G809S may be expected to enhance NF-κB activation in the presence of R837. However, G809S did not increase NF-κB activity like as Y859C [6] (Fig. 4b). Since the *NLRP3* LRR domain plays a central role in mediating inflammation induced by another inflammasome activator, MSU crystals, we examined whether G809S affected NF-κB activation in the presence of MSU [14]. Interestingly, G809S and Y859C mutations did not show any NF-κB activity responses by MSU stimulation. In contrast, wild-type, D303N and G755R mutations significantly increased NF-κB activity following MSU stimulation. These data suggest the G809S LRR missense variant, which may diminish the responsiveness to PAMPs as NOD2 LRR variant reported in Crohn's disease, has a pathogenic effect on these pathways [15–17].

Jéru et al. recently identified a pathogenic Y859C mutation in the LRR domain of *NLRP3*, which increased speck formation and pro-caspase 1 processing, but which had no direct effect on *NLRP3* mediated NF-κB signaling. The G809S variant also increased speck formation relative to wild-type *NLRP*3. These results suggest that G809S, as well as Y859C in the LRR domain, may be a gain of function variant. It should be noted that although the assays used in this study are sensitive, our findings may provide limited evidence to prove that the G809S variant is pathogenic. However, these results indicate that the variant alters the function of NLRP3.

The two case studies presented here consistently showed elevated IL-1-related serum cytokines, IL-1ra, during the attack phase. In addition, monocytes from case 1 and 2 secreted high levels of IL-1β, which may indicate a gain of function variant in *NLRP3*, associated with inflammasome activation. Additionally, we previously reported a CINCA/NOMID patient positive for the compound heterozygous gene mutations, E688K and G809S [9]. This patient developed severe a phenotype compared with her mother, who carried a single mutation, E688K. This genotype-phenotype correlation suggests that the G809S variant may act as an additional genetic factor associated with the severity of CAPS.

However, in this study IL-1β was not detectable in the serum of patients, as IL-1β might be rapidly neutralized, metabolized, or captured by a plethora of IL-1 receptors in vivo. Furthermore, although elevated serum IL-18, which is activated by caspase-1 as well as IL-1β, and IL-6 levels were observed in CINCA/NOMID patient [9], the serum IL-18 levels were increased in case 2 but not case 1, and serum IL-6 levels in both cases did not increase during the fever episodes. Thus, it may be considered that the differences of cytokine profiles and disease phenotypes between case 1 and 2 and typical CINCA/NOMID patients result not only from their genetic background, but also environmental factors.

Additional mutation analysis of our patients also revealed heterozygous variant haplotype of *MEFV*, a gene involved in the pathogenesis of FMF, in addition to G809S in *NLRP3*. Case 1 was heterozygous for P369S and R408Q in *cis* and case 2 was heterozygous for E148Q, P369S, and R408Q in *cis*. Allele frequencies of P369S and R408Q in the Japanese population are 3.6 % and 4.8 %, respectively, according to the International HapMap Project (http://www.hapmap.org/). These frequent variant haplotypes were found to be in strong linkage disequilibrium in the Japanese population. In addition, P369S and R408Q variant haplotype are associated with a variable phenotype and are infrequently associated with typical FMF symptoms [18–21]. Heterozygous P369S and R408Q variant haplotype are also associated with other inflammatory diseases, such as Behçet's disease [18], and systemic lupus erythematosus [21]. Moreover, heterozygous E148Q-P369S-R408Q variant haplotype is more rare, which is associated with chronic recurrent multifocal osteomyelitis [20]. In this report, case 1 and case 2 showed the similar phenotypes as FMF or TRAPS, respectively. Although detailed clinical features and cytokine profiles of the two cases are various, they exhibited a long duration of recurrent fever episodes compared with typical FMF. Thus, these findings suggest that P369S and R408Q variant haplotype may have effects on several inflammatory diseases, but the functional evidence of these variant haplotype remains unclear.

The *MEFV* gene codes for pyrin, that can interact with ASC to induce ASC oligomerization and the activation of procaspase-1, which promotes IL-1β and IL-18 processing [12, 22]. In contrast, some reports have described that pyrin inhibited NLRP3-mediated NF-κB activation by disrupting the NLRP3-ASC interaction [23, 24]. In accordance with the reports, co-expression of NLRP3 and pyrin in HEK293T cells indicated less ASC-dependent NF-κB activation than expression of NLRP3 only, whereas there was no difference in the inhibitory capacity of NF-κB activity between pyrin variants and the wild-type protein. Interestingly, a recent study using pyrin deficient and mutated pyrin knock-in mice demonstrated a gain of function with pyrin variants located in B-Box domains, which caused autoinflammatory phenotypes [22]. Thus, research using knock-in mouse experiments with *MEFV* exon3 variants into pyrin deficient mice would help clarify the pathogenic effects of the *MEFV* variant.

In general, hereditary periodic fever syndromes have been considered monogenic diseases. On the other hand, recent reports have described patients with heterozygous low penetrance variants in two recurrent fever genes [2, 25, 26]. These indicate that oligogenic inheritance has been related to pathogenesis of autoinflammatory diseases. In some cases, patients presented with specific symptoms of both diseases or with a more severe phenotypes. Although the patients in this study were positive for the *NLRP3* variant, they did not present with typical symptoms of CAPS, such as deafness or cold-induced rash. In addition, variants in *MEFV* have been detected in both cases, but they also lacked typical FMF symptoms. However,

both cases had obviously periodic fever episodes. These suggest the presence of oligogenicity and that variants in *NLRP3* and *MEFV* synergistically modify the symptoms of the atypical autoinflammatory diseases.

There are two important limitations in this study when discussing the pathogenicity of low penetrance rare variants. The first limitation is the limited number of patients in the study. Further study using a large number of patients is necessary to confirm our results. Secondly, we only analyzed a limited number of genes. In this study, we concluded that the presence of an *NLRP3* variant with the co-existence of *MEFV* variants contributed to atypical autoinflammatory disease. However, the patients may have had alternative genetic mutations or other rare variants of inflammasome related genes such as *CARD8* [27] elsewhere in the genome, which are truly disease causing, and the two variants described in these patients may be unrelated.

Conclusions

This study describes the molecular analysis of two cases with heterozygous low penetrance variants in exon5 of *NLRP3* and exon3 of *MEFV*. The findings provide in vivo and in vitro evidence for the effect of an *NLRP3* missense variant. Importantly the mutations are within the same signaling pathway and are associated with inflammasome activation. Our observations suggest that oligogenic inheritance may occur in patients with atypical autoinflammatory syndrome. It is therefore important to consider that the phenotypes could be modified by synergistic effects with plural autoinflammatory-associated gene mutations when the patients have atypical autoinflammatory disease.

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Conflict of Interest The authors have declared no conflicts of interest.

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CASE REPORT

Recurrent bacterial meningitis by three different pathogens in an isolated asplenic child

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Abstract Isolated congenital asplenia (ICA) is a rare condition at risk for overwhelming infection. When complicated by invasive infection, the mortality remains high, at greater than 60%. We describe a girl with ICA who developed recurrent meningitis by three different pathogens. The first, meningitis by *Escherichia coli*, occurred 4 days after premature birth. The other two pathogens were serotype 6B *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib), at 18 and 25 months of age, respectively. The patient was successfully treated with prompt antimicrobial therapy in all episodes. Serum anti-polyribosylribitol phosphate (PRP) and anti-6B-type pneumococcal antibodies were below the levels for protective activity after natural infections. Although anti-PRP antibody was significantly increased after Hib vaccination, two (6B and 19F) of seven serotype-specific pneumococcal antibodies were not elevated to protective levels after the second 7-valent pneumococcal conjugate vaccine (PCV7). We, therefore, added a third PCV7. To our knowledge, this is the first neonatal ICA patient with invasive infection and the first case of bacterial meningitis occurring three times. Our findings indicate that monitoring of immune responses after natural infections and vaccinations, and reevaluations of vaccine schedule, are important for ICA patients to prevent subsequent invasive infections.

