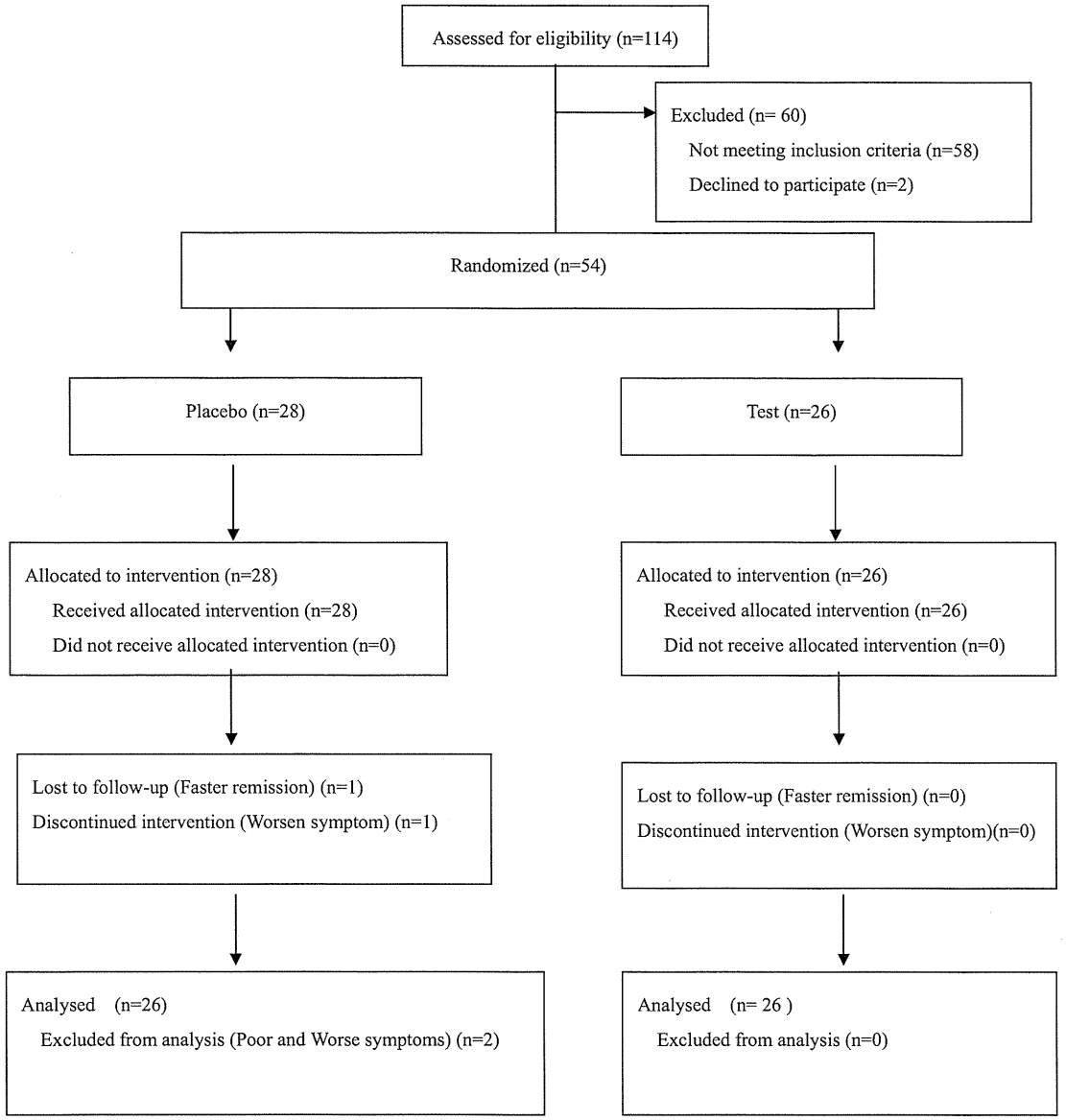

Fig. 2. Diagram showing the decision flow on study subjects throughout the study.

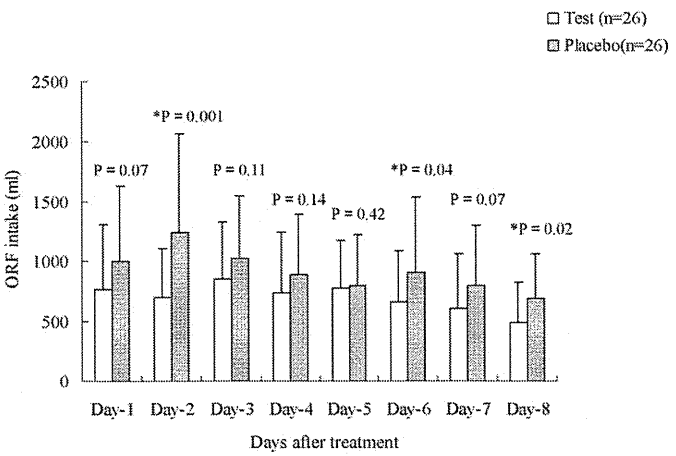

Fig. 3. Comparative analysis of mean daily oral fluid intake in study children between Rotamix IgY and placebo IgY groups. *: Significant differences between the Rotamix IgY and placebo IgY groups (*$p \leq 0.05$, Student's *t*-test).

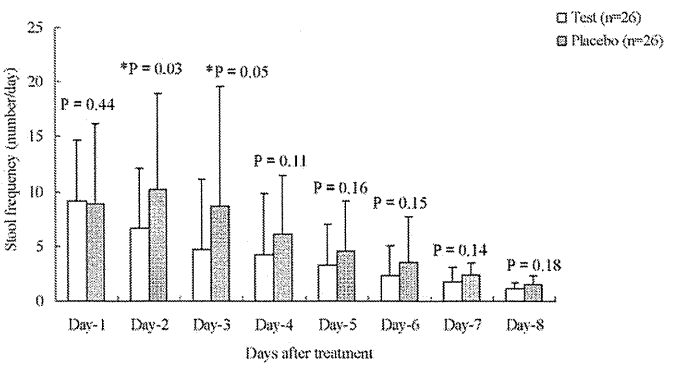

Fig. 4. Comparison of stool frequency (number/day) between children treated with Rotamix IgY or placebo IgY. *: Significant differences compared with placebo and Rotamix IgY groups (*$p \leq 0.05$, Student's *t*-test).

Table 6
Comparative analysis of Rotamix IgY and placebo effects on study groups according to outcome measures.

Parameters	Placebo IgY group	Rotamix IgY group	Statistics
1. Daily oral rehydration fluid volume	Day 2 = 1244.1 ± 818.4 ml Day 6 = 912.5 ± 623.0 ml Day 8 = 688.5 ± 372.7 ml	Day 2 = 704.4 ± 403.8 ml Day 6 = 660.6 ± 429.8 ml Day 8 = 493.9 ± 329.8 ml	Day 2; $p = 0.001^*$ Day 6; $p = 0.04^*$ Day 8; $p = 0.02^*$ (See Fig. 3)
2. Total oral rehydration fluid volume from day of admission	919.1 ± 171.31 ml	699.3 ± 111.1 ml	$p = 0.004^*$
3. Mean duration of intravenous fluid administration	8 days	5 days	$p = 0.03^*$
4. Mean volume of intravenous fluid administered daily	93.3 ± 196.7 ml	77.4 ± 121.4 ml	Not significant ($p = 0.42$)
5. No. of stools/day	Day 2 = 10.2 ± 8.8 Day 3 = 8.7 ± 11.2	Day 2 = 6.7 ± 4.3 Day 3 = 4.7 ± 4.1	Day 2; $p = 0.03^*$ Day 3; $p = 0.05^*$ (See Fig. 4)
6. Total duration of diarrhea from day of admission	185.5 ± 41.7 h	135.3 ± 42.0 h	$p = 0.01^*$
7. Daily frequency of viral shedding	Day 3 = 88 Day 6 = 25 Day 7 = 20 Day 8 = 25	Day 3 = 42 Day 6 = 0 Day 7 = 0 Day 8 = 0	Day 3; $p = 0.005^*$ Day 6; $p = 0.02^*$ Day 7; $p = 0.04^*$ Day 8; $p = 0.02^*$ (See Fig. 5)
8. Total duration of viral shedding from day of admission	4.2 ± 2.9 days	3.0 ± 1.6 days	$p = 0.05^*$

