combined with diphtheria toxoid and tetanus toxoid, *Haemophilus influenzae* type b vaccine, or influenza vaccine), treatment with antibiotics during the previous 3 months, and seasons when specimens were collected (winter, January to March; spring, April to June; summer, July to September; autumn, October to December).

Statistical analysis: The association of the prevalence of pneumococcal carriage with potential risk factors of the streptococcal infection was analyzed first by univariate analysis, and then by multivariate analysis using logistic regression models. The estimates of the association were expressed as odds ratios (OR) and 95% confidence intervals (CI). The STATA software, version 11 (STATA Corp., College Station, Tex., USA) was used for all data analyses.

RESULTS

The demographic characteristics of the study participants are shown in Table 1; further, 97 (42%) participants were less than 1 year of age, majority (55%) of the participants constituted boys, 53 (23%) participants were attending day-care centers, and 105 (46%) had older siblings.

Table 1. Characteristics of the 229 study children

Characteristic	No. (%) of children
Age (yr)	
<1	97 (42)
1–2	75 (33)
3–6	57 (25)
Sex	
Boy	125 (55)
Girl	104 (45)
Day-care attendance	
Yes	53 (23)
No	176 (77)
Presence of older siblings	
Yes	105 (46)
No	121 (53)
Not known	3 (1)
Presence of younger siblings	
Yes	37 (16)
No	179 (78)
Not known	13 (6)
Other vaccination history[1)]	
Yes	195 (85)
No	32 (14)
Not known	2 (1)
Treatment with antibiotics during the previous 3 months	
Yes	82 (36)
No	141 (62)
Not known	6 (2)
Seasonality	
Winter (January–March)	75 (33)
Spring (April–June)	30 (13)
Summer (July–September)	75 (33)
Autumn (October–December)	49 (21)

1): acellular pertusis vaccine combined with diptheria toxoid and tetanus toxoid, *Haemophilus influenzae* type b vaccine, and influenza vaccine.

S. pneumoniae was isolated from 51 of the 229 children (22%). The association between pneumococcal colonization and the potential risk factors are shown in Table 2. The prevalence of carriage appeared to increase with age (19%, 23%, and 28% in the <1 year old, 1–2 years old, and 3–6 years old age groups, respectively), although without statistical significance. Crude analysis showed statistical significance with respect to day-care attendance, the presence of older siblings, treatment with antibiotics during the previous 3 months, and specimen collection during summer. However, multivariate analysis after including these factors and age, showed statistically significant associations only with respect to day-care attendance ($P = 0.049$), the presence of older siblings ($P = 0.006$), and specimen collection during summer ($P = 0.028$). The adjusted OR (95% CI) for day-care attendance and the presence of older siblings were 3.11 (1.00–9.65) and 2.64 (1.32–5.27), respectively. The prevalence of pneumococcal carriage was lowest in summer (12%); the prevalence in winter was 27%, in spring, 37%; and in autumn, 22%. The adjusted OR (95% CI) was 0.36 (0.14–0.90) for summer compared to winter, even after controlling other factors such as day-care attendance.

DISCUSSION

None of the participants had received prior PCV7 immunization. All were healthy at the time of specimen collection and were thus suitable subjects for the evaluation of *S. pneumoniae* colonization. A 22% prevalence of pneumococcal carriage was noted among the study participants.

Previous reports demonstrated a 38–87% prevalence of *S. pneumoniae* colonization in children in Japan (9,10), which is higher than the rate we found. This discrepancy may be explained by the fact that these previous studies specifically targeted children attending day-care centers, i.e., children exposed to one of the risk factors for pneumococcal colonization. In our study, the prevalence of pneumococcal colonization in children attending day-care centers was 34%, closer to the previously reported data (9,10). In Japan, few studies have analyzed groups that have not been exposed to risk factors for pneumococcal carriage. Previous studies have reported a 14% pneumococcal prevalence among children aged 0–14 years on routine immunization visits (11), 46% among infants undergoing routine public medical checkups at age 18 months (3), and 17–48% among infants undergoing routine medical checkups at 3 or 4 months to 18 months (2). In the present study, the pneumococcal prevalence showed a tendency to increase with age: 19% at <1 year, 23% at 1–2 years, and 28% at 3–6 years. In a study carried out in the Netherlands, Bogaert et al. reported that nasopharyngeal colonization of *S. pneumoniae* in children varied with age, with a peak prevalence of pneumococcal colonization of 55% aged 3 years. Thereafter, there was a steady decline of *S. pneumoniae* colonization until a stable prevalence of 8% was attained in children over the age of 10 years (1).

In this study, day-care center attendance was found to be significantly associated with increased pneumococcal carriage; the prevalence of pneumococcal colonization

Table 2. Univariate and multivariate analysis of potential risk factors associated with *Streptococcus pneumoniae* colonization

Variable	No./total no. (%)	Crude OR (95% CI)	*P*	Adjusted OR (95% CI)	*P*
Age (yr)					
<1	18/97 (18.6)	1		1	
1-2	17/75 (22.7)	1.29 (0.61-2.71)	0.507	0.89 (0.38-2.09)	0.783
3-6	16/57 (28.1)	1.71 (0.79-3.71)	0.172	0.74 (0.22-2.54)	0.633
Sex					
Girl	22/104 (21.2)	1			
Boy	29/125 (23.2)	1.12 (0.60-2.11)	0.711	—	—
Day-care attendance					
No	33/176 (18.8)	1		1	
Yes	18/53 (34.0)	2.23 (1.13-4.41)	0.021	3.11 (1.00-9.65)	0.049
Presence of older siblings					
No	18/121 (14.9)	1		1	
Yes	33/105 (31.4)	2.70 (1.37-5.01)	0.004	2.64 (1.32-5.27)	0.006
Presence of younger siblings					
No	41/179 (14.9)	1			
Yes	7/37 (32.0)	0.79 (0.32-1.92)	0.596	—	—
Other vaccination history					
No	6/32 (18.8)	1			
Yes	45/195 (23.1)	1.30 (0.50-3.36)	0.588	—	—
Treatment with antibiotics during the previous 3 months					
No	26/141 (18.4)	1		1	
Yes	25/82 (30.5)	1.94 (1.03-3.66)	0.041	1.54 (0.73-3.25)	0.253
Seasonality					
Winter (January-March)	20/75 (26.7)	1		1	
Spring (April-June)	11/30 (36.7)	1.59 (0.65-3.92)	0.312	1.56 (0.60-4.02)	0.358
Summer (July-September)	9/75 (12.0)	0.38 (0.16-0.89)	0.026	0.36 (0.14-0.90)	0.028
Autumn (October-December)	11/49 (22.4)	0.80 (0.34-1.85)	0.596	0.82 (0.33-2.02)	0.664

in children attending day-care centers was higher than in those who did not attend (34% versus 19%). In other countries, children attending day-care centers have been shown to be at higher risk of pneumococcal infection (1), with an OR (95% CI) of day-care attendance with pneumococcal colonization in healthy children of 2.27 (1.89-2.75) reported (7). In Japan, a study of healthy children undergoing routine public medical checkups revealed an OR (95% CI) for pneumococcal colonization among those attending day-care of 1.7 (1.3-2.3)-3.5 (2.4-5.1) (2). Takeuchi et al. found that almost all children were colonized with *S. pneumoniae* within a year of starting kindergarten (12). In crowded settings like day-care centers, there appear to be frequent opportunities for contact with pneumococcal reservoirs, and nasopharyngeal colonization of *S. pneumoniae* increases because of horizontal spread among those present at such places.

