immune response to PCV remains elusive.

Methods. In this prospective study, 53 children who received 7-valent PCV were enrolled. Antipneumococcal immunoglobulin G (IgG) levels and opsonization index (OI) titers, along with lymphocyte subsets, were investigated in immunocompromised and immunocompetent hosts. Immunocompromised patients comprised 8 hematopoietic stem cell transplant recipients (group A) and 9 immunosuppressive therapy recipients (group B), and controls consisted of 14 children aged >1 year (group C) and 22 infants (group D).

Results. Serotype-specific IgG concentrations and OIs in group A were lower than those in group C. These did not differ among groups B, C, and D. The rates of achieving immunity (defined as an IgG level of 1.0 μg/mL and an OI of 8) in group A were also lower than in group C. Despite the sustained numbers of total T cells and B cells, $CD27^+$ B-cell and $CD4^+$ T-cell counts in group A were lower than those in group C. In group B, the immunoglobulin D–expressing $CD27^-$ B-cell count was only lower than that in group C.

Conclusions. Circulating numbers of $CD27^+$ B cells, rather than $CD4^+$ T cells, may predict the effective PCV responses in immunocompromised children.

Keywords. 7-valent pneumococcal conjugate vaccine; after hematopoietic stem cell transplantation; antipneumococcal immunoglobulin G antibody; opsonophagocytic activity; memory B cell.

The frequency of invasive pneumococcal disease (IPD) in childhood was dramatically decreased as a result of the introduction of pneumococcal conjugate vaccine (PCV) [1, 2]. In Japan, the incidences of pneumococcal meningitis and bacteremia were markedly decreased in infants and children after the introduction of 7-valent pneumococcal conjugate vaccine (PCV7) in 2010 [3]. Immunocompromised hosts are at high risk for the development of IPD: in Japan, recommendations for pneumococcal vaccination are stronger for immunocompromised children than for healthy children. The US Advisory Committee on Immunization Practices recommends routine use of PCV for all children with an immunocompromised status, and the efficacy of this vaccine has been demonstrated [4, 5]. It is recommended that preterm infants receive vaccinations according to their chronological but not gestational age, as with term infants. The antibody responses in preterm infants may be suboptimal but are considered to provide protective immunity in the majority [6]. The immune response to PCV in immunocompromised children was also reportedly lower than that in healthy children [7, 8]. The effective duration of protective immunity against IPD remains elusive. However, immunocompromised conditions arise from heterogeneous therapy-related events and underlying diseases. Furthermore, the other chronological factor of immune reconstitution should be taken into account for patients who have undergone hematopoietic stem cell transplantation (HSCT), to establish the effective vaccination schedule. The production of antipneumococcal antibodies might account for the function of T-cell–dependent and –independent pathways [9–11]. Memory (immunoglobulin D $[IgD]^+CD27^+$ or IgD^-CD27^+) B cells have an important role in the primary and secondary antibody responses to pneumococcal conjugate or polysaccharide vaccines [10–12]. On the other hand, the in vivo effect of memory B cells on the immune response to conjugate vaccine remains elusive. There is little information about the acquisition of PCV immunity in immunocompromised infants and children.

In the present study, to verify the PCV program in immunocompromised children, we investigated the lymphocyte

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subpopulations at vaccination and the antipneumococcal immunoglobulin G (IgG) antibody production and opsonophagocytic activity after vaccination in disease-free children after HSCT, as well as in pediatric patients receiving immunosuppressive therapy.