* Statistically significant.

derived their genomic segments from parental strains of different genogroups and/or serotypes. The HRV 408 was shown to have subgroup II specificity (I1 genotype according to recent nomenclature) [30], and its RNA profile is long which is characteristic of subgroup II rotaviruses [21]. HRV 408 has G3 specificity, but could also be neutralized by polyclonal antisera to G1, G2, and G3, and may induce neutralizing antibodies to at least G1 and G2 [30]. The HRV 248 was shown to have G4P[4] specificity, subgroup II specificity (I1 genotype of VP6), and a long RNA pattern. By RNA–RNA hybridization assay, it was found that 7 of 11 RNA segments of the HRV 248 are derived from Wa genogroup and the remaining 4 are from DS-1 genogroup. Thus, HRV 248 is shown to be an intergenogroup reassortant [31] strain.

In order to determine cross reactivity profile, several rotavirus serotypes originally derived from human, horse, cattle and swine were reacted in vitro against Rotamix IgY. Results revealed that Rotamix IgY strongly cross-neutralized all the major HRV serotypes (G1, G2, G3, and G4) along with other human and animal strains (Table 3). In addition to a broad spectrum of cross-reactivity, synergism was observed in vitro with Rotamix IgY relative to the lower titers of IgY developed individually from HRV 408 and HRV 248 strains. This in vitro cross-reactivity profile suggests that Rotamix IgY might provide a general multi-serotypic passive protection in vivo particularly among rotavirus infected infants and young children considering the broad serotypic diversity of human rotaviruses in different regions of the world.

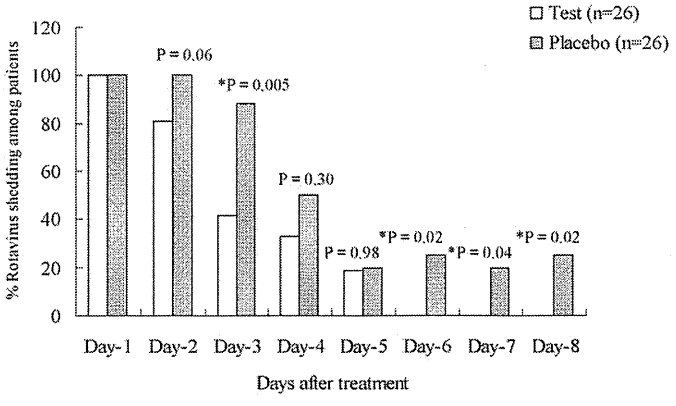

Fig. 5. Effect of Rotamix IgY on daily frequency of rotavirus shedding in stools of children. *: Significant differences between Rotamix IgY and placebo IgY groups (*$p \leq 0.05$, Chi-square test).

As a first step towards assessment of protection against HRV-induced diarrhea in pediatric patients using Rotamix IgY, we used the latter IgY in a placebo-controlled mouse challenge experiment. There was significant reduction in the frequency of diarrheal episodes among challenged mice using the dual-specificity Rotamix IgY. The rate of protection was specific based on the dose-dependent protection rate afforded to mice with protection rate reaching up to 90% at the highest IgY concentration used (Fig. 1). These findings are consistent with previous applications of IgY as a therapeutic agent in cat [17] and calves [16,32] and as prophylactic agent in mice [33]. However, these earlier studies were based on the use of IgY with single rotavirus strain specificity and are therefore of limited application in clinical settings with multi-serotypic etiology. The results on dose-dependent protection effect together with the safety of Rotamix IgY in infected suckling mice encouraged us to evaluate the therapeutic activity of Rotamix IgY among children with diarrhea concurrently infected with HRV and other enteric pathogens during their hospital confinement.

Out of 114 young children and infants admitted with diarrhea and other complaints in a Myanmar hospital, 54 of them (47.4%) were initially entered in this study using our inclusion criteria but 2 of them were excluded later due to incomplete data (Fig. 1). This frequency of rotavirus infection is almost similar to earlier studies in Myanmar (57% and 55% in 2004 and 2005 respectively) [11]. Of the remaining 52 subjects, 92% had a second enteric non-cholera pathogen (Table 5). Mixed infection with different species of enteric pathogens as observed in this study may be representative of clinical conditions in developing countries as earlier observed [27,34].

Rotamix IgY, with a neutralization titer of 10,240–40,960 against a broad range of human rotavirus strains known to be currently circulating worldwide, was administered orally at the rate of 500 mg × 4 doses daily (containing about 30 mg of IgY/day) together with standard medical treatment (Table 2) to control bacterial infections, replace fluid loss and/or provide nutritional supplements to mitigate the effects of diarrhea. As such, Rotamix IgY was used as an adjunct to standard supportive treatment of rotavirus diarrhea with an intention to directly neutralize or suppress rotavirus infectivity in the gut and thereby prevent further cell-to-cell spread of infection and resulting fluid loss.

Since the first 1–3 days in the hospital is usually the most severe period of the illness, it follows that any beneficial effect arising from Rotamix IgY administration within this period (as seen in Fig. 4) signifies clinical relevance [20]. From the public health standpoint, neutralizing rotavirus infectivity in acute enteritis and reducing the duration of viral shedding by specific IgY may potentially prevent further spread of infection in a community or hospital setting. It is worth noting that Rotamix IgY influenced the above clinical parameters despite the presence of co-infecting enteric pathogens in 92% of the subjects indicating an important role played by Rotamix IgY's as an adjunct to supportive therapy for infant rotavirus diarrhea. This finding also highlights the contribution of rotavirus as a pivotal virulence factor in determining the course of infection during mixed infection with other non-cholera enteric pathogens.

While certain observations were statistically significant only on certain days as shown in Figs. 3–5, the totality of data over the whole 8-day observation period will bear out statistically significant differences in observations between the two groups particularly in terms of total oral rehydration fluid volume from day of admission, mean duration of intravenous fluid administration, total duration of diarrhea starting from the day of admission, and total duration of viral shedding from day of admission (Table 6). These significant observations may translate into real benefits in terms of earlier termination of IV fluid by 3 days, earlier recovery from diarrhea by 2 days, and earlier cessation of rotavirus shedding via stool by 1 day.

Data in Tables 2, 4 and 5 indicate that patients in both test and placebo groups had similar demographics, had comparable frequency and type of co-infecting pathogen burden as well as comparable treatments received during the clinical trial. Among test group patients, oral administration of Rotamix IgY containing antibodies known to neutralize or disable a broad spectrum of rotavirus serotypes in vitro (Table 3), some of which were circulating in the study area [11], significantly improved the clinical picture as outlined in Table 6. This means the favorable outcomes among Rotamix IgY treated patients were mainly due to the specific action of the IgY received as can be gleaned from the lack of similar outcomes among placebo patients.

A 2009 report showed that among children less than 5 years of age who were admitted to Yangon Children's Hospital in Myanmar, the most common serotypes were G3, P[4] and P[8] while the most common G and P combinations were G3P[8] and G1P[8] [11]. Representatives of these strains tested in this study (Table 1) could be cross-neutralized by Rotamix IgY (Table 3) which may further account for the adjunctive value of oral Rotamix IgY administration in our clinical setting.

None of the children in either test or placebo group in this study recovered before day 5 from the day of admission to hospital. This is inconsistent with an earlier observation wherein rotavirus diarrhea in children normally lasts for 1–6 days, is self-limiting and children usually recover before day 5 [35]. The generally longer episode of diarrhea observed in this study may be explained by: 1) the presence of a co-infecting pathogens in almost all subjects examined, dual infection being known to produce a more severe illness [36], and 2) malnutrition. In developing countries, diarrhea has been observed to last longer in children with less than ideal nutrition [37].