The present study also revealed a significantly higher prevalence of pneumococcal colonization in children with older siblings than in those without (31% versus 15%). In Alaska, Moore et al. found that a high OR (95% CI) of pneumococcal colonization in healthy children with siblings (1.55 [1.12-2.16]-2.27 [1.83-2.81]) (6). In contrast, a study of healthy children in Japan attending routine public medical checkups demonstrated an OR (95% CI) of 3.5 (2.6-4.7)-3.9 (2.8-5.3) in children with older siblings (2). A likely explanation for this is that the increased range of social activities undertaken by the older siblings' increases the opportunities for nasopharyngeal colonization with *S. pneumoniae* and transmission to their younger siblings (13,14).

This study demonstrated a seasonal variation in pneumococcal colonization, with a significant decrease among participants in summer. Gray et al. also found that the acquisition rate was seasonal, with a marked peak during winter peak and drop during summer (15). Dowell et al. reported that seasonal patterns of IPD prevalence correlated with changes in temperature and daylight hours (16). However, Hashida et al. reported that nasopharyngeal carriage of *S. pneumoniae* in infants at day-care centers in Japan did not differ between summer and winter (9). Meanwhile, Matsubara et al. reported a bimodal peak (spring and autumn) in childhood IPDs. They also associated the spring peak with children entering day-care centers (the fiscal and academic years start in April in Japan) (17). Our multivariate analysis showed that pneumococcal colonization decreased significantly in summer, independent of day-care attendance. It has been suggested that respiratory viruses, especially rhinovirus and respiratory syncytial virus (RSV), are associated with IPDs in children that do not attend day-care centers (18). Hasegawa et al. have reported that RSV is detected mainly during late summer to winter in cases of pediatric community-acquired pneumonia in Japan (19). The increasing prevalence of pneumococcal colonization we identified in autumn may have been associated with the prevalence of such respiratory infections. However, it should be noted that our study participants were healthy when pneumococcal colonization was surveyed. Whether seasonal variation in pneumococcal colonization in healthy children (as opposed to those with IPDs) is independent of day-care attendance, and whether it is associated with

respiratory infection, and/or changes in temperature and daylight hours, needs to be studied further.

The main limitation of our study is its lack of statistical power because of the relatively small sample size.

In summary, we found a 22% prevalence of *S. pneumoniae* among healthy children in Japan, which tended to increase with age. We identified day-care attendance and the presence of older siblings as the major risk factors associated with pneumococcal carriage. A significant decrease in pneumococcal colonization was observed in summer. In addition, seasonal variation was independent of various factors. This is the first report of seasonal variation in pneumococcal colonization among healthy children in Japan. Our findings suggest that prior immunization with PCV7 within the current recommended schedule is required for children exposed to these risk factors.

Conflict of interest None to declare.

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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.

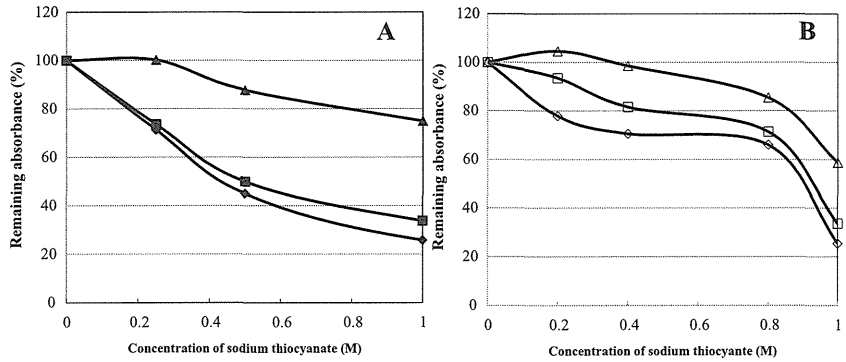

Fig. 1. Avidity of serotype 19F-specific IgG (A) and serotype 6B-specific IgG (B) in sera from pediatric patients with invasive pneumococcal diseases. Two serum samples from Case 1 (closed diamond) and Case 2 (closed square), and the positive control serum (closed triangle) from Case 6 (four months after 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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Analysis of Rotavirus Antigenemia and Extraintestinal Manifestations in Children With Rotavirus Gastroenteritis

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ARTICLE

Analysis of Rotavirus Antigenemia and Extraintestinal Manifestations in Children With Rotavirus Gastroenteritis

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The authors have indicated they have no financial relationships relevant to this article to disclose.

What's Known on This Subject

Rotavirus antigen is detected in acute-phase serum collected from patients with rotavirus gastroenteritis.

What This Study Adds

An association between rotavirus antigenemia and clinical features, particularly extraintestinal manifestations, and the association between serum cytokine levels and rotavirus antigen quantity are clarified.

ABSTRACT

OBJECTIVE. This study was conducted to examine the association between rotavirus antigenemia and clinical features, particularly extraintestinal manifestations, and the association between serum cytokine levels and rotavirus antigen quantity.

METHODS. Sixty hospitalized children who received a diagnosis of acute rotavirus gastroenteritis were enrolled in this study. Paired serum samples were collected from the 60 children when admitted to and discharged from the hospital. Associations among viral antigen levels and fever, elevated transaminase levels, and seizures were evaluated to determine whether antigenemia correlated with disease severity. Viral antigen was measured by using an in-house enzyme-linked immunosorbent assay that detected VP6 antigen. A flow-cytometric bead array was used to measure serum cytokine levels.

RESULTS. Rotavirus antigen levels were significantly higher in serum collected at the time of hospital admission than at the time of discharge. Serum rotavirus antigen levels peaked on day 2 of the illness (2.02 ± 0.73), followed by a gradual decrease in antigen levels to nearly undetectable levels by day 6. The quantity of rotavirus antigen was significantly higher in serum collected from patients with fever than those without fever. The presence or absence of elevated transaminase levels and seizures was not associated with serum rotavirus antigen levels. A weak but significantly positive association was observed between interleukin 8 levels and antigenemia. A weak but significantly negative association was observed between interleukin 10 levels and antigenemia.