MATERIALS AND METHODS

Subjects

This prospective study involved 53 children who received PCV7 between 1 November 2010 and 31 October 2013 at the Department of Pediatrics, Kyushu University Hospital, or at Tagawa Municipal Hospital. They were classified into immunocompromised groups (groups A and B) and control immunocompetent groups (groups C and D), as follows. Group A comprised 8 surviving hematopoietic stem cell transplant recipients (median age, 5.8 years; range, 1.4–16.1 years) whose underlying diseases were acute lymphoblastic leukemia (in 2), severe combined immunodeficiency (in 2), chronic granulomatous disease (in 1), Wiskott–Aldrich syndrome (in 1), myelodysplastic syndrome (in 1), and familial hemophagocytic lymphohistiocytosis (in 1; Supplementary Table 1). The median interval from HSCT to vaccination was 23 months (range, 13–72 months). At the time of vaccination, all patients were disease free after control of graft versus host disease without immunosuppressive agents for >1 year. Group B consisted of 9 patients (median age, 3.4 years; range, 1.5–10.2 years) receiving immunosuppressive therapy. They included 3 liver transplant recipients, 2 kidney transplant recipients, 2 patients with nephrotic syndrome, 1 with juvenile idiopathic arthritis, and 1 with bronchial asthma. Corticosteroids (to 8), tacrolimus (to 6), cyclosporine (to 2), mycophenolate mofetil (to 2), and methotrexate (to 1) were administered as treatment. All liver and kidney transplantations were successful. The underlying diseases were all controlled. Group C comprised 14 healthy children beyond the first year of life (median age, 3.4 years; range, 1.0–12.3 years). Group D was composed of 22 infants aged <1 year, of whom 13 were term (median age, 0.3 years; range, 0.2–0.9 years) and 9 were preterm (median age, 0.3 years; range, 0.3–0.4 years). Informed consent was obtained from all patients' parents. This study was approved by the institutional review boards of Kyushu University and Tagawa Municipal Hospital.

Vaccine

PCV7 (Prevnar; Pfizer, Pearl River, New York) contains polysaccharides of pneumococcal serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F, which are conjugated to the protein carrier CRM197, a nontoxic variant of the diphtheria toxin. PCV7 was subcutaneously administered at a dose of 0.5 mL, according to the standard Japanese immunization schedule. Children aged ≤6 months receive PCV7 3 times every 4 weeks as a primary series, followed by a fourth injection as the booster dose after 1 year of age. Children aged 7–11 months receive PCV7 twice every 4 weeks as a primary series, followed by a third injection as the booster dose after 1 year of age (and at least 2 months after the second injection). Children aged 12–23 months receive PCV7 once as a primary series, followed by a second injection as the booster dose after 2 months. Children aged >24 months receive PCV7 only once.

Collection of Samples

The present study was designed to examine the immune response to a primary series of PCV7 vaccinations because the immunocompromised children were >2 years of age. Peripheral blood specimens were collected from subjects prior to the first vaccination and 4–6 weeks after the last vaccination of the primary series. Flow cytometry was performed on whole-blood specimens, using ethylenediaminetetraacetic acid as the anticoagulant, within 24 hours after collection. Serum samples were immediately separated by centrifugation and stored at −80°C until the following analyses. Because nasopharyngeal carriage of *S. pneumoniae* affects the serotype-specific hyporesponsiveness [13], nasopharyngeal swab samples were collected for culture from each subject at the time of first vaccination. Serotyping of *S. pneumoniae* was performed when *S. pneumoniae* was isolated from the culture samples.

Measurement of Antipneumococcal IgG Antibody

The serum concentrations of antipneumococcal IgG antibodies were measured by World Health Organization (WHO)–approved enzyme-linked immunosorbent assay using standard reference serum (89-SF or 007sp) and C-polysaccharide and 22F polysaccharide absorption, as previously described [14, 15]. The cutoff for the assay was 0.05 μg/mL. The levels of serotype-specific IgG for 7 serotypes (4, 6B, 9V, 14, 18C, 19F, and 23F) were determined according to the WHO protocol (available at: http://www.vaccine.uab.edu/ELISA).

Measurement of Opsonophagocytic Activity

A multiplexed opsonophagocytic killing assay specific for PCV7 serotypes was performed as previously described [16]. The quality control in each assay was performed by using the pooled sera of adults vaccinated with PPV23 or PCV7. The opsonization index (OI) was defined as the serum dilution capable of killing 50% of the bacteria, which was determined by using Opsotiter3 software in accordance with the WHO protocol (available at: http://www.vaccine.uab.edu/UAB-MOPA). The cutoff for the assay was a serum dilution of 1 : 2. An enzyme-linked immunosorbent assay and a multiplexed opsonophagocytic killing assay were performed by members who were blinded to the vaccine allocation.

Analysis of B-Cell and T-Cell Subpopulations

The lymphocyte subsets prior to the vaccination were analyzed by flow cytometry. Fluorescein isothiocyanate (FITC)–conjugated anti-CD19 antibody (BD Biosciences, Mountain View, California) and phycoerythrin (PE)–conjugated anti-CD3 antibody

(Beckman Coulter, Miami, Florida) were used to define the proportions of B cells and T cells, respectively, on gated lymphocytes. FITC-conjugated anti-CD4, PE-conjugated anti-CD3, and phycoerythrin-cyanin 5.1 (PC5)–conjugated anti-CD8 antibodies (Beckman Coulter) were used to define the proportions of helper and cytotoxic T cells. FITC-conjugated IgD antibody (BD Biosciences), PE-conjugated CD27, and PC5-conjugated CD19 antibodies (Beckman Coulter) were used to identify B-cell subsets. A 2- or 3-color flow cytometric analysis was performed by using EPICS XL (Beckman Coulter). The analysis gate was set within the lymphocyte by using forward and side scatters as previously described [17]. Specificity of staining was assessed using fluorochrome-conjugated isotype-matched monoclonal antibodies. Each analysis was performed using at least 20 000 cells.