Keywords Isolated congenital asplenia · Bacterial meningitis · Immunological response · Recurrence · Neonate · Vaccine

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Introduction

Congenital asplenia often occurs as part of a recognized malformation syndrome with anomalies of the heart, great vessels, and viscera [1]. The best known among these syndromes is the asplenia/polysplenia syndrome associated with visceroarterial heterotaxy, and its incidence is estimated at approximately 1/10,000 to 1/40,000 live births [2]. In contrast, isolated congenital asplenia (ICA) occurs fairly more infrequently. A recent French nationwide study indicated that the prevalence is 0.51 per million births [2]. Both conditions have an increased susceptibility to overwhelming invasive infections, carrying considerable mortality. However, the diagnosis of ICA is sometimes difficult because of the lack of other anomalies; therefore, such individuals may be unrecognized until postmortem autopsy.

Practice guidelines for the prevention of life-threatening infections in children with hyposplenia and asplenia advocate antibiotic prophylaxis and immunizations against *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (Hib), the most common causative organisms for

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these patients [3]. However, given that several asplenic cases of overwhelming infections that could be considered as vaccine failures have been documented [4, 5], the immunogenicity of vaccination for asplenic patients is still an important concern.

We present here a girl with ICA who developed multiple episodes of meningitis caused by three different pathogens, namely, *Escherichia coli*, *S. pneumoniae* (serotype 6B), and Hib. She was successfully treated with prompt initiation of antibiotics in all episodes. We also present the details of immune responses to natural infections by Hib and serotype 6B *S. pneumoniae* and those to immunizations of Hib conjugate vaccine and 7-valent pneumococcal conjugate vaccine (PCV7).

Case report

A 4-day-old girl, who was born of nonconsanguineous parents as their first child, weighing 1,742 g at the 34th week of gestation, presented with repetitive apnea during admission because of prematurity. Physical examination showed that heart rate was 135/min and body temperature was 37.2°C. Laboratory data showed WBC of 5.8 × 10^9/l with 28.5% neutrophils, C-reactive protein (CRP) of 4.3 mg/dl, and blood glucose of 95 mg/dl. Cerebrospinal fluid (CSF) examination showed 3,947 cells/μl with 96% polymorphonuclear cells, 197 mg/dl protein, and 44 mg/dl glucose. Two days later, isolates from the CSF and blood were identified as *E. coli* OX:K1:H–, and the same bacterium was also subsequently isolated from the stool of her asymptomatic mother. The patient was diagnosed as having early-onset *E. coli* meningitis that was vertically transmitted. We treated the patient with cefotaxime (CTX) for 21 days. Auditory brainstem response examination at 28 days of age revealed profound hearing impairment at the right ear. The patient was discharged at 38 days of age. Genetic analysis [6, 7] showed that the strain harbored virulence factor genes such as *iroN*, *papG3*, *afa*, and *kps*, but not *cnf1*, *sfa*, or *ibeA*.

At 18 months old, the patient was rehospitalized because of a 6-h history of fever and generalized tonic-clonic convulsion lasting 3 min. On admission, 30 min after the convulsion, heart rate was 170/min and body temperature was 39.4°C. Her consciousness had become clear. Laboratory findings showed WBC of 21.7 × 10^9/l and CRP of 6.0 mg/dl. CSF examination showed no pleocytosis, with normal concentrations of protein (10 mg/dl) and glucose (85 mg/dl). Treatment with intravenous CTX was empirically initiated under the tentative diagnosis of occult bacteremia. The day after admission, serotype 6B *S. pneumoniae* was isolated from the blood but not from the CSF. Resistance to penicillin was established by microbiological [minimum inhibitory concentration (MIC), 2 μg/ml] and genotypic (mutations in *pbp1a*, *pbp2X*, and *pbp2b* [8]) analyses, and CTX was substituted with panipenem–betamipron. On day 3, prolonged fever and frequent vomiting led us to perform a second CSF examination, showing 14,500 cells/μl, protein of 58 mg/dl, and glucose of 63 mg/dl. The CSF was positive for *S. pneumoniae* antigen test (Binax NOW *S. pneumoniae*; Binax), but yielded no organisms in culture. The blood WBC and CRP were elevated to 21.7 × 10^9/l and 22.1 mg/dl, respectively. We diagnosed her disease as pneumococcal meningitis following bacteremia and increased the doses of panipenem–betamipron with good clinical response. She received antimicrobial therapy for 14 days and was discharged without any additional sequelae.

At 25 months of ages, the patient was referred to the emergency department in another hospital with a 2-h history of fever, vomiting, and tonic–clonic convulsion of 2-min duration. At arrival, heart rate was 180/min and body temperature was 39.4°C. Her consciousness soon became clear. Laboratory examination showed WBC of 3.5 × 10^9/l and CRP of 0.6 mg/dl. After blood culture was obtained, the patient received intravenous sulbactam/ABPC. On day 3, the blood culture yielded β-lactamase-non-producing ABPC-resistant (BLNAR) Hib, and the laboratory examinations showed marked deterioration: WBC of 26.6 × 10^9/l and CRP of 21.5 mg/dl. CSF examination showed 4,992 cells/μl, 164 mg/dl protein, and 34 mg/dl glucose with positive culture for Hib. Thus, the diagnosis of a third bacterial meningitis was made. The patient thereafter received intravenous meropenem for 14 days and was discharged on day 16 after onset without any additional sequelae. Molecular analysis of the strain identified three amino acid substitutions: His-517, Thr-385, and Ile-377, in *ftsI* [9]. This substitution pattern was classified as subgroup III BLNAR by a recent nationwide study of childhood meningitis in Japan [9].

The multiple episodes of meningitis prompted us to evaluate immunological functions. The results after the second episode of meningitis showed that serum levels of IgG (639 mg/dl), IgA (65 mg/dl), IgM (97 mg/dl), IgG_2 (80 mg/dl), C3 (140 mg/dl), C4 (24 mg/dl), and CH50 (36.1 U/ml) were within normal limits. T/B-cell subsets (65/28%), CD3/CD4/CD8 lymphocyte subsets (61%/44%/14%), natural killer cell activity (25%), neutrophil phagocytic activity using fluorescence bead test by flow cytometry (70.0%), and neutrophil bacteriocidal activity (93.4%) were also normal. Computed tomography (CT) of the skull and inner ears did not show any deformity or defects. To screen interleukin-1 receptor-associated kinase 4 deficiency and myeloid differentiation primary response protein 88 deficiency, we performed flow cytometric analysis [10], resulting in normal intracellular tumor necrosis factor-α

production of monocytes after lipopolysaccharide stimulation. After the third meningitis, ultrasonography and CT of the abdomen finally revealed asplenia without visceroarterial anomalies. Howell–Jolly body-containing RBCs were exceedingly rarely found (<0.1% of RBCs) in peripheral blood. Ultrasonographic examinations of her parents detected normal size and normal position of the spleen.

Since the diagnosis of ICA at 26 months of age, chemoprophylaxis with amoxicillin of 20 mg/kg/day was introduced as well as vaccinations of Hib vaccine and PCV7. Subsequent to the introduction of these strategies, the patient has not suffered from any invasive infections for more than 2 years. At 36 months of age, we assessed her neurodevelopmental status using the New Edition of the Kyoto Scale of Psychological Development, indicating a normal developmental quotient of 88 (normal range, >80).

We evaluated immune responses to natural infections with Hib and serotype 6B pneumococcus and those to immunizations of Hib vaccine and PCV7 (Table 1). Despite natural infections, serum anti-polyribosylribitol phosphate (PRP) (0.60 μg/ml) and anti-serotype 6B (0.191 μg/ml) antibodies were below the levels of long-term protective activity (1.0 μg/ml [11] and 0.34 μg/ml [12, 13], respectively) 4 and 6 months after each infection, respectively. At 1 month after administration of the second Hib and PCV7 vaccination, anti-PRP antibody was significantly elevated to 3.15 μg/ml, but two (6B and 19F) of seven serotype-specific pneumococcal antibodies were still below the protection levels. We therefore added a third PCV7. Because antibodies to pneumococcal capsular polysaccharide protect the host by opsonizing pneumococci for phagocytosis, we concomitantly performed the opsonophagocytic killing assay (OPA) [14] after the third PCV7. Table 1 shows significantly high OPA titers against types 6B and 19F were observed, findings inconsistent with the low anti-6B and anti-19F IgG antibody levels. OPA titers against five other types were also elevated to the levels for protection (>8) [12, 13].