CONCLUSIONS. Rotavirus antigenemia is frequently observed in a patient's serum during the acute phase, and viral antigen levels change dramatically during the acute phase of the illness. Because patients with fever had higher rotavirus antigen levels, antigenemia severity might contribute to fever. The host immune response plays an important role in controlling antigenemia levels. *Pediatrics* 2008;122:392–397

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Key Words
rotavirus, antigenemia, cytokine

Abbreviations
ELISA—enzyme-linked immunosorbent assay
PBST—phosphate-buffered saline that contains Tween 20
CBA—cytometric bead array
OD—optical density
IL—interleukin

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ROTAVIRUS IS THE major cause of gastroenteritis in young children worldwide. Severe dehydration caused by rotavirus-induced diarrhea and vomiting can be fatal in developing countries. Meanwhile, gastroenteritis induced by rotavirus infection causes a large economic burden in developed countries. Initially, rotavirus replication was thought to be limited to the gastrointestinal tract in patients with gastroenteritis; however, it is widely known that rotavirus gastroenteritis is sometimes complicated by high fever, elevated transaminase levels,[1,2] seizures,[3–5] and encephalitis,[6,7] which may be caused by systemic viral infection. Rotavirus RNA has been detected in the cerebrospinal fluid of patients with convulsions[8]; however, it is unclear whether the RNA was really there. Rotavirus antigens and RNA were detected in the serum of children with rotavirus infection.[9–11] In addition, several investigators have demonstrated that rotavirus antigen is detected not only in serum but also in multiple organs, including the stomach, intestine, liver, lung, spleen, kidney, pancreas, thymus, and bladder, in rotavirus-infected animals.[12]

These findings suggest that rotavirus spreads beyond the intestine in children with rotavirus gastroenteritis, resulting in systemic viral infection.

We have demonstrated that viremia generally correlates with illness severity in children with varicella-zoster virus infection[13] and human herpesvirus 6 infection.[14] Although several lines of evidence suggest that systemic rotavirus infection occurs in infected children,[9–11] it is not well understood whether rotavirus antigenemia levels correlate with disease severity. Only 1 report has postulated that rotavirus RNAemia is associated with high fever, but this study did not present statistical analyses on these findings.[15] Moreover, it has been suggested that cytokines may play an important role in rotavirus gastroenteritis pathogenesis[16–21]; however, no clinical studies have investigated the correlation between levels of rotavirus antigenemia and serum cytokines to date. We hypothesized that the grade of systemic rotavirus replication and cytokine levels induced by viral infection play an important role in extraintestinal manifestations such as fever, elevated transaminase levels, and seizures; therefore, in this study, we examined the association between rotavirus antigenemia and clinical features, particularly extraintestinal manifestations. Moreover, because it has been suggested that cytokines are involved in the pathogenesis of rotavirus gastroenteritis, we also examined the association between serum cytokine levels and rotavirus antigen levels.

METHODS

Patient Characteristics and Sample Collection

Sixty hospitalized children with a diagnosis of acute rotavirus gastroenteritis were enrolled in this study. All patients were admitted to 1 of 4 pediatric departments (Fujita Health University, Kariya Toyota General Hospital, Toyokawa City Hospital, or Showa Hospital) between December 2004 and March 2006. The patients' guardians consented to their participation in this study. This study was approved by the review boards of all 4 institutes. Diagnosis of rotavirus gastroenteritis was confirmed by detection of rotavirus antigen in stool samples by using an immunochromato assay (Dipstick [Eiken, Tokyo, Japan]). Paired serum samples were collected from the 60 children (age: 1.4 ± 1.4 years; gender: 40 boys and 20 girls) at the time of hospital admission (days 1–5; day 1 was defined as the date of onset of symptoms [eg, fever, vomiting, diarrhea]) and discharge (days 4–13). In addition, 20 serum samples were collected from age-matched control children whose stool samples were negative for rotavirus antigen by the immunochromato assay.

Clinical features of the children were examined retrospectively by using medical charts. To assess whether serum rotavirus antigen levels correlated with disease severity, we examined the association between viral antigen levels and fever (>37.5°C), elevated transaminase levels (alanine aminotransferase: >50 IU/L), and seizures, symptoms that are caused by systemic viral infection. The presence or absence of these clinical manifestations was evaluated from the data collected at the time of hospital admission.

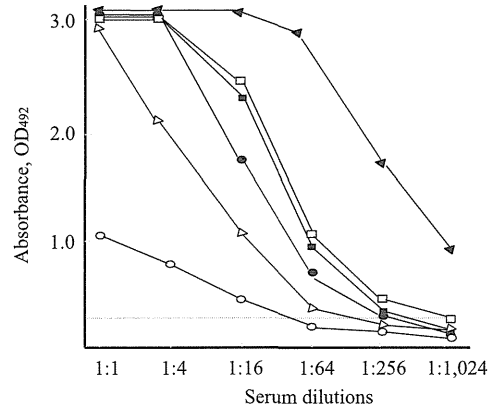

FIGURE 1
ODs of serially diluted serum samples that were collected from 6 patients with rotavirus gastroenteritis. The dotted line indicates the baseline value.

Rotavirus Antigen Detection

Rotavirus antigen was measured using an in-house enzyme-linked immunosorbent assay (ELISA) that detects VP6 antigen of the virus. Fifty microliters of diluted (1:16) serum was used to detect rotavirus antigen. The dilution ratio was determined from preliminary studies by using several different serum samples that were collected from patients with rotavirus gastroenteritis. As shown in Fig 1, a 1:16 dilution was appropriate to measure quantitatively viral antigen in patients' serum. Ninety-six–well plates (Nalgen Nunc International, Rochester, NY) coated with a monoclonal antibody against the VP6 antigen of rotavirus (YO-156)[22] were used for the ELISA.[23] The antibody YO-156 (immunoglobulin G2a subclass) is highly reactive with a common epitope of group A rotaviruses, and the antibody can detect all of the group A rotavirus strains that have been examined. Specificity of the antibody was confirmed by immunoprecipitation assay, immune electron microscopy, and Western blotting analysis.[22] After blocking with 1% bovine serum albumin in phosphate-buffered saline that contains Tween 20 (PBST), the plate was washed with PBST. The plate was then incubated with 50 μL of diluted patients' serum at 4°C overnight. After washing the plate with PBST, 50 μL of antihuman rotavirus hyperimmune rabbit serum diluted 1:5000 with PBST that contained 2.5% skim milk was added to each well. The plate was incubated at 37°C for 1.5 hours. After the plate was washed with PBST, it was incubated with a 1:5000 dilution of peroxidase-conjugated donkey antirabbit immunoglobulin G (Jackson ImmunoResearch Laboratory, Inc, West Grove, PA) at 37°C for 1.5 hours. The quantity of monoclonal antibody bound to rotavirus VP6 antigen was assessed after addition of the substrate. The optical density (OD) was read by spectrophotometry at a 492-nm wavelength. To establish an appropriate cutoff value to distinguish between rotavirus-positive and -negative samples, we tested 20 serum samples that were collected from control subjects. Be-

cause the mean OD of the control samples was 0.084 ± 0.014, we defined 0.13 (mean ± 3 SD) as the baseline value in this study.