Statistical Analysis

The Mann–Whitney *U* test was used to compare the group means between 2 groups. The Fisher exact test was used to determine the distribution between 2 categorical variables. The correlation between the antipneumococcal IgG titer and the OI was determined by the Spearman rank correlation coefficient. *P* values of <.05 were considered to be statistically significant. The SPSS Statistics software program (version 21; SPSS, Chicago, Illinois, and IBM, Armonk, New York) was used for the analysis.

RESULTS

Serotype-Specific IgG Levels and OIs Before and After Vaccination

Demographic characteristics of the 4 groups are shown in Table 1. There was no difference in the age and sex distributions among groups A, B, and C (Table 1). All children categorized into groups A, B, and C received PCV7 once as a primary series because they were >12 months of age. Among samples collected after vaccination, geometric mean concentrations (GMCs) of serotype-specific IgG in group A were lower against 5 of 7 serotypes (4B, $P = .008$; 6B, $P = .015$; 9V, $P = .013$; 18C, $P = .013$; and 23F, $P = .007$), compared with GMCs in group C (Table 2). Median OIs in group A after vaccination were lower against 6 of 7 serotypes (4B, $P = .010$; 9V, $P = .005$; 14, $P = .040$; 18C, $P = .029$;

Table 1. Demographic Characteristics of Subjects, by Study Group

Characteristic	Group A (n = 8)	Group B (n = 9)	Group C (n = 14)	Group D (n = 22)
Age, y, median (range)	5.8 (1.4–16.1)	3.4 (1.5–10.2)	3.4 (1.0–12.3)	0.3 (0.2–0.9)
Male sex, %	44.4	62.5	35.7	54.5
Doses of PCV7 in primary series, no.	1	1	1	2 or 3[a]

Group A comprised hematopoietic stem cell transplant recipients, group B comprised immunosuppressive therapy recipients, group C comprised immunocompetent children, and group D comprised infants.

Abbreviation: PCV7, 7-valent pneumococcal conjugate vaccine.

[a] Children aged ≤6 months received PCV7 3 times every 4 weeks. Children aged 7–11 months received PCV7 twice every 4 weeks.

Table 2. Pneumococcal Serotype–Specific Immunoglobulin G (IgG) Concentrations Among Subjects Before and After Vaccination, by Study Group

	Geometric Mean IgG Concentration, μg/mL (Range)							
	Before Vaccination				After Vaccination			
Serotype	Group A	Group B	Group C	Group D	Group A	Group B	Group C	Group D
4B	0.33 (0.06–1.03)	0.73 (0.18–2.34)	0.38 (0.12–1.65)	0.22 (0.05–0.79)	0.89 (0.09–2.95)[a]	2.14 (0.42–6.01)	2.32 (0.79–5.46)	4.95 (2.57–15.93)[a]
6B	0.53 (0.11–1.70)	1.19 (0.49–4.33)	0.93 (0.28–6.43)	0.33 (0.08–1.63)[a]	0.64 (0.17–1.87)[a]	3.46 (0.51–122.04)	2.84 (0.26–19.14)	1.95 (0.32–20.74)
9V	0.31 (0.04–1.97)	0.56 (0.09–3.31)	0.39 (0.08–5.45)	0.10 (0.02–0.66)[a]	0.86 (0.05–8.04)[b]	3.67 (1.08–10.87)	2.69 (1.43–5.62)	3.64 (0.66–11.37)[b]
14	0.81 (0.13–2.23)	1.89 (0.82–6.14)	0.80 (0.22–5.19)	0.70 (0.13–3.98)	1.58 (0.25–3.91)	5.46 (1.99–15.11)	2.74 (0.45–9.01)	9.65 (1.67–45.12)[a]
18C	0.33 (0.10–1.29)	0.55 (0.18–6.70)	0.40 (0.11–1.84)	0.17 (0.02–0.54)[a]	0.83 (0.11–4.83)[b]	5.09 (1.27–7.39)	2.85 (0.90–7.21)	3.34 (0.43–16.01)
19F	1.48 (0.40–3.05)	2.15 (0.70–12.15)	1.55 (0.40–8.88)	0.51 (0.07–1.69)[a]	1.66 (0.52–3.80)	12.07 (0.95–249.28)	3.00 (0.79–12.27)	5.45 (2.43–19.62)[a]
23F	0.35 (0.10–1.13)	1.03 (0.20–3.37)	0.60 (0.21–1.70)	0.30 (0.04–1.38)[b]	0.68 (0.21–3.11)[a]	2.52 (0.49–52.54)	2.94 (0.44–25.67)	2.84 (0.34–10.46)