Discussion

We report a girl with non-familial ICA with recurrent bacterial meningitis. ICA is a rare anomaly. Mahlaoui et al. [2] recently documented 20 ICA cases in France and reviewed the literature. In addition to the 65 cases in their report and references therein [2], we found reports of 5 other ICA patients [5, 15] in the literature between January 1960 and April 2011 using the Medline database. Thus, we can here review 70 ICA cases in total. Compared with these patients [2, 5, 15], our case is informative and interesting in several respects.

First are the multiple episodes of meningitis caused by three different pathogens. Of the previous 70 cases, 48 (69%) experienced invasive bacterial infection at least once. Of these 48 patients, only 8 had multiple episodes of invasive bacterial infections, two times in 5 cases and three times in 3 cases (Table 2) [2, 16–20]. Our patient is the first described for whom all three episodes were bacterial meningitis. To better understand the underlying pathogenesis, we characterized the causative pathogens by molecular analysis. Penicillin-resistant serotype 6B pneumococcus and BLNAR Hib subgroup III were among the most prevalent strains causing childhood meningitis in Japan [8, 9]. In contrast, *E. coli* is extremely rare among ICA patients, and we are aware of only one such case, which resulted in death at 4 months of age [21]. *E. coli* in our case possessed capsular antigen K1 and the siderophore receptor gene, *iroN*, which contribute to the bacteremic step in *E. coli* neonatal meningitis [7, 22]. Because the same strain was isolated from the stool of her asymptomatic mother, we confirmed the route of contagion. Besides asplenia, prematurity of the host and high pathogenic factors of the *E. coli* strain might have contributed to this infection.

Second is the good prognosis, despite our patient developing meningitis three times, one of which occurred 4 days after premature birth. Our neonatal case is the youngest at the first invasive infection among the previously reported ICA patients. There have been only 3 ICA patients

Table 1 Serum serotype-specific IgG antibody concentrations and opsonophagocytic killing assay titer before and after 7-valent pneumococcal conjugate vaccine

Serotype	4		6B		9V		14		18C		19F		23F	
	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA	IgG conc.	OPA
Before PCV7 (6 months after natural infection)	0.132	NA	0.191	NA	0.062	NA	0.366	NA	4.229	NA	0.295	NA	0.14	NA
1 month after 2-dose PCV7	2.809	NA	0.263	NA	4.040	NA	6.767	NA	3.949	NA	0.356	NA	0.233	NA
1 month after 3-dose PCV7	1.37	536	0.137	557	1.199	326	5.075	2367	1.89	210	0.295	192	0.471	769

PCV7 7-valent pneumococcal conjugate vaccine, *IgG conc.* anti-serotype-specific IgG antibody concentration (μg/ml), *OPA* opsonophagocytic killing assay (titer), *NA* not assessed (under treatment with antimicrobial agents)

Table 2 Isolated congenital asplenia patients with multiple episodes of invasive bacterial infections

Patient number	Gender	Infectious episodes	Age at onset	Type of infection	Organisms	Outcome	Reference
1	F	1	6 months	Meningitis	*Streptococcus pneumoniae*	Survived	[2]
		2	11 months	Meningitis, purpura fulminans	*S. pneumoniae*	Died	
2	M	1	10 months	Meningitis	*S. pneumoniae*	Survived	[2]
		2	11 months	Purpura fulminans	*S. pneumoniae*	Survived	
		3	1 year 7 months	Purpura fulminans	*S. pneumoniae*	Survived	
3	M	1	1 year 9 months	Meningitis	*S. pneumoniae*	Survived	[16]
		2	2 years 3 months	Meningitis	*S. pneumoniae*	Survived	
4	M	1	1 year 2 months	Meningitis	*S. pneumoniae*	Survived	[17]
		2	15 years	Meningitis	Not available	Died	
5	M	1	1 year	Meningitis	*S. pneumoniae*	Survived	[18]
		2	1 year	Meningitis	*S. pneumoniae*	Survived	
		3	1 year	Osteomyelitis	Culture negative	Survived	
6	F	1	6 months	Meningitis	*S. pneumoniae*	Survived	[19]
		2	2 years 6 months	Sepsis	Not available	Died	
7	F	1	1 year 6 months	Arthritis	*S. pneumoniae*	Survived	[19]
		2	1 year 9 months	Arthritis	*Haemophilus influenzae* type b	Survived	
		3	10 years	Sepsis	*S. pneumoniae*	Died	
8	M	1	5 years	Sepsis	*S. pneumoniae*	Survived	[20]
		2	9 years	Meningitis	*S. pneumoniae*	Died	
9	F	1	0 month (4 days)	Meningitis	*Escherichia coli*	Survived	Present case
		2	1 year 6 months	Meningitis	*S. pneumoniae*	Survived	
		3	2 years 1 month	Meningitis	*H. influenzae* type b	Survived	

who had overt infections under 3 months of age, which include 1 fatal case [21] and 2 with major sequelae (central nervous system deficit [23] or loss of foot and fingers [24]). Of the 45 childhood and adult patients with invasive infections whose outcomes were known, 29 (64%) died and 3 (7%) had serious sequelae [2, 5, 23, 24]. In contrast, our patient showed normal neurological development under non-serious sequelae of unilateral hearing loss. Such favorable outcome may be attributable to the early recognition and hospitalization. Fortunately, the first episode developed during the period of hospitalization under close monitoring because of prematurity. In addition, at both second and third infectious episodes, she could receive immediate antimicrobial treatment.

Finally, we meticulously investigated the immunological responses to natural infections with *S. pneumoniae* and Hib and those to vaccinations. Of the 70 cases we can review [2, 5, 15], there has been no report addressing this issue. The spleen is a pivotal organ for the phagocytosis of encapsulated bacteria and for the production of immunoglobulins against these pathogens [3]. Even after natural invasive infections of Hib and serotype 6B pneumococcus, serum antibody levels were not elevated to the levels of long-term protection against the pathogens, which may reflect the immunocompromised status of asplenia. This concept is supported by findings from Mikoluc et al. [25] that the congenital asplenic patients had significantly lower concentrations of serum anti-pneumococcal antibodies and reduced responses to PCV7, especially to serotypes 6B and 23F. Similar findings were also observed in adult asplenic patients with overwhelming infection caused by *S. pneumoniae*, representing vaccine failures [4, 5]. Serum antibody concentrations against 6B and 19F in our patient were significantly lower than those against five other serotypes. In contrast, when we evaluated OPA titers after the third PCV7 vaccination, they were at sufficient levels for protection against all serotypes including types 6B and 19F. OPA might be a more important indicator for protection against *S. pneumoniae* [13].

In conclusion, we described a girl with a rare case of ICA, who presented with recurrent meningitis caused by three different pathogens, and was successfully treated without severe sequelae. Exact determination of serum antibody concentrations of encapsulated bacteria and reevaluation of vaccine schedules should be important to protect against relevant infections in ICA patients.

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ORIGINAL ARTICLE

Endocrine complications in primary immunodeficiency diseases in Japan

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Summary

Background In spite of the accumulating evidence on the interaction between the immune and endocrine systems based on the recent progress in molecular genetics, there have been few epidemiological studies focused on the endocrine complications associated with primary immunodeficiency diseases (PID).

Objective To investigate the prevalence and clinical features of endocrine complications in patients with PID in a large-scale study.

Design and participants This survey was conducted on patients with PID who were alive on 1 December 2008 and those who were newly diagnosed and died between 1 December 2007 and 30 November 2008 in Japan. We investigated the prevalence and the clinical data of the endocrine complications in 923 patients with PID registered in the secondary survey.

Results Among 923 PID patients, 49 (5·3%) had endocrine disorders. The prevalence of the endocrine diseases was much higher in patients with PID than in the general population in the young age group, even after excluding patients with immune dysregulation.

Conclusions Endocrine disorders are important complications of PID. Analysis of the endocrine manifestations in patients with PID in a large-scale study may provide further insights into the relationship between the immune and endocrine systems.