Cytokine Measurements

The flow-cytometric bead array (CBA) was used according to the manufacturer's protocol (Becton Dickinson, San Diego, CA).[24] CBA measured the following cytokines: interleukin (IL)-8, IL-1β, IL-6, IL-10, tumor necrosis factor α, and IL-12. Fifty microliters of sample (standards or test) were added to 50 μL of a cocktail of capture beads and detector antibodies, and the mixture was incubated for 1.5 hours at room temperature in the dark. Excess unbound detector antibody was removed by washing, and 50 μL of reagent was added before data acquisition. Two-color flow-cytometric analysis was performed using a flow cytometer (FACScan [Becton Dickinson, Franklin Lakes, NJ]). A total of 1800 events were acquired by following the protocol supplied. Analysis was performed using CBA dedicated analysis software (CellQuest [Becton Dickinson]). All samples for which the calculated cytokine concentration was below the given sensitivity were treated as undetectable.

Statistical Analysis

Statistical analyses were performed by using Stat View 5.0 (SAS Institute, Cary, NC). Patient gender was compared between the 2 groups by using a χ^2 test. Unpaired comparisons between the patient's age and days of sampling were performed by using a Student's t test. Mean peak absorption levels were compared between the 2 groups by using either a Wilcoxon signed-ranks test or Mann-Whitney U test. Spearman's rank correlation coefficients were used to measure the strength of the association between cytokine levels and rotavirus antigen quantity.

RESULTS

Kinetics of Rotavirus Antigenemia

First, we compared rotavirus antigen levels at the time of hospital admission with levels at the time of discharge. Serum samples that were collected at the time of admission were between days 1 and 5 of the illness, and those that were collected at the time of discharge were between days 4 and 13 of the illness. Rotavirus antigen was significantly higher in serum that was collected at the time of admission (days 1–5) than in those collected at the time of discharge (days 4–13; $P < .0001$; Fig 2). In addition to the antigen levels, detection rate of serum rotavirus antigen was also high in serum that was collected at the time of admission (54 of 60 [90%]) in comparison with the samples that were collected at the time of discharge (19 of 60 [31.7%]). To determine rotavirus antigen kinetics after the onset of illness, we monitored the optical densities in all 120 serum samples. As shown in Fig 3, serum rotavirus antigen levels peaked on day 2 (2.02 ± 0.73), followed by a gradual decrease to nearly undetectable levels by day 6. High detection rates (ranging between 89% and 94%) were also observed in serum samples that were collected until day 5.

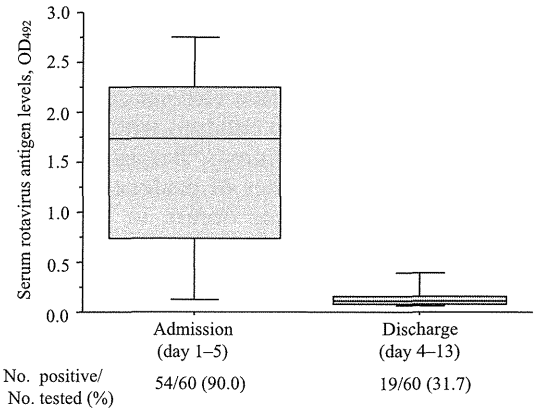

FIGURE 2
Rotavirus antigen levels as assayed by ELISA in 60 paired serum samples that were collected at the time of admission to and discharge from the hospital.

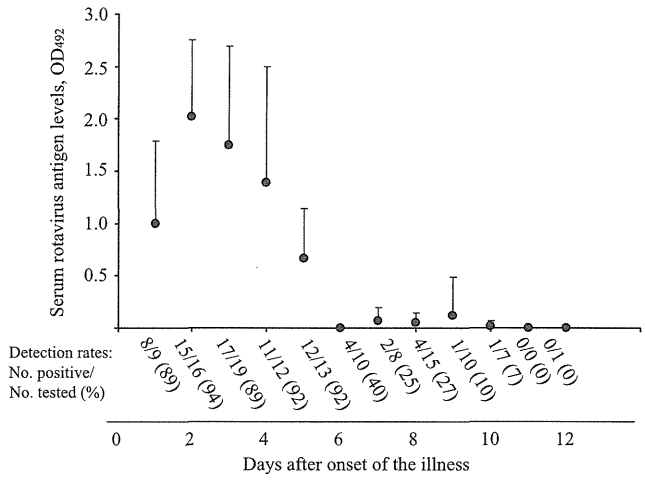

FIGURE 3
Kinetics of serum rotavirus antigen levels and detection rates of the viral antigen.

Association Between Rotavirus Antigenemia Levels and Clinical Symptoms

OD values in serum samples that were collected at the time of admission were compared between patients with and without each clinical manifestation (Fig 4). Although patients' backgrounds were not different between the groups with (gender: 28 boys and 16 girls; age: 1.4 ± 1.3 years; sampling time: 2.7 ± 1.2 days) and without fever (gender: 11 boys and 5 girls; age: 1.4 ± 1.5 years; sampling time: 3.2 ± 1.3 days), the quantity of rotavirus antigen was significantly higher in the serum that was collected from the patients with fever (1.70 ± 0.87) than those without fever (1.07 ± 0.98; $P = .0273$). No febrile episode was observed during admission period in patients without fever at the time of admission to the hospital. Rotavirus antigen levels in patients with (alanine aminotransferase: 84.8 ± 61.8 IU/L) and without elevated transaminase levels were 1.16 ± 0.96 and 1.60 ± 0.92, respectively, with no statistical difference in the antigenemia levels between the 2 groups ($P = .2351$). Finally, we tested whether the level of rotavirus antigenemia was correlated with seizures. The number

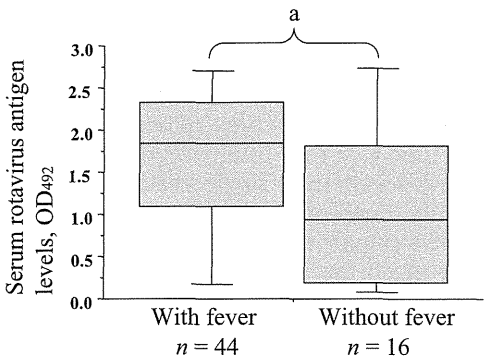

FIGURE 4
Comparison of rotavirus antigen levels between patients with and without fever. [a] P = .0273.

of patients who experienced seizures was small (n = 7). Moreover, 4 of the 7 patients had febrile seizures, whereas the remaining 3 patients had afebrile seizures, which were diagnosed as benign convulsions associated with mild gastroenteritis. No statistical difference was observed in rotavirus antigenemia levels between patients with (1.38 ± 1.04) and without seizures (1.56 ± 0.93; P = .6451).

Association Between Levels of Rotavirus Antigenemia and Cytokines

The associations between the levels of 6 cytokines and the quantity of rotavirus antigen in acute serum samples were examined by Spearman's rank correlation coefficients (Fig 5). We identified a weak (r = 0.36) but significantly (P = .0041) positive association between IL-8 levels and the severity of rotavirus antigenemia. Although a similar correlation was demonstrated between IL-6 levels and quantity of viral antigen (r = 0.23), it was not statistically significant (P = .0697). Meanwhile, there was a weak (r = −0.258) but significantly (P = .0464) negative association between IL-10 levels and rotavirus antigenemia. No statistical correlation was observed between the other 3 cytokines levels and antigen levels.