Passive immunotherapy using IgY has advantages over vaccination due to its: 1) rapid and local onset of action, 2) highly specific activity, 3) applicability to a broader age range of patients from infants to adults including immunodeficient patients, and 4) is non-toxic being a normal part of the human diet [38,39]. Notably, none of the study patients treated with Rotamix IgY or placebo IgY presented with complaints other than the ongoing diarrhea and its resulting dehydration. The absence of any adverse clinical event attributable to or associated with oral IgY administration for 8 days is consistent with our clinical findings in the mouse protection experiment (Fig. 1), as well as in earlier published passive immunization trials with hyperimmune IgY against *Streptococcus mutans* [40] and *Helicobacter pylori* [15] among human subjects. With the recent findings by our group on the application of IgY against *Vibrio cholerae* [41] and Shiga-toxin [42], the present study paves the way toward combined use of IgYs against Shiga toxin, cholera and rotavirus for prevention and treatment of pediatric diarrhea in the future.

Considering that Rotamix IgY is strongly reactive with a broad range of serotypes responsible for the vast majority of rotavirus-associated diarrhea in infants and young children worldwide, and may ameliorate patient condition when administered orally even to those with mixed (rotavirus plus non-rotavirus) enteric infections, it comprises a potentially useful adjunct to general supportive therapy of acute rotavirus infection in pediatric patients.

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Contributors: SR participated in the design, organization and coordination of the study, participated in the statistical analysis and drafted the manuscript. KHM supported the in vitro analysis, KWH carried out the clinical work, participated in the clinical data analysis. KT provided his lab facilities to carry out in vitro analysis, advised on overall data analysis; FCI advised on data interpretation and did critical revision and finalizing of manuscript; TT coordinated the working team. YK NVS, KU, NNDF KO participated in drafting of the manuscript and coordinated with the working team. HNO, YYM, TH, SSM, KT participated in the clinical works and coordinated with the working team. All authors have read and approved the final manuscript. *Conflict of interest statement*: The authors declare that they have no competing interests.

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Analysis of rotavirus antigenemia in hematopoietic stem cell transplant recipients

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Abstract : Systemic rotavirus infection, such as rotavirus antigenemia, has been found in immunocompetent rotavirus gastroenteritis patients. However, the pathogenesis of rotavirus infection in immunocompromised transplant recipients remains unclear. Enzyme-linked immunosorbent assay was used to measure rotavirus antigen levels in serially collected serum samples obtained from 62 pediatric patients receiving allogeneic hematopoietic stem cell transplants (HSCT). Rotavirus antigen was detected in 43 (6.8%) of 633 serum samples (8 of 62 patients). The duration of rotavirus antigenemia ranged between 1 and 10 weeks, and diarrhea was concurrent with rotavirus antigenemia in Cases 3, 6, 7, and 8. The level of viral antigen in the transplant recipients (0.19 ± 0.20) was significantly lower than that observed in serum samples collected from immunocompetent patients on either day 1 (0.49 ± 0.18, $P = 0.0011$) or day 3 (0.63 ± 0.09, $P = 0.0005$). A patient who received a graft from a human leukocyte antigen (HLA)-mismatched donor was at significant risk for rotavirus antigenemia ($P = 0.024$; odds ratio = 9.44) in comparison to patients who received grafts from HLA-matched donors. Although the duration of antigenemia was clearly longer in HSCT patients than in immunocompetent rotavirus gastroenteritis patients, the levels of viral antigen were not as high. Therefore, mismatched HLA may be a risk factor for rotavirus antigenemia after HSCT.

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Key words: rotavirus; antigenemia; hematopoietic stem cell transplantation; pediatric

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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. In addition, the gastroenteritis induced by rotavirus infection causes a large economic burden in developed countries. Rotaviral infection is generally benign and self-limited in immunocompetent children. In contrast, it has been reported that rotavirus can cause severe diarrhea resulting in fatal outcomes for immunocompromised transplant recipients (1–5). Thus, the pathogenesis of rotaviral infection may differ between immunocompetent and immunocompromised individuals.

Initially, rotavirus replication was thought to be limited to the gastrointestinal tract in patients with rotavirus gastroenteritis. However, recently, rotavirus antigen and RNA were detected in the sera of rotavirus-infected children (6–9). In addition, rotavirus antigen was detected not only in the serum but also in several organs, including the stomach, intestine, liver, lung, spleen, kidney, pancreas, thymus, and bladder in rotavirus-infected animals (10). These findings suggest that rotavirus spreads beyond the intestine in children with rotavirus gastroenteritis, resulting in systemic viral infection. Recently, we found that rotavirus antigenemia was frequently observed during the acute phase of rotavirus

gastroenteritis (11). Rotavirus antigen peaked on day 2 of the illness, with the amount of viral antigen gradually decreasing to nearly undetectable levels by day 6. We also found that cytokines were involved in controlling antigenemia levels. The results of this study, together with those from previous studies (7, 8, 12, 13), suggested that host immune responses have important roles in regulating viral replication. Therefore, the kinetics of rotavirus antigenemia in transplant recipients may be different from those in immunocompetent rotavirus gastroenteritis patients. In this study, we sought to elucidate the kinetics of rotavirus antigenemia in hematopoietic stem cell transplant (HSCT) recipients. We measured rotavirus antigen levels in serum samples serially collected from pediatric HSCT recipients and analyzed the associations between antigenemia and clinical features.

Patients and methods

Patient characteristics

Between September 2004 and February 2007, 62 patients received allogeneic HSCT (17 with allogeneic bone marrow transplant [BMT] from human leukocyte antigen [HLA]-matched siblings, 9 with allogeneic BMT from HLA-mismatched siblings, 22 with allogeneic BMT from unrelated donors, 2 with peripheral blood stem cell transplants, and 12 with umbilical cord blood transplants) at the Division of Hematology-Oncology at the Children's Medical Center, the Japanese Red Cross Nagoya First Hospital, or the Department of Pediatrics at the Nagoya University Graduate School of Medicine. The patients' guardians provided written consent for their participation in this study. This study was approved by the review boards of the 3 institutes. Patient characteristics are summarized in Table 1 and include age, gender, underlying diseases, type of graft, HLA matching, having received total body irradiation (TBI) or anti-thymocyte globulin (ATG) in conditioning regimen, and occurrence of acute graft-versus-host disease (GVHD).

Patient management

Details of the conditioning regimen and GVHD prophylaxis have been previously described (14, 15). In brief, patients with hematologic malignancies were conditioned with high-dose chemotherapy consisting of melphalan (180 mg/m^2) plus busulfan (16 mg/kg) or TBI (12 Gy). Patients with severe aplastic anemia were conditioned with 200 mg/kg cyclophosphamide and 10 mg/kg rabbit ATG for transplantation from a matched sibling donor. For patients transplanted with an unrelated bone marrow donor, TBI (5–10 Gy) was added (16). GVHD prophylaxis consisted of cyclosporine or tacrolimus with short-term methotrexate. All patients received trimethoprim–sulfamethoxazole orally or inhaled pentamidine as prophylaxis against *Pneumocystis jirovecii*. The standard doses of oral amphotericin B and acyclovir were administered as prophylaxis for fungal and herpes simplex virus infections. Intravenous γ-globulin preparations were administered weekly during the first 3 months as prophylaxis for cytomegalovirus (CMV) infection. In addition, ganciclovir was given as preemptive therapy against CMV infection following a positive result from a CMV antigenemia assay. Acute and chronic GVHD was diagnosed and graded according to established criteria.

Experimental design

Serum samples were collected from 62 recipients at the time of HSCT, weekly for 3 or 4 months post transplant. Ultimately, 633 serum samples were analyzed in this study. In addition to these samples, 15 serum samples were collected from rotavirus gastroenteritis patients on days 1, 3, and 5 of illness and used as controls. Clinical data were collected retrospectively and assessed to determine associations with rotavirus antigenemia.