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Introduction

A wide variety of clinical complications have been described in primary immunodeficiency diseases (PID).[1,2] PID have been reported to be associated with an increased risk of cancer, in particular non-Hodgkin lymphoma,[2] and the contribution of immune dysfunction in PID to cancer risk is receiving much attention. It is also well known that patients with PID often have complications such as autoimmune and allergic disorders.[1,3] Recently, the interaction between the immune and endocrine systems has been getting increasing attention.[4,5] However, there have so far been no reports focusing on the endocrine complications associated with PID in a large-scale survey.

Many endocrine disorders in patients with PID are thought to be due to the development of the autoimmunity, which is closely related to the pathophysiology of PID.[6] However, it is not known how the immunological and molecular defects in individual PID contribute to the development of various autoimmune endocrine disorders. In addition, the genetic defects in some PID can lead to these complications directly or indirectly via nonimmunological mechanisms.[6]

We analysed the endocrine complications in PID from the information obtained from the nationwide PID survey in Japan conducted in 2008. This is the first large-scale survey focusing on the endocrine complications in PID.

Materials and methods

This survey was performed according to the nationwide epidemiological survey manual of patients with intractable diseases (2nd edition 2006, Ministry of Health, Labour and Welfare of Japan) as described previously.[7] PID classification was based on the criteria of the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee in 2007.[8] The survey was conducted on patients with PID who were alive on 1 December 2008 and those who were newly diagnosed and died between 1 December 2007 and 30 November 2008 in Japan. The initial survey covered 1224 paediatric departments and 1670 internal medicine departments, which were randomly selected according to the number of beds among the 2291 paediatric departments and 8026 internal medicine departments in Japan. Primary questionnaires regarding the number of patients and the disease names based on the PID classification

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were sent to the selected hospitals. The initial survey was conducted to investigate the prevalence of the respective PID. The secondary survey was performed to study the detailed clinical features of individual patients with PID. Secondary questionnaires regarding age, gender, clinical manifestations and complications other than those related to haematopoietic stem cell transplantation of individual patients with PID were sent to the respondents who answered that they observed at least one PID patient with characteristics listed in the primary questionnaires. The details of the methods of the questionnaire investigation, the response rates and the breakdown of the number of patients in both paediatric and internal medicine departments were described elsewhere.[9] The questionnaires were designed to elucidate the clinical characteristics including the manifestations and laboratory data of the patients. In this study, all endocrine manifestations in patients with PID were included as complications of PID, even if they were well known major symptoms of PID.

Results

Detailed clinical information was available from 923 (secondary survey) out of 1240 patients with PID (initial survey).[9] Among the 923 patients with PID, 49 (5·3%) had endocrine disorders. As shown in Table 1, more than two thirds of the patients with PID were <20 years old and the prevalence of endocrine diseases was much higher in the young population of patients with PID than that in the general young population,[7,10–14] even after excluding patients with immune dysregulation (PID category IV). As expected, hypoparathyroidism was the most common endocrine disorder, because it is very frequently observed in patients with DiGeorge syndrome. Endocrine manifestations were also common in patients with diseases of immune dysregulation, such as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Although the number of patients with defects in innate immunity was small, endocrine complications seemed to be more common than expected. Interestingly, endocrine disorders were not observed in patients with complement deficiencies. In addition, Graves' disease and Addison's disease were not observed in any of the patients with PID in this study.

Type 1 diabetes mellitus (T1D) was observed in six patients with PID (Tables 1 and 2) including four with type 1A (autoimmune) and two with type 1B (autoantibody-negative, idiopathic). Type 1A diabetes mellitus occurred frequently in patients with IPEX or IPEX-like syndrome (two of six patients, 33·3%) (Table 1). One patient of unknown aetiology in PID category IV showed type 1A diabetes and Hashimoto's thyroiditis along with recurrent viral infections (Tables 1, 2 and S1). In the cases of type 1A diabetes mellitus, anti-glutamic acid decarboxylase (GAD) autoantibodies and anti-insulin autoantibodies (IAA) were positive in all patients and in two of four patients, respectively (Table 2). The patients with IPEX and IPEX-like syndrome had a history of diabetic ketoacidosis with poor glycaemic control, and they developed T1D at a younger age than the other patients with PID. The first case of warts, hypogammaglobulinaemia, infections, and myelokathexis (WHIM) syndrome with T1D and hypothyroidism was included (Tables 2 and S2).[15] With regard to type 1B diabetes mellitus, the patient with hypogammaglobulinaemia of unknown aetiology had diabetic ketoacidosis (Table 2). On the other hand, type 2 diabetes mellitus (T2D) was observed in two patients with PID (Table 1).

Hashimoto's thyroiditis was observed in five patients with PID (Tables 1 and S1). The onset was very early in the patient with IPEX syndrome (at birth). All patients had at least 1 autoantibody among the anti-thyroid peroxidase (TPO), anti-thyroglobulin (Tg) and thyroid stimulating hormone receptor autoantibodies (TRAb).

Nonautoimmune hypothyroidism was reported in seven patients with PID (Tables 1 and S2). Anti-thyroid autoantibodies were all negative when measured. Among these, three patients with X-linked agammaglobulinaemia (XLA), IgG subclass deficiency or WHIM syndrome had primary (congenital) hypothyroidism detected by newborn mass screening. Hypothyroidism in the other four patients with normal TSH levels was considered to be due to central hypothyroidism, a disorder of the pituitary, hypothalamus or hypothalamic-pituitary portal circulation. Two patients with severe combined immunodeficiency (SCID) developed hypothyroidism before they received haematopoietic stem cell transplantation.

Growth hormone deficiency (GHD) was observed in six patients with PID (Tables 1 and S3), whose heights at the diagnosis of GHD ranged from −11·3 SD to −2·5 SD. Five patients were treated with growth hormone. One patient with SCID received cord blood transplantation when she was 20 months old, without conditioning chemotherapy or radiation.

Hypogonadism was observed in three patients with PID (Tables 1 and S4). Among them, two had hypergonadotrophic (primary) hypogonadism, whereas the other had hypogonadotrophic (central) hypogonadism. None of the patients received haematopoietic stem cell transplantation.

One common variable immunodeficiency disease (CVID) patient had isolated ACTH deficiency (Table 1). The other endocrine complications included hypophosphataemia, pseudohypoaldosteronism, adrenal crisis, hypoglycaemia and hypophosphataemic rickets as shown in Table 1.

Discussion

This is the first nationwide survey focusing on the endocrine complications of PID. Among these, hypoparathyroidism was the most common, observed in patients with DiGeorge syndrome and APECED.[16,17] In APECED, the calcium-sensing receptor has been reported to be the autoantigen responsible for hypoparathyroidism.[18] Although it has been reported that 79% of patients with APECED have hypocalcaemia due to hypoparathyroidism,[17] only 1 (25%) among four patients with APECED developed hypoparathyroidism in this study, which might be one of the clinical characteristics of patients with APECED in Japan.