DISCUSSION

An in-house ELISA that used a monoclonal antibody YO-156 directed to group A common epitope on VP6 was used to measure serum rotavirus antigen in this study. VP6 is an inner capsid protein in rotavirus particle and is the most abundant protein detected in rotavirus particle and in the cells that are infected with rotavirus. The VP6 carries group-specific and subgroup-specific antigens. Our monoclonal antibody can detect both of the 2 subgroups (I or II). VP4 and VP7 are the outer capsid proteins, and they are associated with P-type and G-type specificities, respectively. In human rotavirus, the presence of at least 10 P types and 10 G types has been reported. Thus, to detect rotavirus antigen efficiently, it is reasonable to use monoclonal antibody that commonly is reactive to VP6 of any human rotavirus strain. To determine the appropriate serum dilution to clarify differences in viral antigen levels, we measured OD values in serially diluted serum samples and found that serum diluted 16-fold was the best to quantify viral antigen (Fig 1). As shown in Fig 3, the detection rate of rotavirus antigen in patients' serum ranged between 89% and 94% until 5 days after illness onset, suggesting a high frequency of rotavirus antigenemia during the acute phase of rotavirus gastroenteritis. This result supports previous studies that demonstrated frequent detection of rotavirus antigen in acute-phase serum; however, the detection rate of rotavirus antigen was higher in our study than in 2 previous studies.[10,11] The previous studies used undiluted serum to measure viral antigen, whereas we used serum diluted 16-fold, suggesting that our ELISA system has greater sensitivity than previously published ELISA methods. Indeed, in our comparison of the sensitivity between our in-house ELISA and a commercial kit (Rotaclone [Meridian Bioscience, Inc, Cincinnati, OH]) by using same serum samples, our in-house ELISA exhibited clearly higher sensitivity than the commercial kit (data not shown).

Previous reports demonstrated that rotavirus antigen levels decreased to baseline levels during a 3- to 4-week interval by testing paired serum samples[10]; however, this study demonstrated that rotavirus antigen levels were significantly lower at the time of discharge from the

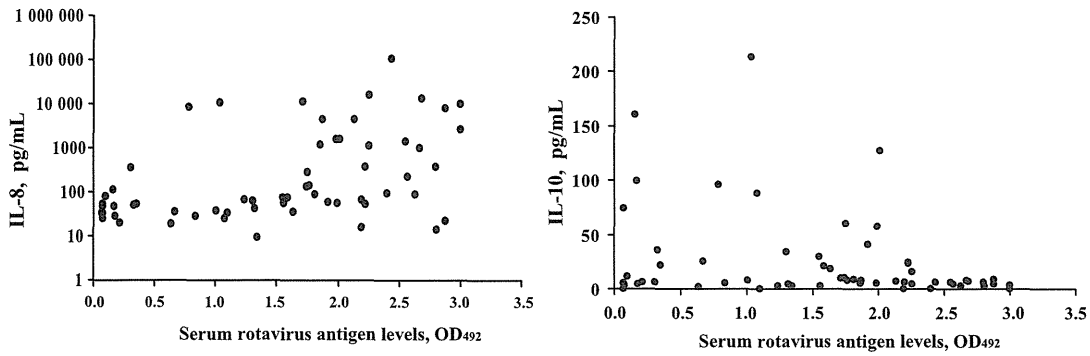

FIGURE 5
Association between rotavirus antigen levels and serum cytokine concentrations. A weak (r = 0.36) but significantly (P = .0041) positive association was observed between IL-8 levels and rotavirus antigenemia. A weak (r = −0.258) but significantly (P = .0464) negative association was also observed between IL-10 levels and rotavirus antigenemia.

hospital than at the time of hospital admission ($P < .0001$). In addition, the viral antigen kinetic analysis showed that viral antigen peaked on day 2 of the illness, and antigen levels quickly returned to almost undetectable levels by day 6. Fischer et al[10] also examined viral antigen levels after disease onset but demonstrated unclear viral antigen kinetics. Thus, to our knowledge, this is the first study to demonstrate the kinetics of rotavirus antigenemia during the acute phase of rotavirus gastroenteritis. The high sensitivity of our ELISA system and sampling schedule allowed us to clarify such a dynamic change of viral antigen during acute illness. Because both innate and adaptive immunities play an important role in viral clearance, an analysis of the antirotavirus host immune response and rotavirus antigenemia would clarify the pathogenesis of rotavirus gastroenteritis.

Although 3 extraintestinal manifestations were evaluated in this study, only fever was statistically associated with viral antigen levels. It has been demonstrated that younger patients generally have higher viral antigen levels than older patients, because viral antigen levels are higher in patients with primary rotavirus infections than those reinfected with the virus.[11] Moreover, sampling time affects rotavirus antigen detection in serum, as shown in Fig 3. When we compared the mean age and sampling time between patients with and without fever, no significant difference was observed between these 2 groups. Thus, we believe that serum rotavirus antigen levels are associated with the occurrence of fever in patients with rotavirus gastroenteritis. There was no correlation between rotavirus antigen levels and 2 additional manifestations: elevated transaminase levels and seizures. The elevated transaminase levels that occurred in these children was mild, as demonstrated previously.[2] It seems that systemic spread of rotavirus antigen is not involved in mild elevated transaminase levels in patients with rotavirus gastroenteritis. It is widely known that rotavirus can cause both febrile seizures[5] and benign convulsions associated with mild gastroenteritis.[3,4] Because the number of patients with seizures was small and the patients with seizures had 2 different types of convulsions, it is difficult to analyze conclusively the correlation between rotavirus antigenemia and seizures. A large number of patients with rotavirus gastroenteritis should be analyzed to determine an association between central nervous system complications, such as seizures and encephalopathy, and viral antigen levels. Moreover, rotavirus antigen should be measured in the cerebrospinal fluid to determine whether the virus can directly invade the central nervous system, as was previously suggested by reverse transcriptase–polymerase chain reaction analysis.[8] Furthermore, because other variables, including genotype, primary or secondary infection, and coinfection with other pathogens, were not evaluated in this study, an association between these factors and antigenemia levels should be analyzed in future study.

It is widely known that cytokines play an important role in the pathogenesis of viral infections. Several in vivo and in vitro studies have suggested that cytokines are involved in the pathogenesis of rotavirus infection.[16–21] Although Jiang et al[20] demonstrated that several cytokines are associated with the severity of rotavirus gastroenteritis, a correlation between cytokine production and rotavirus antigen levels has not been studied. Six cytokines were measured in serum samples that were collected at the time of hospital admission by CBA, a method that can simultaneously measure multiple cytokine levels in a single reaction and therefore requires only small amounts of serum. Two (IL-8 and Il-10) of the 6 cytokines were statistically correlated with rotavirus antigen levels in this study. It has been demonstrated that rotavirus infection can induce the production of several chemokines, including IL-8, in intestinal cell lines and small animal models.[18,21] IL-8 may play an important role in generating the mucosal immune response to rotavirus infection. This study showed that significantly higher IL-8 levels were observed in patients with higher levels of rotavirus antigenemia. The results of this study together with previous in vitro and in vivo studies[18,21] suggest that increased systemic spread of the virus could trigger a strong immune response in the host.