Group A comprised hematopoietic stem cell transplant recipients, group B comprised immunosuppressive therapy recipients, group C comprised immunocompetent children, and group D comprised infants.

[a] $P < .01$, compared with group C.

[b] $P < .05$, compared with group C.

Table 3. Pneumococcal Serotype–Specific Opsonization Index (OI) Among Subjects Before and After Vaccination, by Study Group

	OI, Median (Range)							
	Before Vaccination				After Vaccination			
Serotype	Group A	Group B	Group C	Group D	Group A	Group B	Group C	Group D
4B	2 (2–5)	2	2 (2–217)	2 (2–765)	325.5 (2–1168)[a]	2171 (2–17 496)	1323 (78–5039)	1334 (2–6274)
6B	2 (2–173)	2 (2–990)	3 (2–2640)	2 (2–6)	133 (2–1670)	2788 (2–17 496)	2607 (2–17 496)	2411 (2–17 496)
9V	2 (2–94)	2 (2–28)	6.5 (2–4459)	2 (2–5)[b]	41.5 (2–2190)[b]	1419 (2–17 496)	1131 (99–4497)	732 (24–7763)
14	2 (2–111)	22 (2–4317)	6 (2–1445)	2 (2–4916)	738 (237–17 496)[a]	17 496 (11–17 496)	4480.5 (19–34 992)	5974 (113–17 496)
18C	3.5 (2–11)	5 (2–701)	7 (2–450)	2 (2–39)[a]	132.5 (6–586)[a]	812 (2–4105)	730 (30–2523)	864 (21–17 496)
19F	2 (2–32)	2 (2–60)	2 (2–249)	2 (2–172)	52 (2–1544)[a]	1539 (2–17 496)	485 (25–3527)	461 (12–17 496)
23F	2 (2–172)	2 (2–223)	3 (2–3515)	2 (2–11)[a]	340.5 (2–6469)[b]	4133 (2–17 496)	5579 (11–34 992)	1971 (2–17 496)

Group A comprised hematopoietic stem cell transplant recipients, group B comprised immunosuppressive therapy recipients, group C comprised immunocompetent children, and group D comprised infants.

Abbreviation: OI, opsonization index.

[a] $P < .05$, compared with group C.

[b] $P < .01$, compared with group C.

19F, $P = .020$; and 23F, $P = .009$), compared with OIs in group C (Table 3). There were no differences in the GMCs of IgG and median OIs for any serotype between group B and group C. Among samples collected before vaccination, GMCs of IgG against 5 serotypes (6B, 9V, 18C, 19F, and 23F) and median OIs against 3 serotypes (9V, 18C, and 23F) in group D were significantly lower than those in group C. On the other hand, after vaccination, GMCs of IgG against 4 serotypes (4B, 9V, 14, and 19F) in group D were significantly higher than those in group C. There were no differences in the GMCs of IgG and median OIs against any serotypes between preterm and term infants (data not shown). Antipneumococcal IgG levels positively correlated with the OIs in each serotype after PCV receipt (Table 4).

Rates of Achieving the Thresholds of IgG Levels and OIs After Vaccination

More than 0.2 μg/mL of serotype-specific IgG is recommended for effective protection after the administration of conjugate vaccines. On the other hand, >1.0 μg/mL of IgG against serotypes 4, 14, and 19F was reported to give a better correspondence between the serological response rate and vaccine effectiveness [18]. When the thresholds of IgG levels and OIs are defined as ≥1.0 μg/mL and ≥8 in any serotype, respectively [19], the rates of achieving the threshold of IgG levels against serotypes 6B ($P = .026$), 9V ($P = .010$), 18C ($P = .011$), and 23F ($P = .026$) in group A after PCV receipt were lower than those in group C (Figure 1). The rates of achieving the OI threshold in group A after PCV receipt tended to be lower in group A than in group C, although this did not reach statistical significance. The rates of achieving the threshold IgG level and OI after PCV receipt did not differ between group B and group C.