The prevalence (33·3%) of T1D in patients with IPEX syndrome in this study seemed to be lower than that (>70%) of the previous reports.[19,20] The low prevalence of T1D might be due to

Table 1. Endocrine complications in PID patients

PID category	Hypopara-thyroidism	Diabetes mellitus			Thyroid disease		GHD	Hypogonadism	Isolated ACTH deficiency	Others	The number of PID patients			
		T1D			Autoimmune hypothyroidism (Hashimoto's thyroiditis)	Non-autoimmune hypothyroidism					*n*	0–19 years	Total	Percent in total
		1A	1B	T2D										
I. Combined T and B cell immunodeficiencies											**4**	**67**	**75**	**5·3**
RAG1 deficiency						1					1	6	6	16·7
CD4 deficiency					1						1	2	2	50·0
Undetermined											2	10	10	20·0
T-B-SCID						1	1				2	4	4	50·0
II. Predominantly antibody deficiencies											**13**	**231**	**378**	**3·4**
X-linked agammaglobulinaemia						1				2*	3	93	138	2·2
Common variable immunodeficiency disorders			1		1††		1		1	2†	6	29	93	6·5
IgG subclass deficiency						2					2	45	50	4·0
Undetermined			1				1**	1**			2	9	9	22·2
III. Other well-defined immunodeficiency syndromes											**20**	**126**	**165**	**12·1**
Hyper-IgE syndrome							1	1		1‡	3	31	46	6·5
DiGeorge syndrome	14										14	29	32	43·8
Ataxia telangiectasia				1							1	8	13	7·7
Chronic mucocutaneous candidiasis					1††						1	9	13	7·7
ICF syndrome								1			1	0	1	100·0
IV. Diseases of immune dysregulation											**6**	**31**	**38**	**15·8**
IPEX syndrome		2			1					1§	4	5	6	66·7
APECED	1										1	3	4	25·0
Undetermined		1**			1**						1	2	2	50·0
V. Congenital defects of phagocyte number, function or both											**3**	**106**	**153**	**2·0**
Chronic granulomatous disease						1	1				2	54	87	2·3

Table 1. *(continued)*

PID category	Hypopara-thyroidism	Diabetes mellitus: T1D 1A	T1D 1B	Diabetes mellitus: T2D	Thyroid disease: Autoimmune hypothyroidism (Hashimoto's thyroiditis)	Thyroid disease: Non-autoimmune hypothyroidism	GHD	Hypogonadism	Isolated ACTH deficiency	Others	The number of PID patients: *n*	0–19 years	Total	Percent in total
Shwachman–Diamond syndrome							1				1	2	2	50·0
VI. Defects in innate immunity											**2**	**9**	**12**	**16·7**
NEMO deficiency										1[¶]	1	7	7	14·3
WHIM syndrome		1**				1**					1	2	3	33·3
VII. Autoinflammatory disorders											**1**	**54**	**74**	**1·4**
Familial Mediterranean fever				1[††]							1	23	36	2·8
VIII. Complement deficiencies											**0**	**18**	**23**	**0**
IX. Undetermined											**0**	**3**	**5**	**0**
Total	15	6		2	5	7	6	3	1	7	49	645	923	5·3
Estimated prevalence per 10 000 in the young population (0–19 years) of PID patients (95% CI)	232·6 (141·4–380·1)	93·0 (42·7–201·5)		15·5 (2·7–87·3)	46·5 (15·8–135·9)	108·5 (52·7–222·3)	93·0 (42·7–201·5)	46·5 (15·8–135·9)	15·5 (2·7–87·3)					
Prevalence per 10 000 in the general young Japanese population	0·072[‡‡]	1·19		0·461[§§]	30·0[§§]	13·5[¶¶]	1·47	ND	0·035					
References	[7]	[10]		[10]	[11]	[12]	[13]	ND	[14]					

SCID, severe combined immunodeficiency; ICF, immunodeficiency with centromeric instability and facial anomalies; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; APECED, autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy; NEMO, NF-κB essential modulator; WHIM, warts, hypogammaglobulinaemia, infections, and myelokathexis; T1D, type 1 diabetes; T2D, type 2 diabetes; GHD, growth hormone deficiency.

*Hypophosphatemia 1, Obesity 1.

†Obesity 2.

‡Pseudohypoaldosteronism 1.

§Adrenal crisis, Hypoglycaemia 1.

¶Hypophosphatemic rickets 1.

**Two endocrine disorders were observed in the same patient.

††the case whose onset age of a endocrine complication is 20 years or older, *n*: number of PID patients who had endocrine disorders, CI: confidence interval.

‡‡prevalence in all age groups.

§§incidence data.

¶¶prevalence in the United States, ND: no data available.

Table 2. Clinical data of T1D patients

Case		1	2	3	4	5	6
Disease		IPEX syndrome	IPEX-like syndrome	Immune dysregulation (undetermined)	WHIM syndrome	CVID	Hypogammaglobulinaemia (unknown aetiology)
Genetic mutations (gene name)		+ (*FOXP3*)	Unknown	Unknown	+ (*CXCR4*)	Unknown	NT
HSCT		–	–	–	–	–	–
Sex		M	M	F	F	F	M
Present age		8 years 5 months	14 years 5 months	21 years 8 months	18 years 9 months	19 years 1 month	25 years 3 months
Onset age of T1D		3 months	10 months	7 years 9 months	5 years 7 months	7 years 9 months	6 years 5 months
Type of T1D		1A	1A	1A	1A	1B	1B
Clinical symptoms		Polydipsia, polyuria	Polydipsia, weight loss	ND	Polydipsia, polyuria	None	None
Diabetic ketoacidosis		+ (pH 7·112)	+ (pH 7·012)	–	–	–	+ (urine ketone body (4+))
Laboratory data	Normal range						
Fasting blood glucose (mmol/l)	3·9–6·1	31·7	29·1	6·1*	7·6	8·3	7·7
HbA1c (%)	4·3–5·8	7·9	8·3	8·7*	8·9	5·6	9·1
Plasma CPR (nmol/l)	0·33–0·93	ND	0·27	0·10*	ND	0·27	ND
Urinary CPR (μg/day)	20–100	ND	ND	2·5*	15	NT	ND
Anti-GAD Ab							
Result		+	+	+*	+	None	None
Value (U/ml)	<1·5	69·1	4860	9·3*	92	ND	ND
Anti-IAA Ab							
Result		–	ND	+*	+	ND	ND
Value (nIU/ml)	<125	2·8		ND	ND		
Treatment							
Age at the start		3 months	10 months	7 years 9 months	5 years 7 months	8 years 1 month	6 years 5 months
Content		Insulin	Insulin	Insulin	Insulin	Insulin	Insulin

NT, not tested; ND, no data available; FOXP3, forkhead box P3; CXCR4, CXC chemokine receptor 4; HSCT, haematopoietic stem cell transplantation; CPR, C-peptide immunoreactivity; GAD, glutamic acid decarboxylase; IAA, insulin autoantibody.
*Post-treatment data.

some genetic factor, because the Japanese have been have reported to be one of the races with the lowest incidence of T1D.[21] With regard to the patient with WHIM, Takaya *et al.*[15] have reported that mutations of *CXCR4*, the gene responsible for WHIM syndrome, might be closely related to the development of T1D, because recent findings have suggested that impaired CXCR4 signalling is involved in the pathogenesis of T1D. The prevalence of T1D in patients with CVID was 1·1% (one in 93 patients) in our study, which was almost equal to that in the previous report.[3]

The development of T2D was observed in only one of 13 patients with ataxia telangiectasia (AT) (7·7%) in contrast to the high prevalence of T2D in the previous report (five of eight patients),[22] suggesting the unique clinical characteristics of patients with AT in Japan.

Hashimoto's thyroiditis is a relatively common endocrine manifestation in patients with IPEX syndrome.[19,20] The prevalence of Hashimoto's thyroiditis in patients with CVID in our study was 1·1% (one in 93 patients), which was similar to that of the previous report.[23] There have been only a few reports of Hashimoto's thyroiditis in patients with (S) CID.[24,25] Interestingly, this was the first report of Hashimoto's thyroiditis in a patient with CD4 deficiency, while autoimmune cytopenia is frequently associated with this disease (19%).[26] The patient with a patient with CD4 deficiency and Hashimoto's thyroiditis did not receive stem cell transplantation, suggesting that this complication was caused by autoimmunity based on the combined immunodeficiency. Nagpala *et al.*[25] reported an infant with autoimmune thyroiditis and hypothyroidism with SCID due to adenosine deaminase deficiency despite an extremely low number of T cells and a low level of IgG, which suggested that the leaky SCID phenotype permitted the survival of a few T cells with autoimmune potential.[27]

Central hypothyroidism (no TSH elevation) was observed in two patients with SCID before they received haematopoietic stem cell transplantation (Table S2), also suggesting the possibility that this complication was related to the combined immunodeficiency itself. In addition, this was the first report of primary hypothyroidism (elevated TSH levels at birth) in patients with

XLA or IgG subclass deficiency, although the aetiologies remain to be determined.