Note that IL-10 levels were negatively correlated with rotavirus antigen levels. This cytokine has modulatory effects on both T-helper 1 and 2 cytokines and plays a regulatory role in inflammatory processes. It has been demonstrated that plasma IL-10 levels increase in the acute phase of rotavirus gastroenteritis[17,20]; however, the role of this cytokine in disease pathogenesis remains obscure.

CONCLUSIONS

The results of this study demonstrate that IL-10 levels were significantly lower in patients with high rotavirus antigen levels, which suggests that this cytokine plays an important role in the immune response against systemic rotavirus infection.

ACKNOWLEDGMENTS

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BIRTHS FUELING HISPANIC GROWTH: IMMIGRATION NO LONGER TOP FACTOR

"Births, not immigration, now account for most of the growth in the nation's Hispanic population, a distinct reversal of trends of the past 30 years. The Hispanic baby boom is transforming the demographics of small-town America in a dramatic way. Some rural counties where the population had been shrinking and aging are growing because of Hispanic immigration and births and now must provide services for the young. 'In all of the uproar over immigration, this is getting missed,' says Kenneth Johnson, demographer at the University of New Hampshire's Carsey Institute. 'All the focus is on immigration, immigration, immigration. At some point, it's not. It's natural increase.' This natural increase—more births than deaths—is accelerating among Hispanics in the USA because they are younger than the US population as a whole. Their median age is 27.4, compared with 37.9 overall, 40.8 for whites, 35.4 for Asians and 31.1 for blacks. Because they are younger and likely to have more children, Hispanics are having an impact that far outlasts their initial entry into the country."

El Nasser H. *USA Today*. June 30, 2008
Noted by JFL, MD

Analysis of Rotavirus Antigenemia and Extraintestinal Manifestations in Children With Rotavirus Gastroenteritis

Ken Sugata, Koki Taniguchi, Akiko Yui, Fumi Miyake, Sadao Suga, Yoshizo Asano, Masahiro Ohashi, Kyoko Suzuki, Naoko Nishimura, Takao Ozaki and Tetsushi Yoshikawa

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Correlation Between Serum Matrix Metalloproteinase and Antigenemia Levels in Patients Infected With Rotavirus

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Rotavirus (RV) antigenemia has been reported in patients with gastroenteritis; however, the exact mechanism remains unclear. In order to elucidate the mechanism of RV antigenemia, an association between RV antigenemia and matrix metalloproteinase (MMP) were analyzed. The object of this study was to elucidate the role of MMPs and tissue inhibitors of metalloproteinases (TIMPs) in the pathogenesis of RV antigenemia. Forty children admitted to hospital with RV gastroenteritis were enrolled in this study. Paired serum samples were collected at the time of admission and discharge. Enzyme-linked immunosorbent assays (ELISA) were used to detect serum concentrations of viral antigens, MMP-1, -2, -9, -13, TIMP -1, and -2. Cytokines were measured using flow cytometric beads array. RV antigens were significantly higher in serum collected at the time of admission than discharge ($P < 0.001$). MMP-9 concentrations were significantly higher in serum collected at the time of admission than discharge ($P < 0.001$). MMP-2 concentrations were significantly lower in serum collected at the time of admission than discharge ($P < 0.001$). A weak but a significantly positive association ($P = 0.034$) was observed between RV antigen and MMP-9 in serum collected at the time of admission, and inverse association was observed between RV antigen and MMP-2. In addition, a weak but significantly positive association ($P = 0.002$) was observed between IL-6 and MMP-9. These data suggest that MMPs may contribute to the pathogenesis of RV antigenemia. ***J. Med. Virol. 84:986–991, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: rotavirus; antigenemia; MMP; TIMP

Rotavirus (RV) is the major cause of gastroenteritis in young children worldwide. Severe dehydration caused by RV-induced diarrhea and vomiting could be fatal in developing countries [Parashar et al., 2003; Parashar et al., 2009; Widdowson et al., 2009]. Gastroenteritis induced by RV infection causes a large economic burden in developed countries [Glass et al., 1996; Dennehy, 2008]. Although RV replication is considered to be limited to the gastrointestinal tract, it has been reported that RV antigen and/or RNA were detected in the serum obtained from patients with RV gastroenteritis [Blutt et al., 2003; Fischer et al., 2005; Ray et al., 2006]. An association between RV antigen concentrations and extraintestinal manifestations or serum cytokine concentrations in children with RV gastroenteritis have been demonstrated and that data also suggested that cytokines play an important role in the development of RV antigenemia [Sugata et al., 2008]; however, the pathogenesis of RV antigenemia is not fully understood.

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Conflicts of interest: None.

This study was approved by the review boards of all four institutions (#08-177).

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Matrix metalloproteinases (MMPs), which include the collagenases, stromelysins, gelatinases, and membrane-type MMPs are a family of structurally related zinc-dependent proteases that are involved in cellular infiltration, cell migration, tissue damage, remodeling, and repair. [Joni and Zena, 2004] Particularly, MMPs can cleave extracellular matrix proteins, and have an important role in the regulation of extracellular matrix turnover [Nagase et al., 2006]. It has been suggested that dysregulated expression of MMPs plays a pathogenetic role in a number of diseases including inflammatory bowel disease (IBD) [Ravi et al., 2007; Meijer et al., 2007a, c; Makitalo et al., 2010] and pathogen induced colitis [Stallmach et al., 2000; Medina and Radomski, 2006]. These data suggest that MMPs and their inhibitor, tissue inhibitors of metalloproteinases (TIMPs), contribute to the pathogenesis of various types of enterocolitis. The primary object of this study was to elucidate role of MMPs and TIMPs in the pathogenesis of RV antigenemia. The concentrations of RV antigen, MMPs, TIMPs, and cytokines were measured in paired serum samples collected from patients with RV gastroenteritis.

PATIENTS AND METHODS

Patients

Forty hospitalized children (age: 1.9 ± 1.2; gender: 28 boys and 12 girls) admitted to hospital with a diagnosis of acute RV gastroenteritis were enrolled in this study. All patients were admitted to one of four pediatric departments (Fujita Health University, Kariya Toyota General Hospital, Toyokawa City Hospital, and Konan Kosei Hospital) between January 2006 and March 2006. Informed consent was obtained from the patients' guardians prior to participation in this study. This study was approved by the review boards of all four institutions.

Diagnosis of RV gastroenteritis was confirmed by detection of RV antigen in stool samples using an immunochromato-assay [Dipstick (Eiken Chemical, Tokyo, Japan)]. Paired serum samples were collected at the time of hospital admission [days 1–5; day 1 was defined as the date of onset of symptoms (e.g., fever, vomiting, diarrhea)] and at the time of discharge (days 4–11). In addition, 31 serum samples were collected from age-matched healthy control children.