Rotavirus antigen detection

Rotavirus antigen was measured using a previously described enzyme-linked immunosorbent assay (ELISA) for the detection of VP6 antigen of the virus (11). Diluted serum (1:16, 50 μL) was used to detect rotavirus antigen. Ninety-six–well plates (Nalgen Nunc, Rochester, New York, USA) coated with a monoclonal antibody against the VP6 antigen of rotavirus (YO-156) (17) were used for the ELISA (18). The plates were incubated with 50 μL of diluted serum at 4°C overnight. Then, 50 μL of anti-human rotavirus hyperimmune rabbit serum (diluted 1:5000 with phosphate buffered saline [PBS] containing 0.05% Tween-20 [PBST] and 2.5% skim milk) was added to each well. Peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) (diluted

Patient characteristics and risk factors for rotavirus antigenemia after hematopoietic stem cell transplantation

Categories	Rotavirus antigenemia		Odds ratio (95% CI)	*P*
	Yes (*n* = 8)	No (*n* = 54)		
Age (years)	7.4 ± 5.6	8.3 ± 5.1	...	0.652
Sex				
Male	4	37	0.46 (0.10–2.06)	0.312
Female	4	17		
Underlying disease*				
Malignancy	4	36	2.0 (0.44–8.93)	0.368
Non-malignancy	4	18		
Total body irradiation				
Yes	6	41	1.05 (0.18–5.85)	0.955
No	2	13		
Anti-thymocyte globulin				
Yes	4	11	0.25 (0.05–1.19)	0.080
No	4	43		
Acute graft-versus-host disease (grade 2–4)				
Yes	3	17	0.77 (0.16–3.58)	0.705
No	5	37		
Source of the graft				
Related donor	3	23	1.0	
Unrelated donor	3	19	1.21	0.827
CBT	1	11	0.70	0.760
PBSCT	1	1	7.67	0.206
HLA matching				
Match	1	31	9.44 (1.09–82.11)	0.024
Mismatch	7	23		

*Acute lymphoblastic leukemia, 23; aplastic anemia, 12; acute myeloid leukemia 11; myelodysplastic syndrome, 4; rhabdomyosarcoma, 1; malignant lymphoma, 2; neuroblastoma, 1; others, 8.
CI, confidence interval; CBT, cord blood transplant; PBSCT, peripheral blood stem cell transplant; HLA, human leukocyte antigen.

Table 1

1:5000, Jackson ImmunoResearch Laboratory Inc., West Grove, Pennsylvania, USA) was used as a secondary antibody. Finally, the amount of rotavirus VP6 antigen bound to specific monoclonal antibody was assessed by adding substrate. The optical density (OD) was read using spectrophotometry at 492 nm, and an appropriate cut-off value was established based on data from 20 serum samples collected from control subjects. As the mean OD of the control samples was 0.084 ± 0.014, we defined 0.13 (mean + 3 standard deviations [SD]) as the baseline value in this study.

Measurement of rotavirus-specific IgG

Serum anti-rotavirus IgG antibody titer was determined using sandwich ELISA. Briefly, 96-well plates were coated with rabbit anti-rotavirus serum diluted 1:10,000 in 10 mM PBS overnight at 4°C. After the plates were washed twice with 10 mM PBS containing 0.05% PBST, then 1% bovine serum albumin in PBST was added, and the plates were incubated for 4 h at 4°C. The plates were washed twice with PBST. SA-11-infected culture fluid was then added to the plates and incubated for 1 h at 37°C. Serum samples diluted with 2.5% skim milk in

PBST were allowed to react for 1 h at 37°C. After washing 4 times with PBST, donkey anti-human IgG (H+L) conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratory Inc.) diluted 1:1000 in 2.5% skim milk in PBST was added. The plates were incubated for 1 h at 37°C, then washed 4 times with PBST, and o-phenylenediamine-2HCl substrate was added. The OD was read by spectrophotometry at 492 nm. As the mean OD of the control samples was 0.047 ± 0.026, we defined 0.124 (mean + 3 SD) as the baseline value in this study.

Statistical analyses

Statistical analyses were performed using JMP7 (SAS Institute, Cary, North Carolina, USA). A Mann–Whitney *U*-test was used to compare the levels of rotavirus antigenemia between sera collected from transplant recipients and immunocompetent rotavirus gastroenteritis patients (days 1, 3, and 5). The antigen levels in 16-fold and 4-fold diluted serum samples were compared using a Wilcoxon's signed-ranks test. The anti-rotavirus IgG antibody levels with and without rotavirus antigenemia were compared using a Student's *t*-test.

To elucidate risk factors for rotavirus antigenemia in transplant recipients, pre-transplant variables and transplant variables were compared between recipients with and without antigenemia. Pre-transplant variables included age, gender, and underlying diseases. Transplant variables included TBI, ATG, HLA matching, type of graft source, and acute GVHD. The ages of the recipients with and without rotavirus antigenemia were compared using a Student's *t*-test. Odds ratios (and 95% confidence intervals) were based on 2 × 2 contingency tables and were calculated to assess the association between rotavirus antigenemia and demographics. The significance of measurement was determined by chi-square and Fisher's exact tests.

Results

Rotavirus antigen was detected in 43 (6.8%) of 633 serum samples (8 of 62 patients). The kinetics and season of rotavirus antigenemia are shown in Figure 1. Rotavirus antigenemia lasted between 1 and 10 weeks. Rotavirus antigenemia started within 4 weeks of the transplant in all 8 recipients. Although the endemic seasons for rotavirus gastroenteritis are generally in the winter and spring in Japan, rotavirus antigenemia was observed in non-endemic periods in Cases 1, 2, 3, 4, and 7. Figure 1 also shows a temporal relationship between rotavirus antigenemia and diarrhea. Diarrhea was concurrent with rotavirus antigenemia in Cases 3, 6, 7, and 8. Meanwhile, rotavirus antigenemia persisted after the cessation of diarrhea in Cases 6 and 7. Moreover, diarrhea was not observed during rotavirus antigenemia in Cases 1, 2, and 5.

To determine whether the amount of serum rotavirus antigen was higher in HSCT recipients than in immunocompetent rotavirus gastroenteritis patients, the antigen levels were compared between the 2 groups (Fig. 2). As expected, rotavirus antigen peaked on day 3 after illness onset in the serum samples collected from immunocompetent rotavirus gastroenteritis patients. The levels of rotavirus antigenemia in the transplant recipients (0.22 ± 0.19) and day 5 serum samples collected from immunocompetent rotavirus gastroenteritis patients (0.19 ± 0.20) were similar ($P = 0.9060$). The level of viral antigen in the transplant recipients was significantly lower than that observed in either day 1 (0.49 ± 0.18, $P = 0.0011$) or day 3 (0.63 ± 0.09, $P = 0.0005$) of serum samples collected from immunocompetent rotavirus gastroenteritis patients. Although a remarkable peak in rotavirus antigen levels was observed in immunocompetent rotavirus gastroenteritis patients (11), no such peak was seen in the kinetics of rotavirus antigenemia in HSCT recipients (data not shown).

As rotavirus antigenemia levels were low in HSCT recipients, antigen level was measured using less dilute

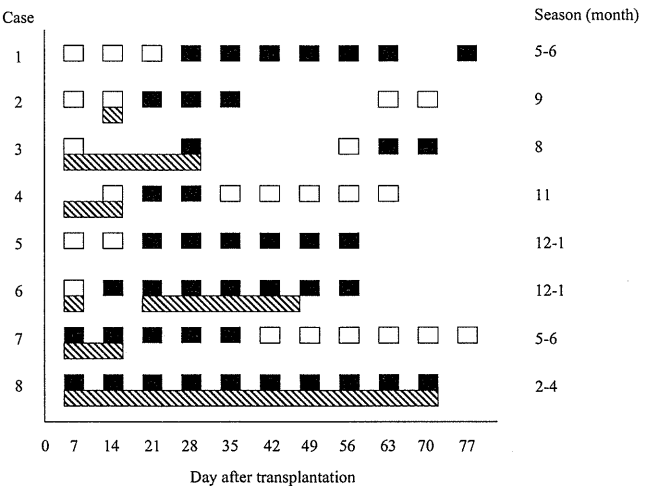

Fig. 1. Associations between rotavirus antigenemia (black boxes) and diarrhea (shaded bars) are shown. White boxes indicate antigenemia-negative serum samples.