Table 4. Correlation Between Immunoglobulin G Level and Opsonization Index After Vaccination

Serotype	R	*P* Value
4B	0.619	<.001
6B	0.796	<.001
9V	0.581	.001
14	0.477	.007
18C	0.511	.003
19F	0.390	.03
23F	0.426	.017

Lymphocyte Subpopulations at the Time of the First Vaccination

Peripheral leukocyte and lymphocyte counts and the proportions and absolute numbers of T-cell and B-cell subpopulations at the time of the first vaccination are shown in Table 5. Both the proportion and the absolute number of $CD4^+$ T cells in group A were significantly lower than those in group C ($P = .037$ and $P = .015$, respectively). The proportion but not the number of $CD8^+$ T cells in group D was lower than that in group C ($P = .016$). Irrespective of the presence of IgD expression, both the proportions and the numbers of $CD27^+$ B cells in group A were lower than those in group C (IgD^+ memory B cells: proportion, $P = .028$, and number, $P = .015$; IgD^- memory B cells: proportion, $P = .020$, and number, $P = .050$). The proportion and number of IgD^-CD27^+ B cells ($P < .0001$ and $P = .0007$, respectively) and the proportion but not the number of IgD^+CD27^+ B cells ($P = .014$) in group D were lower than those in group C. Both the proportions and numbers of IgD^+CD27^- naive B cells in group A and group D were not lower than those in group C. Total B-cell counts and IgD^+CD27^- B-cell counts but not the other B-cell subset counts in group B were lower than those in group C.

T-Cell and B-Cell Subpopulations in Subjects With a Protective IgG Level and OI

We then investigated the relationships between the immune response to PCV and the numbers of $CD4^+$ T cells and IgD^+ and