Of note, the prevalence of GHD in patients with PID seemed much higher than that in the general population (Table 1). Until now, GHD has been reported in patients with several diseases in PID including SCID, CVID and Shwachman–Diamond syndrome, as shown in our study.[28–30] However, to the best of our knowledge, this was the first report of GHD in patients with hyper-IgE syndrome (HIES) and chronic granulomatous disease (CGD). Some SCID patients with GHD have been reported to have *STAT5b* gene mutations.[31] However, the gene was not investigated in our patient with SCID. With respect to the mechanism underlying the development of GHD in patients with CVID, common impairment in the IGF-1 and IgG pathways has been suggested as a cause of the growth retardation in some patients with CVID.[32] In addition, anti-pituitary antibodies have been detected in some of these patients.[33] The patient with congenital agammaglobulinaemia had various other complications in addition to GHD (Table S3), suggesting that this patient might have had a novel primary immunodeficiency.

Hypogonadism in patients with immunodeficiency with centromeric instability and facial anomalies (ICF) syndrome has been reported previously[34], although the mechanism is unclear. On the other hand, this was the first report of hypogonadism in patients with congenital agammaglobulinaemia and HIES. It is possible that hypogonadism has not been a major concern in PID for clinicians.

Isolated ACTH deficiency usually occurs during adult life, and only a few cases have been reported in childhood.[35] However, the development of isolated ACTH deficiency in a 14-year-old girl with CVID has been reported[35], in addition to the present case (Table 1). Therefore, a common pathological background is suspected in some of the patients with CVID.

Several limitations of this study should be considered. First, there were only a small number of adult patients with PID reported in this study, from which we could not estimate the accurate prevalence of endocrine manifestations in adults. Second, not all of the patients with PID were given sufficient examinations by endocrinologists and different examination methods were used at the respective hospitals.

There has been growing evidence of the interaction between the immune and endocrine systems.[4,5] In this study, we have found an increased prevalence of endocrine complications in patients with PID, which appear to be caused by immune dysregulation or by the underlying genetic disorders of the respective PID. Although various endocrine abnormalities have been reported to occur after stem cell transplantation,[36] therapy-related endocrine abnormalities were not included in the present study. A large-scale study such as a nationwide survey, focusing on the endocrine diseases, may have the potential to provide further insights into the mechanisms or pathophysiology of endocrine disorders in non-PID as well as patients with PID.

Conflicts of interest/financial disclosure

We declare that we have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical data of patients with Hashimoto's thyroiditis.

Table S2. Clinical data of patients with nonautoimmune hypothyroidism.

Table S3. Clinical data of patients with GHD.

Table S4. Clinical data of patients with hypogonadism.

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Low opsonic activity to the infecting serotype in pediatric patients with invasive pneumococcal disease

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ABSTRACT

Serotype-specific protective immunity in pediatric patients with invasive pneumococcal disease (IPD) has not been fully investigated. To determine the protective immunity to the infecting serotype, the serotype-specific immunoglobulin G (IgG) levels and opsonization indices (OIs) were examined in 24 Japanese pediatric patients whose serum was collected within one month of an IPD episode between May 2008 and June 2011. The median age (range) of IPD patients was 17 (10–108) months and 63% were boys. In all 17 patients tested, the levels of serotype-specific IgG to the infecting serotype were higher than 0.2 μg/ml, but the OIs to the infecting serotype were <8. The avidities of 19F- or 6B-specific IgG in patients with levels higher than 5.0 μg/ml, but with undetectable OIs, were confirmed to be lower than those in patients with high OIs. Our data demonstrated that although the levels of serotype-specific IgG to the infecting serotype were higher than 0.2 μg/ml in sera of pediatric patients with IPD, the OIs were low one month after the IPD episode. Low opsonic activities in these patients may, in part, be explained by the low avidity of serotype-specific IgG.

1. Introduction

Streptococcus pneumoniae is a leading human pathogen that causes a wide variety of diseases, ranging from otitis media to pneumonia, bacteremia, and meningitis in both children and adults [1]. Antibodies to pneumococcal capsular polysaccharide (CPS) and complement provide protection against pneumococcal strains with homologous or cross-reactive capsular serotypes [2]. Seven-valent pneumococcal conjugate vaccine (PCV7; Prevnar®, Pfizer) has been used for children in the USA since 2000 [3], and the incidence of invasive pneumococcal disease (IPD) caused by the seven vaccine serotypes (VTs) has declined markedly, although the incidence of non-VT infection has not declined [4–6]. A recent study reported that the incidence rate of IPD in children less than 5 years old was 12.6–13.8 per 100,000 in Chiba prefecture, Japan, before the introduction of PCV7 [7]. However, no information is available regarding a possible high-risk population for IPD in Japan, as was reported for Navajo children in the United States [8].

PCV7 was licensed in Japan in October 2009, and a 3 + 1 schedule (three doses for the primary series and one booster) was approved and implemented (http://idsc.nih.go.jp/vaccine/dschedule.html). Further, the Japanese government decided in November 2010 to subsidize PCV7 for children below 5 years of age.

Vaccine-induced protective immunity is currently estimated by measuring the concentrations of serotype-specific immunoglobulin G (IgG) using enzyme-linked immunosorbent assay (ELISA) [9] and the opsonization index (OI) using a multiplex-opsonophagocytic assay (MOPA) [10]. The World Health Organization working group suggested a serotype-specific IgG of

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concentration of 0.35 μg/ml as a putative measure of protection at a population level against invasive disease in infants after immunization with pneumococcal conjugate vaccine [11]. This working group also reported that antibody concentrations of 0.2–0.35 μg/ml measured with the ELISA using serum without serum absorption with 22F polysaccharide correlated best with an OI of 8, which in turn correlates best with protective efficacy. Henckaerts et al. proposed a protective threshold concentration of 0.20 μg/ml assessed with ELISA using serum absorption with 22F polysaccharide as a measure of the serotype-specific IPD efficacy for the pneumococcal conjugate vaccine [12], with the exception of serotype 19F [13]. A recent study also reported that the serological response rate following a three-dose PCV7 primary vaccination as determined using a threshold of ≥0.2 μg/ml IgG and an OI ≥ 8 corresponded well with overall effectiveness against IPD [14]. Although this threshold may not be necessarily applicable to individual patients, it is of interest to determine the protective immunity to the infecting serotype in sera collected during the acute phase in pediatric patients with IPD.

In this study, we therefore examined the IgG levels and OIs to the infecting serotype in sera of pediatric patients within one month of an IPD infectious episode. We report that the opsonic activity to the infecting serotype is low in sera obtained within one month of an episode of IPD.

2. Materials and methods

2.1. Patients

Thirty-two pediatric patients, whose cultures from sterile sites, such as blood or cerebrospinal fluid, were positive for *S. pneumoniae* between May 2008 and January 2012 at 22 hospitals in Japan, were investigated in this study. All patients were enrolled in this study when their attending doctors requested the measurement of the antipneumococcal antibodies in their sera. Sera were obtained from these 32 patients after the episode of IPD. All of the pneumococcal isolates were serotyped using coagglutination tests with rabbit antisera (Statens Serum Institute, Copenhagen, Denmark) at the Department of Bacteriology I, National Institute of Infectious Diseases. Serotype 6C was confirmed by an in-house factor antiserum [15]. All eight patients were excluded from our studies of the protective immunity to the infecting serotype: six patients for whom sera were collected more than one month after the onset of the IPD, one patient who received intravenous immunoglobulin as a treatment of IPD, and one patient with an underlying hypogammaglobulinemia. Consequently, we evaluated antipneumococcal IgG and the OIs to the infecting serotype in 24 pediatric patients with IPD. This study was reviewed and approved by the Ethics Committee of the RIMD, Osaka University, and conducted according to the principles expressed in the Declaration of Helsinki.

2.2. ELISA

Antipneumococcal IgG antibodies were measured with the WHO approved ELISA using a standard reference serum (89-SF) and C-polysaccharide and 22F polysaccharide absorptions as previously described [9,16]. The levels of serotype-specific IgG for the infecting serotypes including 6B, 9V, 14, 19F and 23F were determined according to the WHO protocol [a detailed protocol is available at www.vaccine.uab.edu/ELISAProtocol (89SF)].