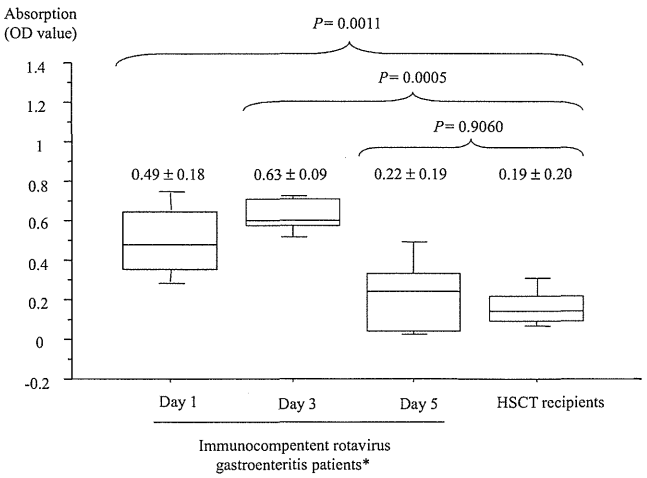

Fig. 2. Comparison of rotavirus antigen levels between immunocompetent rotavirus gastroenteritis patients and hematopoietic stem cell transplant (HSCT) recipients. *Days after onset of the illness. OD, optical density.

sera (1:4) to determine whether these positive samples contained low levels of rotavirus antigen. Forty-three antigen-positive serum samples and 40 randomly selected antigen-negative samples were used in this experiment (Fig. 3). The lower dilutions of antigen-positive serum samples (1:4) demonstrated significantly higher rotavirus antigen levels than the 1:16 diluted sera ($P < 0.0001$). However, no statistical difference was observed between 1:4 and 1:16 dilutions of antigen-negative samples ($P = 0.2733$). In addition, the immune response against rotavirus was also examined to confirm rotavirus infection; only one recipient with rotavirus antigenemia demonstrated a marked increase in rotavirus IgG antibody titers (Fig. 4).

Table 1 summarizes the results of statistical analyses identifying risk factors for rotavirus antigenemia in HSCT recipients. No statistical difference was seen between the ages of recipients with (7.4 ± 5.6 years) and without (8.3 ± 5.1 years) rotavirus antigenemia ($P = 0.652$). Moreover, neither gender ($P = 0.312$) nor underlying disease ($P = 0.368$) correlated with occurrence of rotavirus antigenemia. Of the 4 transplant-related variables, neither having received TBI ($P = 0.955$), having received ATG ($P = 0.080$), complications from acute GVHD ($P = 0.705$), nor type of graft source (related vs. unrelated; $P = 0.827$, related vs. cord blood; $P = 0.760$, related vs. peripheral blood stem cell transplant; $P = 0.206$) were associated with occurrence of rotavirus antigenemia. However, a patient who received a graft from an HLA-mismatched donor was at significant risk for rotavirus antigenemia ($P = 0.024$; odds ratio = 9.44) in comparison to patients who received a graft from an HLA-matched donor.

Discussion

Although it has been reported that rotavirus can cause severe clinical manifestations in immunocompromised transplant recipients (1–4), few studies have been conducted to elucidate the full spectrum of

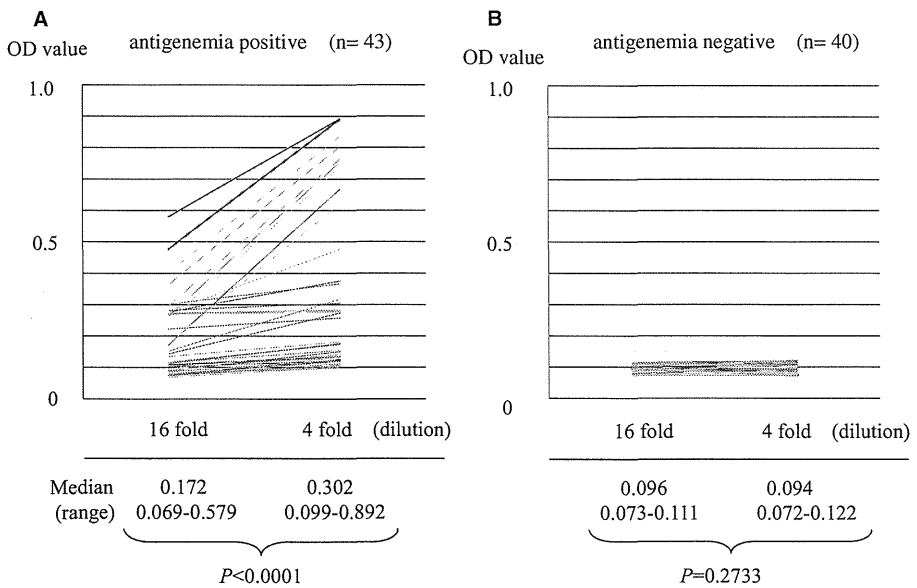

Fig. 3. Comparison of rotavirus antigen levels between 16-fold and 4-fold diluted serum samples to determine rotavirus antigenemia. OD, optical density. (A) Antigenemia positive (n = 43). (B) Antigenemia negative (n = 40).

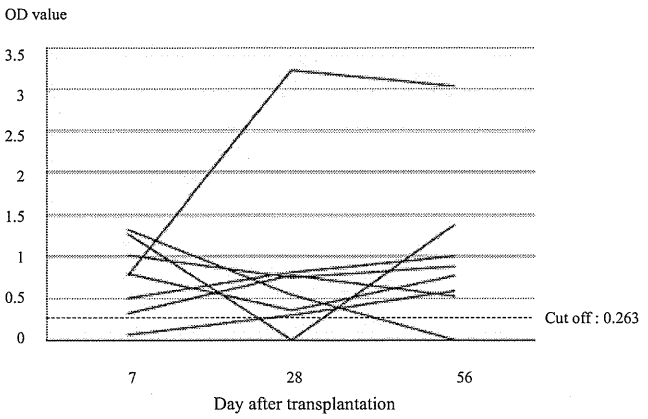

Fig. 4. Kinetics of anti-rotavirus immunoglobulin G (IgG) antibody titers in rotavirus antigenemia-positive patients (n = 8). IgG antibody titers were determined using enzyme-linked immunosorbent assay. OD, optical density.

rotavirus infection in transplant recipients (4, 19–21). In this study, rotavirus antigen was detected in 43/633 (6.8%) serum samples, and rotavirus antigenemia was observed in 8/62 (12.9%) transplant recipients. To the best of our knowledge, this is the first study that demonstrates rotavirus antigenemia in transplant recipients. Stelzmueller et al. (4) demonstrated that rotavirus infection was observed in 1.5% of solid organ transplant (SOT) recipients, and the highest frequency of rotavirus infections was observed in pediatric liver transplant recipients (52%) based on conventional rotavirus antigen detection analysis of stool samples. In addition, previous reports have identified rotavirus infection in 10–12% of pediatric BMT recipients (1, 20). Thus, although the clinical specimens used to detect rotavirus antigen were different, the frequency of antigenemia in our pediatric HSCT recipients was similar to previous studies (1, 20).