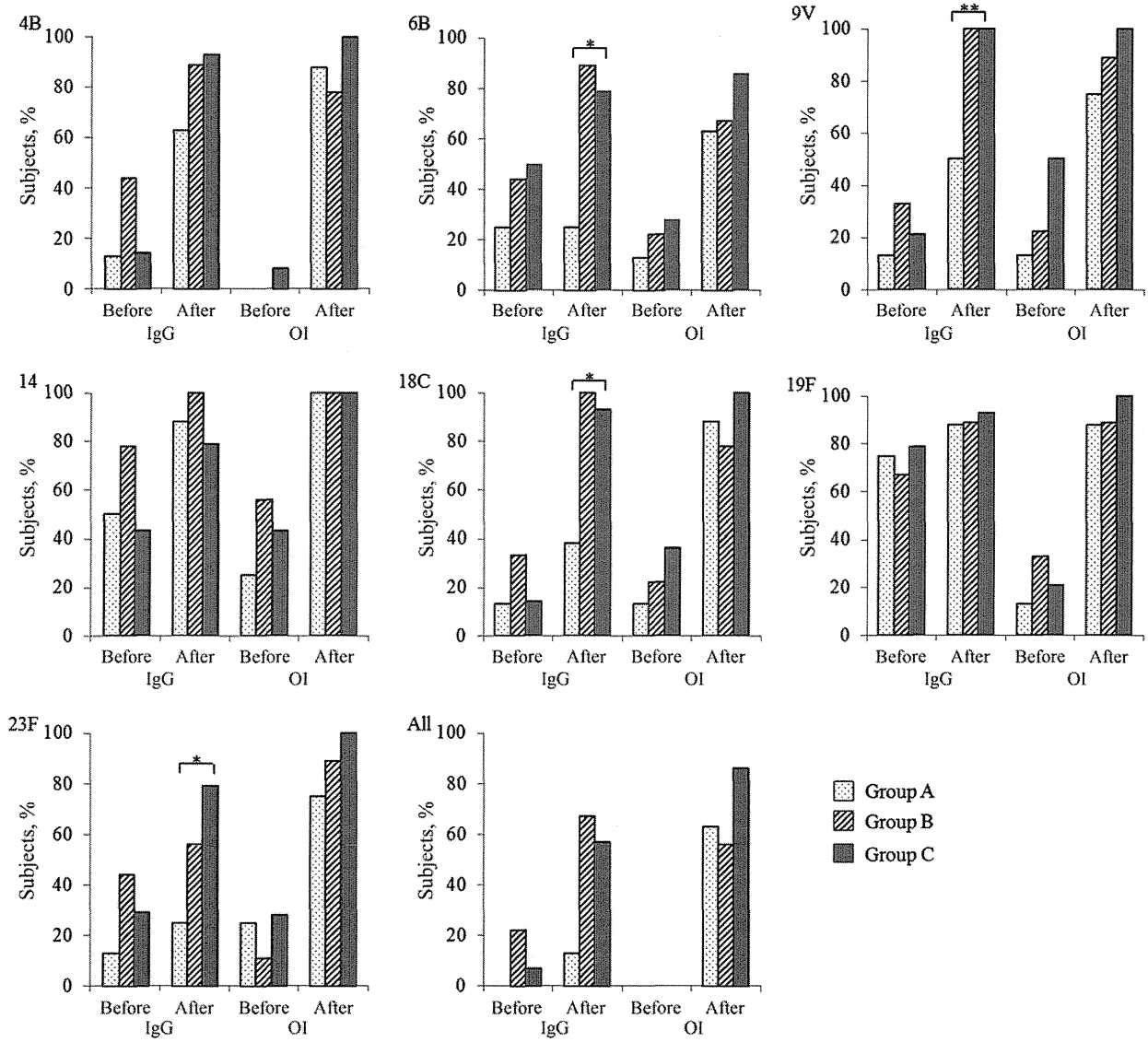

Figure 1. Proportions of hematopoietic stem cell transplant recipients (group A), immunosuppressive therapy recipients (group B), and immunocompetent children (group C) who exceeded the protective levels of immunity against each pneumococcal serotype, defined as an immunoglobulin G (IgG) titer of ≥1.0 μg/mL and an opsonization index (OI) of ≥8. Children in all groups were >1 year old. "All" denotes all 7-valent pneumococcal conjugate vaccine serotypes. *$P < .05$ and **$P < .01$.

IgD^- memory B cells in 31 children beyond the first year of life (groups A, B, and C). To investigate these relationships, we selected the serotypes with a significant difference in the rate of achieving the threshold IgG level between group A and group C (serotypes 6B, 9V, 18C, and 23F). Protective responders were defined as children reaching the threshold IgG level (≥1.0 μg/mL) and OI (≥8). The proportions of responders against serotypes 6B, 9V, and 18C with an IgD^+ memory B-cell count exceeding the median value were significantly higher than those of nonresponders (Table 6). The proportion of responders against serotype 6B with an IgD^- memory B-cell count exceeding the median value was significantly higher than that of nonresponders (Table 6). The proportion of children with a $CD4^+$ T-cell count exceeding the median value did not differ between responders and nonresponders against the 4 serotypes. In group A, absolute numbers of $CD4^+$ T cells and IgD^- and IgD^- memory B cells in responders tended to be higher than those in nonresponders, although this did not reach statistical significance (Supplementary Table 2).

Isolation of Resident Pneumococcus and Clinical Utility of PCV

S. pneumoniae was isolated from nasopharyngeal swab specimens obtained from 6 of 53 children (11.3%). None of the strains carried the serotypes of PCV7. No PCV7-associated

Table 5. Absolute Cell Counts and Proportions of Leukocyte and Lymphocyte Subpopulations Among Subjects Before Vaccination, by Study Group