2.3. MOPA

A multiplexed opsonophagocytic killing assay (MOPA) for the infecting serotype based on antibiotic-resistant target bacteria was performed at the Research Institute for Microbial Diseases, Osaka University, as previously described [10]. The quality control serum was prepared from pooled sera of adults vaccinated with the 23-valent pneumococcal polysaccharide vaccine (PPV23; Pneumovax®, MSD), and this was used in each assay. The OI was defined as the serum dilution that killed 50% of bacteria, and the OIs were determined using opsotiter3 software according to the WHO protocol (at www.vaccine.uab.edu/UAB-MOPA). Only the OI results for the infecting serotypes including 6B, 6C, 14, 19A, 19F and 23F were used in this study.

2.4. Measurement of protective immunity

Neither the serotype-specific IgG nor the OI was available in one patient with serotype 15B and another with serotype 24F infection. Only the OI was available in three patients with serotype 19A and two patients with serotype 6C infection. The OIs were not determined in another five patients because their sera contained antibiotics. Consequently, the level of serotype-specific IgG or OI to the infecting serotype was measured in 17 patients, and both the levels of serotype-specific IgG and OIs were measured in only 14 patients.

2.5. Avidity of serotype-specific IgG

The avidity of the serotype-specific IgG in sera was evaluated using ELISA by the previously published method with a minor modification [17]. Serum samples that had been preadsorbed C-polysaccharide and 22F CPS were added to the coated microtiter plates, and the plates were incubated for 1 h at 37 °C. After washing the plates, sodium thiocyanate (NaSCN) at concentrations from 0 to 1.0 M was added to each well and the plates were incubated for 15 min at room temperature. After washing of the plates, diluted goat anti-human IgG HRP-conjugate was added to each well. After incubation for 1 h at room temperature, the substrate solution was added to the plates, followed by incubation for 20 min at room temperature. The optical density at 405 nm was measured. The avidity of serotype-specific IgG was expressed as the percentage of absorbance remaining after treatment with different concentrations of NaSCN.

3. Results

The clinical characteristics of the 24 pediatric patients with IPD are shown in Table 1. The diagnosis of these patients included meningitis ($n = 11$), bacteremia ($n = 10$), and bacteremic pneumonia ($n = 2$) and septic arthritis ($n = 1$). The median age (range) was 17 (10–108) months, and 63% were boys. Four patients (17%) had associated comorbid conditions including immune thrombocytopenia and splenectomy, meningoencephalocele, asplenia and single ventricle, and hydrocephalus (V-P shunt). In the 24 examined, the most common infecting serotype was 6B (9 isolates, 38%), followed by 19F (4 isolates, 17%), 19A (3 isolates, 13%), 6C and 14 (2 isolates each 8%) and one isolate each of 9V, 15B, 23F and 24F (4%). The median (range) period from the onset of IPD to the time of serum collection was two (0–23) days.

Three patients received PPV23 due to pre-existing medical conditions (Table 1). Before their episode of IPD, two patients infected with serotype 19F and one patient infected with serotype 9V received PPV23. Because PPV23 contains serotypes 19F and 9V, all three cases were considered PPV23 vaccine failure (VF). Ten patients received one to three doses of PCV7 at various ages as shown in Table 1. Only one patient (Case 18) completed a course of three doses of PCV7 between 2 and 6 months of age. The other nine patients were immunized with PCV7 during the catch-up phase. PCV7 breakthrough infection (BTI) was defined where a patient who received at least one dose of PCV7 had an episode

Table 1
Clinical characteristics of 24 pediatric patients with invasive pneumococcal disease (IPD).

No.	Age (months)	Sex	Diagnosis	Comorbid condition	Infecting serotype	Serum obtained days after IPD	Antibody to the infecting serotype		Vaccination before IPD (doses)	Age at each dose (month)	Category of IPD after PPV23	Category of IPD after PCV7	Outcome
							IgG (μg/ml)	OI					
1	108	M	Meningitis	ITP, splenectomy	19F	10	6.53	2	PPV23(1)	62	Vaccine failure	NA	Alive
2	50	M	Meningitis	Meningoencephalocele	19F	17	5.1	2	PPV23(1)	42	Vaccine failure	NA	Alive
3	75	M	Bacteremia	Asplenia, single ventricle	9V	1	0.57	NT	PPV23(1)	24	Vaccine failure	NA	Dead
4	14	M	Bacteremia	None	6B	11	0.34	2	None	–	NA	NA	Alive
5	38	M	Meningitis	None	19F	4	1.08	2	None	–	NA	NA	Alive
6	14	M	Bacteremia	None	14	5	2.1	5	None	–	NA	NA	Alive
7	13	M	Bacteremia	None	6B	4	2.25	NT	None	–	NA	NA	Alive
8	12	M	Meningitis	None	6B	20	1.81	7	PCV7(1)	10	NA	Breakthrough infection	Alive
9	10	M	Meningitis	None	19F	0	0.85	NT	None	–	NA	NA	Alive
10	17	M	Bacteremic pneumonia	None	19A	2	NA	NT	None	–	NA	NA	Alive
11	30	M	Bacteremic pneumonia	None	6B	0	0.53	2	PCV7(1)	28	NA	Vaccine failure	Alive
12	17	F	Meningitis	None	24F	1	NA	NA	PCV7(1)	16	NA	Non-VT infection	Alive
13	12	F	Meningitis	None	6B	12	0.78	2	None	–	NA	NA	Alive
14	10	M	Meningitis	None	15B	2	NA	NA	None	–	NA	NA	Alive
15	30	F	Bacteremia	None	6B	0	1.18	2	PCV7(1)	26	NA	Vaccine failure	Alive
16	26	F	Bacteremia	None	19A	1	NA	2	None	–	NA	NA	Alive
17	15	F	Bacteremia	None	14	0	1.75	2	None	–	NA	NA	Alive
18	10	M	Bacteremia	None	19A	0	NA	2	PCV7(3)	4, 5, 6	NA	Non-VT infection	Alive
19	30	F	Meningitis	Hydrocephalus (V-P shunt)	6B	23	0.92	2	PCV7(1)	28	NA	Vaccine failure	Alive
20	17	F	Meningitis	None	6B	0	1.38	2	PCV7(2)	9, 11	NA	Breakthrough infection	Alive
21	11	F	Septic arthritis	None	23F	0	0.55	2	PCV7(3)	7, 8, 9	NA	Breakthrough infection	Alive
22	16	F	Bacteremia	None	6B	0	5.62	2	None	–	NA	NA	Alive
23	49	M	Meningitis	None	6C	1	NA	2	PCV7(1)	36	NA	Non-VT infection	Alive
24	14	M	Bacteremia	None	6C	7	NA	NT	PCV7(2)	9, 10	NA	Non-VT infection	Alive

OI, opsonization index; ITP, immune thrombocytopenia; PPV23, 23-valent pneumococcal polysaccharide vaccine; PCV7, 7-valent pneumococcal conjugate vaccine; NA, not applicable; NT, not tested because of antibiotic use; VT, vaccine type.

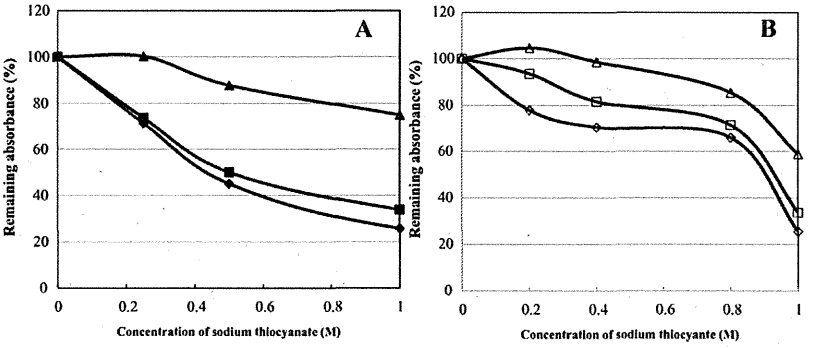

Fig. 1. Avidity of serotype 19F-specific IgG (A) and serotype 6B-specific IgG (B) in sera from pediatric patients with invasive pneumococcal diseases. Two serum samples from Case 1 (closed diamond) and Case 2 (closed square), and the positive control serum (closed triangle) from Case 6 (four months after the episode of IPD and one month after two doses of PCV7 vaccination) were examined for the avidity of serotype 19F-specific IgG. Two serum samples from Case 22 before (open diamond) and after (open square) two doses of PCV7 vaccination, and a positive control serum (open triangle) collected from Case 6 after two doses of PCV7 were used to test the avidity of serotype 6B-specific IgG.

of IPD for which the pneumococcal isolate was a PCV7 serotype, and PCV7 VF was defined as the subset of BTI in which the patient had completed the Advisory Committee on Immunization Practice (ACIP)-recommended PCV7 vaccine schedule at least two weeks before the IPD [18,19]. An instance of an IPD patient who had had at least one dose of PCV7 and for whom the pneumococcal isolate was not a PCV7 serotype was defined as PCV7 non-VT infection. Of 10 patients who received PCV7 previously, three cases (Cases 11, 15 and 19) were classified as PCV7 VF, and three cases (Cases 8, 20 and 21) were classified as PCV7 BTI. The other four cases (Cases 12, 18, 23 and 24) were classified as PCV7 non-VT infection.