Both rotavirus-specific neutralizing antibodies and $CD8^+$ cytotoxic T cells have been shown to play important roles in terminating rotavirus infection (22–25). In addition to adaptive immunity, it has been suggested that the innate immune response is also important for protecting the host from rotaviral infection (13, 26). Thus, it is likely that rotaviral infection would be more severe in transplant recipients with severe immunosuppression than in immunocompetent children. Persistent rotavirus excretion in the stool has been documented in children with a congenital T-cell deficiency (27) and BMT recipients (5). We previously reported that the duration of rotavirus antigenemia in immunocompetent rotavirus gastroenteritis patients was short (11). Our present study suggests that rotavirus antigenemia persists for a longer period in transplant recipients, and this corresponds to rotavirus excretion in the stool (5).

To confirm specificity of the low levels of antigenemia in transplant recipients, antigen levels were measured in less diluted serum samples (Fig. 3). As less dilute (1:4) sera demonstrated statistically higher levels of antigen than that of a higher dilution (1:16), we considered that serum demonstrating low level of antigen really contained low levels of rotavirus antigen. Ray et al. (8) and Blutt et al. (9) reported that serum rotavirus antigen levels were negatively associated with rotavirus antibody levels in children with rotavirus antigenemia. Moreover, serum rotavirus antigen levels were significantly lower in patients who had a subsequent infection than in those with primary infection (7). The ages of the patients in this study ranged between 8 months and 23 years old. Thus, most of the recipients in this study could have previously had a primary rotavirus infection, which may have caused their lower levels of rotavirus antigen. Further detailed immunologic analysis of rotavirus infection is needed to clarify the pathogenesis of the characteristic kinetics of rotavirus antigenemia (low levels and long duration) observed in HSCT recipients.

Seven of 8 recipients with rotavirus antigenemia failed to demonstrate an antibody response against rotavirus. There are 2 possible explanations for such a low immune response rate. One is low immunogenicity of the low levels of antigenemia, and another is severe immunosuppression in transplant recipients. Although it is not clear whether positive antigenemia indicates active rotaviral infection, a serological assay is insufficient for monitoring rotavirus antigenemia in HSCT recipients.

It has been suggested that rotavirus can cause severe diarrhea (1, 3–5) and toxic megacolon (28), and may be confused with enteric GVHD (29), which results in significant morbidity in transplant recipients. As shown in Figure 1, persistent diarrhea and rotavirus antigenemia were concurrent in Cases 3, 6, 7, and 8. Meanwhile, rotavirus antigenemia persisted for a few weeks after the resolution of diarrhea in Cases 3, 6, and 7. Diarrhea was not observed in the 2 cases with persistent rotavirus antigenemia. Although it would be difficult to prove an association between rotavirus antigenemia and persistent diarrhea, because no complete examinations were carried out to exclude all other pathogens that would cause diarrhea, the current findings suggest that rotavirus antigenemia may be involved in the persistent diarrhea in HSCT recipients in some recipients. It was difficult to determine how many patients without rotavirus antigenemia had

diarrhea during the observation period following transplantation, because this study was a retrospective study using stored serum samples. Therefore, future prospective study is needed to elucidate the precise association between rotavirus antigenemia and diarrhea.

Moreover, asymptomatic rotavirus antigenemia was demonstrated in these patients. Asymptomatic rotavirus excretion in stool has also been reported in HSCT recipients, which could potentially make them index cases for nosocomial rotavirus infections (19). Thus, it is important to elucidate the ability of virus to be transmitted from asymptomatic rotavirus antigenemia patients. A prospective study that concurrently monitors rotavirus antigenemia and viral excretion in the stool is currently underway.

Notably, the timing after transplantation and occurrence of rotavirus antigenemia in non-endemic seasons in HSCT recipients were quite different from expected. According to a previous study based on detecting rotavirus antigen in stool, the median duration of rotavirus infection is 20 days after SOT (4). In our study, rotavirus antigenemia started within 4 weeks after transplant in all 6 cases except for Cases 7 and 8. Six of the 8 recipients were cared for in laminar airflow rooms at the beginning of rotavirus antigenemia. Although medical personnel may have caused nosocomial transmission, the likelihood of this possibility is very low, because standard precautions were thoroughly followed. Kang et al. (20) detected rotavirus antigen in the stool of an HSCT recipient at the time of pre-transplant screening. From a clinical standpoint, a large-scale molecular epidemiological study is needed to elucidate the route of viral transmission in HSCT recipients. In addition to the timing of rotavirus antigenemia, the seasons in which the rotavirus antigenemia occurred are another remarkable finding in this study. Although the endemic seasons in Japan for rotavirus infection are the winter and spring, rotavirus antigenemia was observed in the summer and fall in several cases (Fig. 1). Recently, a similar finding was demonstrated in SOT recipients (4). One possible mechanism for rotavirus antigenemia outside of its endemic season is the persistence of rotavirus infections in immunocompromised patients. Further human or animal studies are necessary to determine whether rotavirus can persistently infect a host.

We believe that this is the first report to demonstrate HLA mismatches as a significant risk factor for rotavirus antigenemia after HSCT, which is similar to Epstein–Barr virus (EBV) infection (30). ATG administration, which is another well-known risk factor for EBV infection after HSCT (30), tended to be a risk for rotavirus antigenemia, though it did not reach the level of statistical significance. Several characteristic factors have been suggested to pose significant risks for each viral infection in HSCT recipients. The present study suggests that the risk factors for rotavirus antigenemia are similar to those for EBV infection after HSCT. If the clinical significance of rotavirus antigenemia in HSCT recipients is confirmed, predictions about patients at high risk for rotavirus antigenemia would be important for improving their prognosis. Further clinical analysis of rotavirus antigenemia should be continued to determine the significance of rotavirus antigenemia on the morbidity or mortality of HSCT recipients.

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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

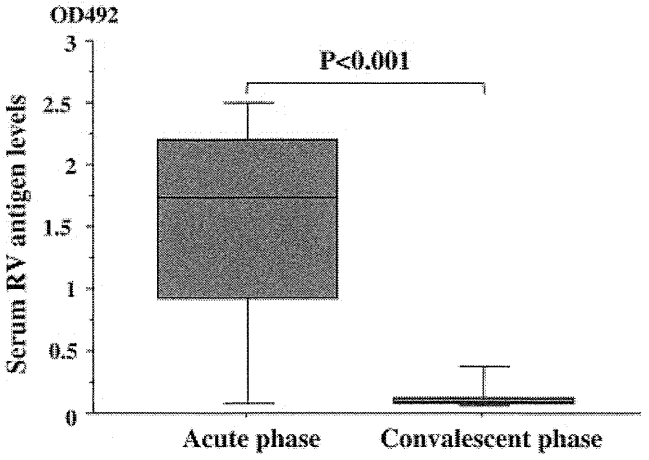

Fig. 1. Comparison of the serum RV antigen concentrations between the time of admission and discharge in patients with RV gastroenteritis (n = 40). Data are shown as box plots, where these boxes represent the first through third quartiles, the lines within these boxes represent the median, and the lines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

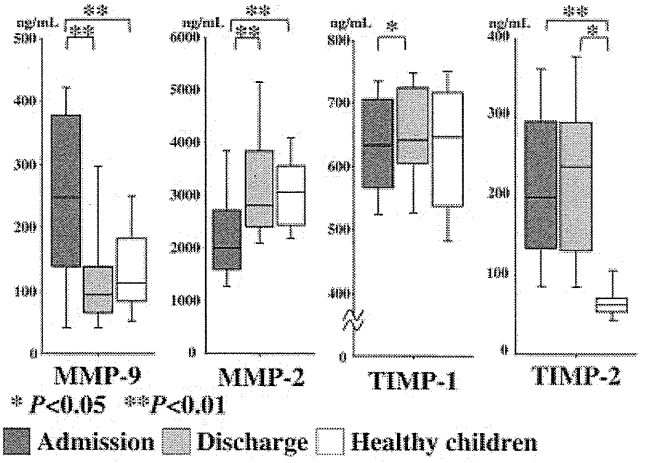

Fig. 2. Comparison of the serum MMP-2, -9, TIMP-1, and -2 concentrations among the time of admission and discharge in patients with RV gastroenteritis (n = 40), and healthy children (n = 31). Data are shown as box plots, where these boxes represent the first through third quartiles, the lines within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