Variable	Group A	Group B	Group C	Group D
Leukocyte count, cells/μL	7600 (5420–10 470)	7800 (4900–9580)	7000 (4440–14 910)	10 300 (7100–15 180)[a]
Lymphocyte count, cells/μL	2713 (2010–4030)	2559 (515–4790)	3669 (309–6777)	4755 (2383–8868)
T cells[b]				
Count, cells/μL	1790 (919–2287)	1928 (205–3332)	2535 (431–4720)	3180 (1537–5579)
Percentage of lymphocytes	56.5 (45.7–72.1)	76.0 (61.1–84.1)	66.1 (50.0–80.8)	67.1 (45.5–87.7)
$CD4^+$				
Count, cells/μL	545 (314–1177)[a]	680 (147–1701)	1313 (309–1960)	1589 (506–3281)
Percentage of T cells	34.2 (24.4–56.3)[a]	50.9 (28.0–60.1)	49.0 (37.7–71.9)	51.7 (17.9–77.9)
$CD8^+$				
Count, cells/μL	556 (331–1127)	546 (41–1071)	732 (87–1276)	590 (291–2273)
Percentage of T cells	39.3 (15.6–65.0)	30.9 (22.2–43.0)	26.8 (18.4–45.1)	18.3 (15.2–40.8)[a]
B cells[c]				
Count, cells/μL	704 (125–1173)	194 (50–641)[a]	660 (179–1431)	965 (179–2475)
Percentage of lymphocytes	18.6 (6.2–31.7)	13.5 (1.4–26.4)	21.4 (10.9–30.9)	12.5 (5.9–46.8)
IgD^+CD27^-				
Count, cells/μL	546 (87–1014)	140 (32–392)[a]	417 (137–994)	832 (162–2263)
Percentage of B cells	84.0 (63.9–88.0)	64.0 (61.0–74.8)	70.4 (62.3–83.2)	91.4 (75.5–97.3)[d]
IgD^+CD27^+				
Count, cells/μL	9 (3–23)[a]	9 (1–57)	14 (9–79)	11 (1–43)
Percentage of B cells	1.3 (0.4–7.4)[a]	5.7 (1.0–9.0)	3.7 (1.3–7.6)	1.5 (0.0–7.1)[a]
IgD^-CD27^+				
Count, cells/μL	30 (9–74)[a]	36 (9–156)	71 (18–423)	11 (2–233)[d]
Percentage of B cells	7.0 (3.7–9.8)[a]	18.3 (14.3–29.1)[d]	10.0 (3.5–29.6)	1.9 (0.6–17.0)[d]

Data are median value (range). Group A comprised hematopoietic stem cell transplant recipients, group B comprised immunosuppressive therapy recipients, group C comprised immunocompetent children, and group D comprised infants.

Abbreviation: IgD, immunoglobulin D.

[a] $P < .05$, compared with group C.

[b] Identified by flow cytometry, using fluorescein isothiocyanate–conjugated anti-CD19 antibody.

[c] Identified by flow cytometry, using phycoerythrin-conjugated anti-CD3 antibody.

[d] $P < .01$, compared with group C.

adverse events or IPD occurred in any children during the study period.

DISCUSSION

The notable finding in this study is that the PCV response and the numbers of $CD27^+$ B cells and $CD4^+$ T cells at vaccination were lower in patients who underwent HSCT than in controls. The rates of acquiring protective immunity were significantly higher in subjects with a IgD^+CD27^+ B-cell count greater than the median value but not in those with $CD4^+$ T-cell count greater than the median value. These results suggest the critical involvement of memory B-cell pools in the PCV responses of immunocompromised children.

Several studies have reported that competence in cellular immunity plays an important role in the immune response to conjugate vaccine [9, 20]. On the other hand, the role of humoral immunity, especially memory B cells, in the immune response to conjugate vaccine is poorly understood. IgD^+ memory B cells, characterized as nonswitched memory or marginal zone–like B cells, mainly produce immunoglobulin M (IgM) and are capable of responding immediately to the antigens of encapsulated bacteria in a T-cell–independent fashion [21]. IgD^- memory B cells, called switched memory B cells, require T-cell costimulation to produce high-affinity IgG and other isotypes of antibody within germinal centers of lymphoid tissue [22]. The proportion of IgD^- memory B cells was positively associated with the antibody response to PCV among adult patients infected with human immunodeficiency virus [10]. In addition, serum IgM antibodies contribute to high levels of opsonophagocytic activities in children vaccinated with PCV, suggesting that IgD^+ memory B cells might be related to the immune response to PCV [23]. In this context, the present results suggest that distinct memory B-cell subsets, rather than $CD4^+$ T cells, might contribute to PCV responses in immunocompromised children.

Individuals who have undergone HSCT have long-term depletion of both IgD^+ and IgD^- memory B cells [24]. Memory B-cell pools are not restored 24 months after HSCT [24]. IgD^- memory B-cell counts are reported to correlate with $CD4^+$ T-cell counts, which indicate the impaired function of germinal centers [25]. Depletion of IgD^+ memory B cells may be a consequence of hyposplenism occurring after HSCT [26]. In the present study, both the proportions and the numbers of $CD4^+$ T cells and memory B cells were reduced in

Table 6. Counts of $CD4^+$ T Cells and B Cells, With or Without Immunoglobulin D (IgD) Expression, Among Subjects, by Response to Pneumococcal Conjugate Vaccine