RV Antigen Detection

RV antigen was measured using an in-house enzyme-linked immunosorbent assay (ELISA) that detects VP6 antigen of the virus as described previously [Sugata et al., 2008]. In brief, 96-well plates (Nalgen Nunc International, Rochester, NY) were coated with a monoclonal antibody against the VP6 antigen of RV (YO-156). Serum samples were diluted (1:16) and 50 μl was applied to the 96-well plate. Anti-human RV hyper immune rabbit serum diluted 1:5,000 in phosphate-buffered saline containing Tween-20 was used for secondary antibody, followed by peroxidase-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories Incorporated, West Grove, PA). The quantity of monoclonal antibody bound to RV VP6 antigen was assessed by OD values after addition of the substrate. Cut off value was 0.13 (mean + 3 SD of control serum samples) in this study.

Detection of MMPs and TIMPs

Serum concentrations of MMP-1 (1:5 dilution), -2 (1:100 dilution), -9 (1:10 dilution), and -13 (1:8 dilution) were determined by ELISA (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The calculated detection limits of undiluted samples were 1.7, 0.37, 0.6, and 0.032 ng/ml for MMP-1, -2, -9, and -13, respectively. TIMP-1 concentration in the serum (1:20 dilution) was also determined by ELISA (Invitrogen, CA) according to the manufacturer's instructions. The minimum detectable concentration of TIMP-1 was 1 ng/ml. Serum TIMP-2 concentration (1:5 dilution) was determined by ELISA (GE Healthcare) according to the manufacturer's instructions. The minimum detectable concentration of TIMP-2 was 3 ng/ml.

Cytokine Detection by Flow Cytometric Bead Array

Flow cytometric bead array (CBA) (Becton Dickinson, San Diego, CA) was used to measure the following cytokines: Interleukin (IL)-8, IL-1, IL-6, IL-10, tumor necrosis factor (TNF), and IL-12. Sera or standards (50 μl) were added to 50 μl of a cocktail of capture beads and detector antibodies, and the mixture was incubated for 1.5 hr at room temperature in the dark. Excess unbound detector antibody was removed by washing and 50 μl of reagent was added before data acquisition. Two-color flow-cytometric analysis was performed using a flow cytometer [FACScan (Becton Dickinson, Franklin Lakes, NJ)]. Analysis was performed using CBA dedicated analysis software [CellQuest (Becton Dickinson)].

Statistical Analysis

Statistical analyses were performed using Stat View 5.0 (SAS Institute, Cary, NC). Each marker was compared between the two groups by using either a Wilcoxon signed-ranks test or Mann–Whitney U test. Spearman's rank correlation coefficients were used to measure the strength of the association between MMPs concentrations and RV antigen or cytokine concentrations.

RESULTS

RV Antigen Concentrations in the Serum

The level of RV antigens in the sera of children diagnosed with RV gastroenteritis was assessed. As we

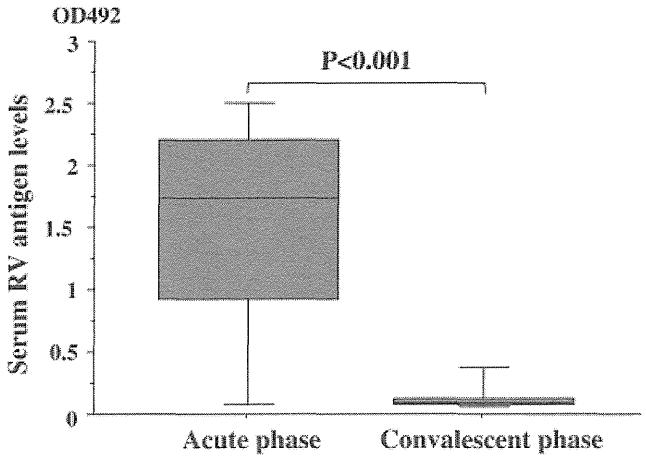

Fig. 1. Comparison of the serum RV antigen concentrations between the time of admission and discharge in patients with RV gastroenteritis (n = 40). Data are shown as box plots, where these boxes represent the first through third quartiles, the lines within these boxes represent the median, and the lines outside the boxes represent 10 and 90 percentiles.

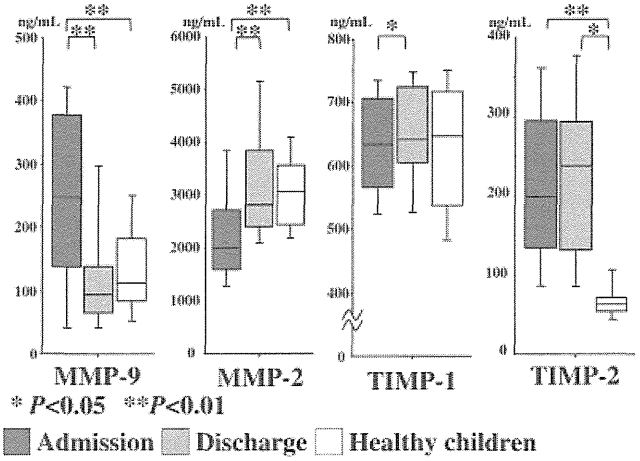

Fig. 2. Comparison of the serum MMP-2, -9, TIMP-1, and -2 concentrations among the time of admission and discharge in patients with RV gastroenteritis (n = 40), and healthy children (n = 31). Data are shown as box plots, where these boxes represent the first through third quartiles, the lines within these boxes represent the median, and the lines outside the boxes represent 10 and 90 percentiles.

expected, RV antigen was significantly higher ($P < 0.001$) in serum that was collected at the time of admission (median, inter quartile range (IQR); 1.56, 1.05–2.20) than in those collected at the time of discharge (median, IQR; 0.26, 0.08–0.12) (Fig. 1). No RV antigen was detected in serum samples collected from healthy controls (data not shown).

The Serum Concentration of MMPs and TIMPs

In order to investigate the potential pathogenic role of MMPs and TIMPs in RV antigenemia, the serum concentrations of MMP-1, -2, -9, and -13, as well as TIMP-1 and -2 were determined. Interestingly, MMP-9 concentrations were significantly higher in serum collected at the time of admission (median, IQR; 248, 142–379 ng/ml) than at the time of discharge (median, IQR; 89, 65–117 ng/ml, $P < 0.001$) or those collected from healthy children (median, IQR; 112, 87–179 ng/ml, $P = 0.002$). In contrast to MMP-9, MMP-2 concentrations were significantly lower in serum collected at the time of admission (median, IQR; 1932, 1600–2686 ng/ml) than at the time of discharge (median, IQR; 2819, 2405–3837 ng/ml, $P < 0.001$) or those collected from healthy children (median, IQR; 3050, 2471–3579 ng/ml, $P < 0.001$). Meanwhile, TIMP-1 concentrations were slightly but significantly lower in serum collected at the time of admission (median, IQR; 636, 573–706 ng/ml) than at the time of discharge (median, IQR; 648, 609–724 ng/ml, $P = 0.043$). Irrespective of sampling period, serum TIMP-2 concentrations in RV gastroenteritis patients [at the time of admission (median, IQR; 192, 131–283 ng/ml, $P < 0.001$) and discharge (median, IQR; 237, 131–289 ng/ml, $P = 0.002$)] were significantly higher than healthy children (median, IQR; 63, 54–70 ng/ml) (Fig. 2). Only five of the 36 serum samples evaluated contained low levels of MMP-1 at the time of admission (mean ± SD; 5.9 ± 26.4 ng/ml) and discharge (mean ± SD; 4.2 ± 16.2 ng/ml). Additionally, low concentrations of MMP-13 were detected in serum samples collected at the time of admission (mean ± SD; 0.09 ± 0.15 ng/ml) and discharge (mean ± SD; 0.06 ± 0.14 ng/ml). A statistical difference in serum MMP-1 and MMP-13 concentrations among the three sample groups (at the time of admission and discharge in RV gastroenteritis patients and healthy children) was not detected.