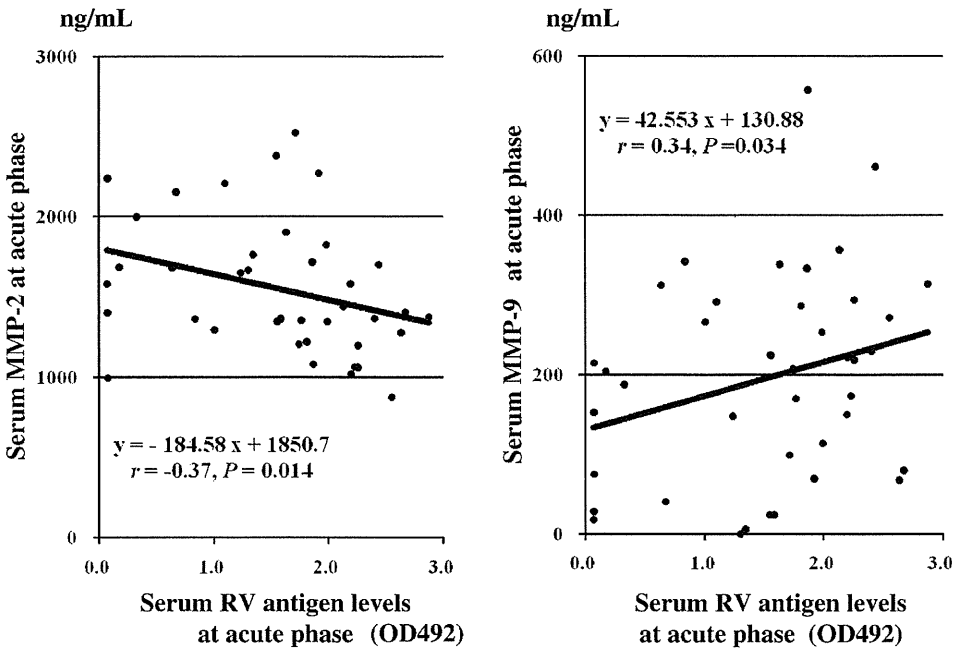

Fig. 3. Association between serum RV antigen and serum MMP concentrations. A weak (r = −0.37) but significantly (P = 0.014) negative association was observed between MMP-2 concentrations and RV antigenemia. A weak (r = 0.34) but significantly (P = 0.034) positive association was also observed between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

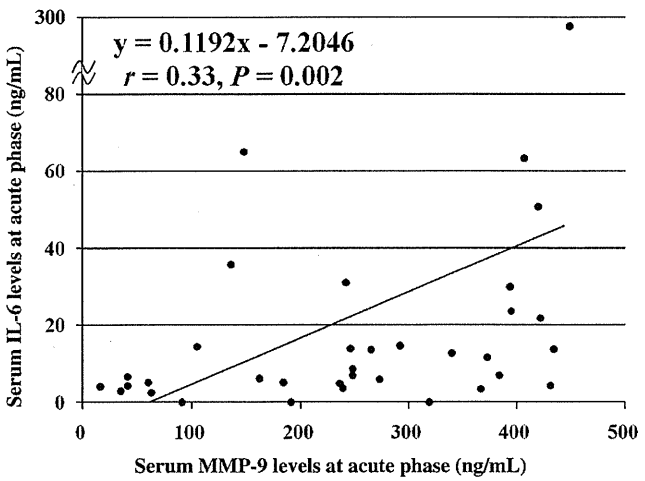

Fig. 4. Association between serum cytokines and MMP concentrations. A weak (r = 0.33) but significantly (P = 0.002) positive association was observed between IL-6 and MMP-9 concentrations.

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.

In accordance with our hypothesis regarding an association between MMPs and RV antigenemia, a positive correlation was identified between MMP-9 and RV antigenemia, while a reverse correlation was found between MMP-2 and RV antigenemia. These data suggest that these two MMPs have important roles in the pathogenesis of RV antigenemia. Since it has been suggested that the level of RV antigenemia is associated with disease severity [Ramani et al., 2010], an association between the serum MMPs concentrations and disease severity should be analyzed in a future study. Additionally, it is important to elucidate which cells are responsible for the local production of the MMPs in RV gastroenteritis, and the regulatory mechanisms for their production. Epithelial cells or infiltrating mucosal lymphocytes have been suggested to synthesize MMP-9 and MMP-2 in inflamed tissues [Castaneda et al., 2005]. As it is difficult to obtain biopsy specimen from RV gastroenteritis patients, pathological analysis of RV infected animals would be helpful to determine which cells in the intestinal tissue secret the MMPs. Since MMPs knockout mice have been useful to analyze role of MMPs in the pathogenesis of IBD [Garg et al., 2006; Munoz et al., 2009], we propose similar experiments to test our hypothesis.

The enzymatic activity of MMPs is controlled by TIMPs [Brew and Nagase, 2010]. Thus, the balance of MMPs and TIMPs within the tissue may be important for the regulation of local tissue damage. Two TIMPs (TIMP-1 and TIMP-2) were investigated in this study. TIMP-1 concentrations were found to be similar in patients with RV gastroenteritis and healthy controls. Meanwhile, TIMP-2 concentrations in RV gastroenteritis patients were significantly increased at the time of admission and discharge compared to healthy controls suggesting that TIMP-2 might be important for the pathogenesis of RV antigenemia. Although TIMP-1 concentration has been shown to increase in IBD patients [Louis et al., 2000; Arihiro et al., 2001; Wiercinska-Drapalo et al., 2003; Kapsoritakis et al., 2008], it has been demonstrated that TIPM-2 concentration remained normal in these patients [Kapsoritakis et al., 2008]. Thus, TIMPs expression may be differentially regulated in RV infection and IBD.

As several cytokines and chemokines have been suggested to be associated with upregulation of MMPs synthesis [Lotz and Guerne, 1991; Saren et al., 1996; Kusano et al., 1998], the correlation between serum cytokine concentrations and MMP-9 or MMP-2 was analyzed in this study. Although many reports have demonstrated a positive correlation between TNF-α and MMP synthesis [Pender et al., 1997; Pender et al., 1998; Louis et al., 2000], no remarkable association between these two biomarkers was found in the present study. It has been suggested that RV infection activates dendritic cells in Peyer's patches resulting in upregulation of TNF-α expression [Lopez-Guerrero et al., 2010]. Therefore, an association between locally produced MMP and TNF-α should be examined to elucidate the precise role of TNF-α in the regulation of MMPs in RV infection. Meanwhile, significant positive association between IL-6 and MMP-9 was identified. It has been suggested that IL-6 released from fibroblasts was responsible for secretion of MMP-9 from dendritic cells in in vitro dermal microenvironment model [Saalbach et al., 2010]. Similar pathophysiological mechanisms should be evaluated in the intestinal tissue of RV gastroenteritis patients.

In conclusion, these results suggest that MMP-9 and MMP-2 play important role in causing RV antigenemia as factors for attack and protection, respectively. Additionally, TIMP-2 might be important for the pathogenesis of RV antigenemia as a controller of MMPs. However, no other cohort such as Norovirus gastroenteritis patients was included in this study, further cohorts analysis is necessary to determine whether kinetics of MMPs and TIMPs were specific for RV gastroenteritis or not.