Serotype, T- or B-Cell Count[a]	Responders, No. (%)	Nonresponders, No. (%)	*P* Value
6B	n = 19	n = 12	
$CD4^+$ T-cell count >697 cells/μL	11 (58)	5 (42)	.473
IgD^+CD27^+ B-cell count >12 cells/μL	14 (74)	2 (17)	.003
IgD^-CD27^+ B-cell count >44 cells/μL	14 (74)	2 (17)	.003
9V	n = 26	n = 5	
$CD4^+$ T-cell count >697 cells/μL	14 (54)	1 (20)	.333
IgD^+CD27^+ B-cell count >12 cells/μL	16 (62)	0 (0)	.018
IgD^-CD27^+ B-cell count >44 cells/μL	15 (58)	1 (20)	.172
18C	n = 23	n = 8	
$CD4^+$ T-cell count >697 cells/μL	13 (57)	3 (38)	.433
IgD^+CD27^+ B-cell count >12 cells/μL	15 (65)	1 (13)	.016
IgD^-CD27^+ B-cell count >44 cells/μL	14 (61)	2 (25)	.113
23F	n = 20	n = 11	
$CD4^+$ T-cell count >697 cells/μL	12 (60)	4 (36)	.273
IgD^+CD27^+ B-cell count >12 cells/μL	12 (60)	4 (36)	.273
IgD^-CD27^+ B-cell count >44 cells/μL	12 (60)	4 (36)	.273

Responders had an immunoglobulin G titer of ≥1.0 μg/mL and an opsonization index of ≥8. A total of 31 subjects >1 year of age were analyzed. All were hematopoietic stem cell transplant recipients, immunosuppressive therapy recipients, or immunocompetent.

[a] Values are median absolute T- or B-cell counts.

patients who underwent HSCT. This is the first report investigating the association between PCV response and detailed B-cell subsets in children who underwent HSCT. Memory B-cell counts and $CD4^+$ T-cell counts might be useful to determine the optimal schedule of PCV vaccination after HSCT.

Despite the decreased B-cell counts in pediatric patients who received immunosuppressive therapy, single-dose PCV induced effective PCV responses. On the other hand, neither the proportion nor the absolute number of memory B cells was lower in these children than in controls. These findings indicate that memory B-cell pools, but not total B-cell counts, are involved in the immune response to PCV. Serotype-specific antibody concentrations after receipt of a single PCV dose in solid organ transplant recipients were reported to be lower than those in healthy children, although the responses depend on the type of organ transplanted; heart transplant recipients showed lower responses than liver transplant recipients or healthy controls [27]. Treatments with rituximab or methotrexate were predictive of an impaired antibody response to PCV. On the other hand, anti–tumor necrosis factor drugs, mycophenolate mofetil, and cyclosporine A did not affect the antibody responses [28–30]. Further study of PCV responses in immunocompromised children should be directed to the types of transplantation and immunomodulation therapy, including biologics.

In infants in the present study (group D), $CD27^+$ memory B cells were lower in terms of their proportion and number. This might be explained by the fact that group D included an appreciable number of preterm infants. The proportions of lymphocyte subsets in infants differ from those in healthy children >1 year of age. On the other hand, the proportion of memory B cells in preterm infants was reportedly similar to that in term infants [31]. Our study verified the effective PCV response in infants after the primary series of boosting schedule. Taking these findings together into consideration, the optimal boost may induce preventive PCV immunity in immunocompromised children showing low $CD27^+$ B-cell counts.

There are several limitations to the present study. First, the total study population might be small for statistical analysis, although different methods were used for the assessment of preventive immunity in the subgroup analysis. Second, the correlations between pneumococcal-specific anti-IgM antibody level and memory B-cell counts and percentages were not assessed in this study. Finally, substantial long-term efficacy of PCV was not evaluated in the present study. The adequate titers in immunocompromised hosts might be waning. Further large-scale and long-term investigation is needed to evaluate the efficacy of PCV for immunocompromised children.

In conclusion, the GMCs of antipneumococcal antibodies and the median OIs in children who underwent HSCT were lower than those in immunocompetent children. Lower immune responses to PCV in children who underwent HSCT might be induced by the depletion of memory B cells, as well as by T-cell dysfunction. Further large-scale study appears to be warranted to determine whether the balance between $CD4^+$ T cells and memory B cells might be useful to decide the schedule of PCV vaccination in immunocompromised children.

Supplementary Data

Supplementary materials are available at http://jid.oxfordjournals.org. Consisting of data provided by the author to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the author, so questions or comments should be addressed to the author.

Notes

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