The level of serotype-specific IgG or the OI for the infecting serotype was determined for 17 of 24 cases. The levels of specific IgG for the infecting serotype ranged widely from 0.34 to 6.53 μg/ml. In all 17 cases, the level of specific IgG for the infecting serotype was higher than 0.20 μg/ml, the putative threshold for preventing IPD [12,14]. The geometric mean concentration for the 17 cases was 1.35 μg/ml. In contrast, the OI for the infecting serotype was <8 in all of 17 cases. In particular, obvious discrepancies were found in two patients with serotype 19F (Cases 1 and 2) and one patient with serotype 6B (Case 22) who had serotype-specific IgG higher than 5 μg/ml and undetectable OI.

To investigate these discrepancies, we next examined the avidities of serotype 19F-specific IgG in sera from Cases 1 and 2, and the avidities of serotype-6B specific IgG in sera from Case 22. The percentages of remaining absorbance to 19F CPS of the positive control serum (IgG 7.25 μg/ml, OI 2336) collected from a patient (Case 6) after two doses of PCV7 vaccination were 100–75% at concentrations of 0.25–1.0 M of NaSCN (Fig. 1A). In contrast, the percentages of remaining absorbance to 19F CPS of sera from Cases 1 (IgG 6.53 μg/ml, OI 2) and 2 (IgG 5.10 μg/ml, OI 2) to 19F CPS were 74–44% and 71–26% at concentrations of 0.25–1.0 M of NaSCN, respectively.

The percentages of remaining absorbance to 6B CPS of the positive control serum (IgG 4.16 μg/ml, OI 4626) collected from Case 6 after two doses of PCV7 99–59% at concentrations of 0.4–1.0 M of NaSCN (Fig. 1B). In contrast, the percentages of remaining absorbance of serum from Case 22 before PCV7 vaccination (IgG 5.62 μg/ml, OI 2) and after two doses of PCV7 vaccination (IgG 2.37 μg/ml, OI 562) were 71–25% and 81–34% at concentrations of 0.4–1.0 M of NaSCN.

4. Discussion

In pediatric patients with IPD, the serum OIs for the infecting serotype within one month after the infectious episode were <8 in all 17 patients tested for OI, although the levels of IgG for the infecting serotype were higher than 0.2 μg/ml in all 17 patients tested for serotype-specific IgG. Undetectable OIs suggest that the serotype-specific IgG in their sera are largely nonfunctional. Soininen et al. similarly reported that sera from unimmunized children without nasopharyngeal carriage contained serotype-specific IgG, but infrequently had serotype-specific opsonic activity [20].

Three patients received PPV23 before PCV7 was licensed in Japan in 2009 because they were at increased risk for pneumococcal disease. Although the current guideline of the ACIP recommends that children aged 2–18 years with underlying medical conditions should receive PPV23 after completing all recommended doses of PCV13 [21], pediatricians should be aware of the possible induction of nonfunctional IgG by PPV23 in high-risk children aged >2 years. Two patients with PCV7 BTI received one or two doses of PCV7 9–11 months after birth, and two patients with PCV7 VF received only one dose of PCV7 26–28 months after birth. All four of these patients comprised the catch-up cases for PCV7. Interestingly, all cases with BTI or VF were caused by serotype 6B. A recent study from the US reported that 155 of 753 (21%) pediatric IPD cases were PCV7 BTIs caused predominantly by serotypes 6B (32%) and 19F (29%) [18]. The PCV7 BTIs caused by serotype 6B were more likely to have occurred in children who received only one or two PCV7 doses (84%) compared with infections caused by other VTs (61%). Rennels et al. also reported a low immune response to 6B and other serotypes, including 9V and 18C in children who received fewer than three doses of PCV7 [22].

Our data demonstrated that sera collected from Cases 1, 2 and 22 containing 19F- or 6B-specific IgG levels higher than 5.0 μg/ml, but lacking opsonic activity, contained lower avidity of serotype-specific IgG than the positive control sera with high OIs. An improvement of the avidity of 6B-specific IgG was confirmed in the sera with a high OI from Case 22 by two doses of PCV7 vaccination. Two previous studies using sera from healthy adults with or without vaccination with PPV23 demonstrated that higher avidity antibodies were more effective than lower avidity antibodies in in vitro complement-dependent opsonophagocytosis and for in vivo protection against pneumococcal infection in mice [23,24]. These data are, partially, in agreement with our findings of high levels of serotype-specific IgG with low avidity in serum from pediatric patients within one month after IPD. The low avidity of serotype-specific IgG levels may explain the undetectable OIs in sera collected from Cases 1, 2 and 22 within one month of an IPD episode.

O'Brien et al. recently reported the pneumococcal antibody status in a child with of PCV7 vaccine failure caused by serotype 14

[25]. In this patient, the serotype-specific IgG and the OIs in serum were 4.98 μg/ml and 1024, respectively, 35 days after the administration of three doses of PCV7. However, this patient developed occult bacteremia at 9.6 months of age, 53 days after the third dose of PCV7. Because of a slightly decreased serotype-specific IgG (4.25 μg/ml) and a significantly decreased OI of 4 in the serum of this patient after this episode of IPD, the authors suggested that the functional antibodies existing during infection with consumed by binding to the serotype 14 antigen. This finding also suggests that the ELISA assay detected some nonspecific or nonfunctional IgG in the serum of this patient, and is in agreement with the findings in the sera of our pediatric patients with IPD.

The limitations of our study are the small number of IPD cases examined and the variable timing of serum collection, although the sera were all collected within one month after the IPD episode. These limitations meant that we were unable to compare the induction of opsonic activity to the infecting serotype between the acute phase and the convalescence phase in pediatric patients with IPD.

In conclusion, in all of 17 patients tested within one month of an IPD episode, the serum OIs to the infecting serotype were <8, whereas the levels of serotype-specific IgG were higher than 0.2 μg/ml. Low avidity of the serotype-specific IgG were confirmed in three patients associated with the serotype-specific IgG levels higher than 5 μg/ml, but with undetectable OIs.

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Appendix A. The Japanese IPD Study Group

In addition to TO, NI, KM, JN, BC, BC, KT, YA, KO, the members of the Japanese IPD Study Group are Kenji Okada (National Fukuoka Hospital), Takashi Nakano (Kawasaki Medical University), Hideki Akeda (Okinawa Prefectural Nanbu Medical Center), Masako Habu (Tokyo Metropolitan Bokutoh General Hospital), Eri Yamaguchi (Chidoribashi Hospital), Kei Komiya (Nihon University School of Medicine), Shinji Kido (Toyota Memorial Hospital), Takahiro Niizuma (Koshigaya Municipal Hospital), Masato Arao (Saitama Medical University), Fumie Ishiwada (Chiba Kaihin Municipal Hospital), Mai Kubota (Shizuoka Children's Hospital), Kenji Furuno (National Fukuoka-Higashi Medical Center), Yoshio Yamaguchi (National Hospital Organization Tochigi Hospital), Kaoru Obinata (Juntendo University Urayasu Hospital), Mikio Yoshioka (KKR Sapporo Medical Center), and Tomomi Naito (Saiseikai Kawaguchi General Hospital).

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