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Journal of Medical Virology 84:832–838 (2012)

Phylogenetic and Computational Structural Analysis of VP7 Gene of Group A Human Rotavirus G1P[8] Strains Obtained in Sapporo, Japan From 1987 to 2000

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Many studies indicate that G1P[8] genotypes are the most prevalent rotavirus strains worldwide. Although two vaccines have been licensed and their value proven in many countries, continuous surveillance for genetic evolution of circulating rotavirus strains before and after the introduction of the vaccines is desirable. G and P typing were carried out on all field strains isolated during 1987–2000 in Sapporo, Japan. Phylogenetic analysis for the VP7 gene of rotavirus G1P[8] strains was performed. Amino acid substitutions were mapped on the predicted three-dimensional VP7 protein image. G1P[8] genotype predominated. One hundred thirteen strains with G1P[8] genotype were analyzed. Phylogenetic studies of the VP7 gene classified these strains into three lineages. The mean estimated substitution rate was 7.25×10^{-4} nucleotide substitutions per site per year. One predominant lineage contained the mutant strains which had VP7 amino acid substitutions at residue 91 and 212 that is in the neutralization domains. They were estimated to locate in or near intersubunit boundary of VP7 trimer. It is suggested that the most prevalent G1P[8] lineage strains in Sapporo obtained some survival advantages by changing the neutralization domains of VP7. ***J. Med. Virol. 84:832–838, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: human rotavirus; VP7; G1P[8]; substitution rate; protein structure prediction

INTRODUCTION

Rotaviruses are the leading cause of severe diarrheal disease of infants and young children worldwide including Japan [Estes and Kapikian, 2007; Nakanishi et al., 2009]. It is estimated that each year rotavirus gastroenteritis causes more than 600,000 deaths of infants and children under 5 years of age, mainly in developing countries [Parashar et al., 2009]. Rotaviruses are classified into seven groups (A–G) of which group A accounts for a large majority of rotavirus infections.

Rotaviruses have a triple layered-structure, which contains 11 segments of double-stranded (ds) RNA. The viral genome encodes six structural proteins (VP) and six nonstructural proteins. Among them the outer capsid proteins, VP7 and VP4, are key antigens used to distinguish strains [Estes and Kapikian, 2007]. The VP7 and VP4 genes provide G and P genotypes, respectively, and at least 12 G genotypes and 15 P genotypes have been identified in human rotavirus strains [Maes et al., 2009; Patel et al., 2011]. Although gene reassortment could theoretically lead to many G and P combinations, mainly five strains are identified, namely, G1P[8], G2P[4], G3P[8], G4P[8], and G9P[8]. Several studies indicate that rotaviruses of the G1P[8] genotype are the most important strains worldwide [Gentsch et al., 2005; Santos and Hoshino, 2005; Annarita et al., 2010].

Recently two rotavirus vaccines have been introduced in many countries [Ruiz-Palacios et al., 2006; Vesikari et al., 2006]. Both have been shown to be efficacious and safe in randomized controlled trials [Linhares et al., 2008; Armah et al., 2010; Madhi et al., 2010; Zaman et al., 2010].

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To develop safe and effective vaccines, an understanding of potential rotavirus antigenic transition is essential. Two recent long-term analyses have reported the diversity of the outer capsid genes of G1P[8] rotaviruses and revealed important amino acid substitutions [Arista et al., 2006; Banyai et al., 2009]. Although there were some short term epidemiological studies on G1 or G3 rotavirus in Asia [Phan et al., 2007a; Mitui et al., 2011], there has been no longitudinal epidemiological analysis—more than 10 years—about G1P[8] strains performed in Japan. To extend a knowledge of G1P[8] strains and the significance of amino acid substitutions prior to vaccine introduction, the mutation sites and the change of characteristics were investigated in the most common rotavirus strain G1P[8] collected in Sapporo, Japan during 1987–2000.

MATERIALS AND METHODS

Specimens, RNA Extraction, Reverse Transcription, and Nucleotide Sequence

Stool samples were obtained from 2,496 hospitalized children with acute gastroenteritis in Sapporo, Japan during 1987–2000. Three hundred ninety-one stool samples were identified to be rotavirus positive by immunochromatography using DIPSTICK "Eiken" ROTA® kit (SA Scientific, San Antonio, TX). The stool samples were diluted with phosphate buffered saline (pH 7.4) to 20% suspensions and clarified by centrifugation at 10,000*g* for 20 min twice.

The viral dsRNA was extracted from 20% stool suspensions using TRIzol® LS reagent (Invitrogen, Carlsbad, CA) and denatured in 33% dimethyl-sulfoxide. The dsRNA was converted into cDNA using SuperScript® Reverse Transcriptase II (Invitrogen) and then PCR amplified with Taq® DNA polymerase (Promega, Madison, WI). KU strain (D16343) and distilled water were used as positive and negative control, respectively. The methods of G and P typing have been previously described [Gouvea et al., 1990; Gentsch et al., 1992] except for using Beg9s (GGCTTTAAAAGAGAGAATTT) and End9s (GGTCACATCATACAATTCTA) primers instead of Beg9 and End9 primers to increase sensitivity. The amplicons were analyzed by 1% SeaKem® LE Agarose (Lonza Rockland, Rockland, ME) gel electrophoresis and visualized by staining with ethidium bromide.

The VP7 gene amplicons determined G1P[8] strains were gel purified by Wizard® SV Gel and PCR Clean-Up System (Promega) and sequenced with the BigDye® Terminator ver3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). In addition to Beg9s and End9s, primers used in sequencing were 5′VP7 (TTGCTTCAGTTGGATAATAC) and 3′VP7 (ACTACGACATGTACTATTCG) for N and C terminal domain sequencing, respectively. These primers were designed for the present study. The dye terminator was removed by using PERFORMA® DTR Gel Filtration Cartridges (Edge BioSystems, Gaithersburg, MD) and sequences were obtained with ABI PRISM® 3100 Genetic Analyzer (Applied Biosystems).

Phylogenetic Analysis and Bayesian Evolutionary Analyses

Nucleotide alignments were edited in BioEdit ver7.0.9.0 [Hall, 1999] and MEGA 4 [Tamura et al., 2007] using ClustalW. Maximum likelihood phylogenetic tree was constructed using PhyML ver3.0 [Guindon and Gascuel, 2003] employing the Hasegawa–Kishino–Yano substitution model (HKY85) and gamma-distributed rate variation among sites. Tree reliability was estimated by the approximate likelihood ratio test method.

Rates of evolution were estimated using the Bayesian Markov chain Monte Carlo method implemented in BEAST ver1.5.4 [Drummond and Rambaut, 2007]. The model of nucleotide substitution that best fitted the data was determined using jModelTest ver0.1.1 [Guindon and Gascuel, 2003; Posada, 2008]. Consequently the date were analyzed using the HKY or general time reversible (GTR) nucleotide substitution models, with or without a gamma-distributed among-site rate variation with four rate categories plus invariant positions depending on the model. Substitution rates were estimated using a lognormal relaxed clock model to accommodate variation in substitution rates among different branches [Drummond et al., 2006]. Analyses were run for 20 million states and sampled every 1,000 states to get adequate effective sample sizes at least over 100. The convergence of the parameters to a stationary distribution was assessed with the Tracer program (http://tree.bio.ed.ac.uk/software/tracer).

In this study 130 human rotavirus VP7 sequences of the G1 references were retrieved from GenBank. They had complete codons or partial codons including over 95% ORF sequences with known collection dates between 1974 and 2009. To reduce excessive computational load, closely related sequences collected at the same country and year were manually removed without compromising the genetic or geographical heterogeneity of each alignment, namely one sequence each country, year, and lineage/sub-lineage.

Computational Analysis of Protein Structure Prediction

The possible three-dimensional VP7 structure of one Sapporo G1P[8] strain, 87SA1133 (GU358424), was estimated using the protein structure homology-modeling server SWISS-MODEL [Arnold et al., 2006]. Rhesus rotavirus structure (pdb#3fmg, G3P[3]) was used as a template [Aoki et al., 2009; Chen et al., 2009]. The quality of this model was evaluated by using the Verify 3D program [Bowie et al., 1991; Luthy et al., 1992]. The putative structure image was generated by using UCSF Chimera-Molecular Modeling System [Pettersen et al., 2004].