Association Between RV Antigen and MMPs Concentrations

Next the relationship between RV antigenemia and MMPs expression was examined. A weak but significantly positive association ($r = 0.34$, $P = 0.034$) was observed between RV antigen concentrations and MMP-9 concentrations. Conversely, a weak but significantly negative association ($r = -0.37$, $P = 0.014$) was observed between RV antigen concentrations and MMP-2 concentrations (Fig. 3). No statistical correlation was observed between the serum concentrations of MMP-1, MMP-13, TIMP-1 and TIMP-2 and RV antigen concentrations.

Association Between Cytokines and MMPs Concentrations

Cytokines have shown to regulate the synthesis of MMPs. Serum cytokine concentrations were evaluated in the serum samples collected at the time of admission to the hospital. A weak but significantly positive association ($r = 0.33$, $P = 0.002$) was observed between IL-6 and MMP-9 concentrations in serum

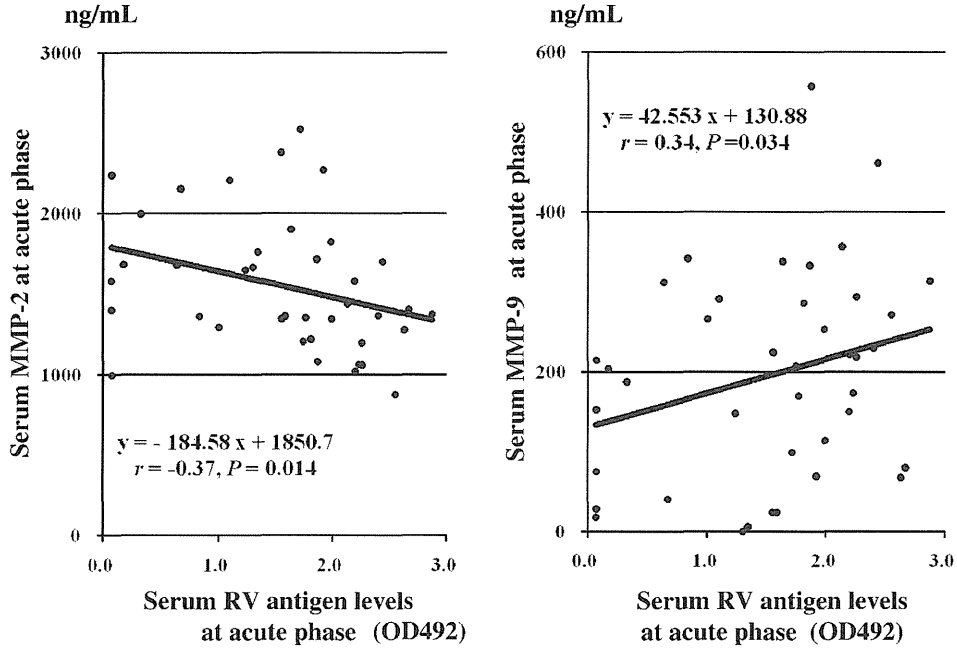

Fig. 3. Association between serum RV antigen and serum MMP concentrations. A weak (r = −0.37) but significantly ($P = 0.014$) negative association was observed between MMP-2 concentrations and RV antigenemia. A weak (r = 0.34) but significantly ($P = 0.034$) positive association was also observed between MMP-9 concentrations and RV antigenemia.

collected at the time of admission (Fig. 4). No correlation was observed between the serum concentrations of MMP-1, -9, -13, TIMP-1 or -2 and the other cytokines evaluated.

DISCUSSION

It has been demonstrated that MMP-2 and MMP-9, referred to as gelatinase, are upregulated in colonic mucosa in IBD [Bailey et al., 1994; Gao et al., 2005; Meijer et al., 2007a, b] or gastric mucosa in *Helicobacter pylori*-associated gastritis [Bergin et al., 2004; Koyama, 2004; Bergin et al., 2008]. Epithelial-derived MMP-9 is an important mediator for tissue injury in colitis, whereas MMP-2 protects against tissue damage and maintains gut barrier function [Garg et al., 2009]. In this present study, the kinetics of MMPs in patients with RV gastroenteritis was clarified. Interestingly, the serum concentration of MMP-9, which can induce tissue injury, was significantly increased, while MMP-2 that protects against tissue damage was significantly decreased in acute phase of the disease. These findings suggest that the upregulation of MMP-9 and downregulation of MMP-2 play important roles in tissue damage in patients with RV gastroenteritis as similar to IBD or *Helicobacter pylori*-associated gastritis. To our knowledge, this is the first report to elucidate the kinetics of MMPs in patient with RV gastroenteritis.

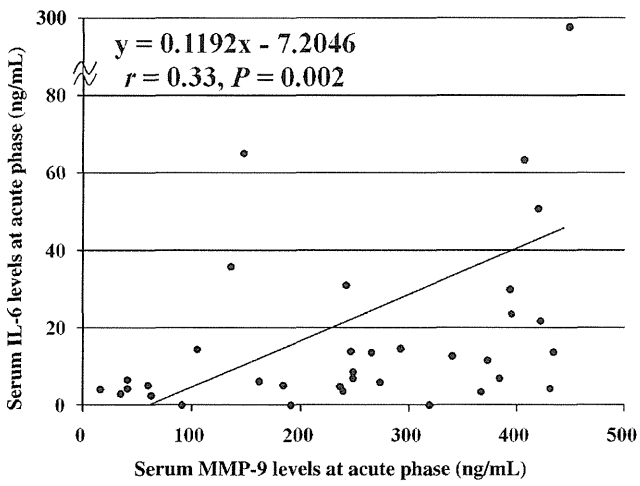

Fig. 4. Association between serum cytokines and MMP concentrations. A weak (r = 0.33) but significantly ($P = 0.002$) positive association was observed between IL-6 and MMP-9 concentrations.

In contrast to MMP-2 and MMP-9, low concentrations of MMP-1 and MMP-13 were detected in a small number of RV gastroenteritis patients and healthy controls in this study. Our findings are in line with previous studies that detected weak MMP-1, but no MMP-13 gene transcripts in colonic epithelial cells isolated from biopsies from IBD patients. Although it has been demonstrated that abundant MMP-1 and MMP-13 gene transcripts were detected in HT-29 cells [Pedersen et al., 2009]. Therefore, further in vitro and in vivo analyses are needed to determine the precise role of MMP-1 and MMP-13 in the pathogenesis of RV gastroenteritis.