Pathophysiological analysis of five severe cases with rotavirus infection

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Introduction: Rotavirus infection is usually localized to the intestine. Severe cases with extraintestinal symptoms are rare, and its pathogenesis remains unclear.

Case presentation: We report severe rotavirus infections in five previously healthy, unvaccinated children that occurred in the 2011 season in Aichi Prefecture, Japan. All cases had short histories of recurrent diarrhoea and vomiting that preceded extraintestinal symptoms. Two cases with prolonged seizures and unconsciousness were diagnosed with encephalitis/encephalopathy; one case died and the other survived with severe neurological sequelae in spite of intensive treatment including anticytokine therapy. Another two cases had rapidly worsening cardiopulmonary dysfunction and seizures, and they died a few hours after admission despite cardiopulmonary support. The last case was transferred to hospital in cardiopulmonary arrest and resuscitation was unsuccessful. All cases had positive stool samples for rotavirus RNA, and the rotavirus antigen was also detected in the serum of four cases, but not in the cerebrospinal fluid (CSF). Serum IL-6 and IL-10 levels were significantly higher in the severe cases than in children with rotavirus gastroenteritis without extraintestinal symptoms, and serum IL-10 levels were significantly higher than CSF levels in the severe cases.

Conclusion: Cytokines appear to mediate the pathogenesis of severe rotavirus infection with extraintestinal involvement. In conclusion, these cases highlight the need for a routine rotavirus immunization programme.

Keywords: anticytokine therapy; encephalitis; encephalopathy; fatal infection; rotavirus; sudden unexpected death.

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Introduction

Rotavirus infection is a common cause of gastroenteritis among children. Severe dehydration caused by rotavirus-induced vomiting and diarrhoea can be fatal, and rotavirus infection causes approximately 350 000 to 590 000 deaths year⁻¹, primarily in developing countries (Ramig, 2004). Although rotavirus-related deaths have declined with improving clinical management, they continue to occur even in developed countries. Prior to

implementation of routine rotavirus immunization, rotavirus gastroenteritis annually caused an estimated 14–60 deaths among children in Europe and the USA (Parashar & Glass, 2006; Van Damme *et al.*, 2006). Infection is mostly localized to the intestine, but severe cases can have extraintestinal involvement, including encephalopathy and sudden death (Ioi *et al.*, 2006; Medici *et al.*, 2011; Minato *et al.*, 1992; Nakano *et al.*, 2011; Salmi *et al.*, 1978). The pathogenesis of these severe infections remains unclear.

Herein, we report five cases of very severe rotavirus infection that occurred during the 2011 season in Aichi Prefecture, Japan and their laboratory characteristics.

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CSF, cerebrospinal fluid; CT, computed tomography; GCS, Glasgow Coma Score; RT-PCR, reverse-transcription PCR.

Case Report

The clinical characteristics of the five cases with severe rotavirus infection are shown in Table 1. No case had been vaccinated against rotavirus infection. No case had amino or organic acids metabolic abnormalities, nor positive family history. All cases were treated with oral or intravenous hydration, and all bacterial cultures were sterile [cerebrospinal fluid (CSF), blood and urine]. CSF specimens from all patients were negative for herpes simplex virus and enterovirus by PCR and by reverse-transcription PCR (RT-PCR), respectively.

Case 1

A previously healthy 18-month-old Japanese girl with a 2 day history of vomiting and fever was admitted with prolonged generalized convulsions following unconsciousness on the second day of her illness. Her Glasgow Coma Scale (GCS) score was 4 (E2, V1, M1), her pupillary light reflex was prompt without anisocoria, and her skin turgor was decreased. Vital signs were as follows: temperature, 40.0 °C; pulse, 180 min⁻¹; respiration, 20 min⁻¹; and blood pressure, 91/70 mmHg. Initial laboratory examinations revealed the following: haemoglobin, 13.0 g dl⁻¹; leukocyte count, $2.3 \times 10^4 \mu\text{l}^{-1}$ (88 % neutrophils); platelet count, $22.2 \times 10^4 \mu\text{l}^{-1}$; aspartate aminotransferase (AST), 144 IU l⁻¹; alanine aminotransferase (ALT), 60 IU l⁻¹; urea, 36.1 mg dl⁻¹; creatinine, 0.4 mg dl⁻¹; blood glucose, 8 mg dl⁻¹; NH₃, 128 µg dl⁻¹; pH, 7.18; HCO₃, 18.7 mEq l⁻¹; and BE, -22.7 mEq l⁻¹. Electrolytes and CSF were normal. Cerebral magnetic resonance imaging with diffusion-weighted sequence demonstrated bilateral areas of increased signal in the subcortical white matter. An electroencephalograph showed the burst-suppression pattern of low voltage activity. She was diagnosed with acute encephalopathy and treated with steroid pulse therapy, immunoglobulin therapy, and anticonvulsants. Despite intensive care, her seizures were intractable, without recovery of her level of consciousness, and she eventually died on day 9.

Case 2

A previously healthy 12-month-old Japanese boy presented with a 4 day history of vomiting, diarrhoea and fever that

progressed to prolonged generalized convulsions and unconsciousness on day 4. He was initially treated with oral/intravenous hydration. On admission, his GCS score was 6 (E4, V1, M1), and his vital signs were as follows: temperature, 39.7 °C; pulse, 200 min⁻¹; respiration, 60 min⁻¹; and blood pressure, 87/40 mmHg. His pupillary light reflex and skin turgor were normal. Initial laboratory examination revealed the following: haemoglobin, 13.1 g dl⁻¹; leukocyte count, $1.8 \times 10^4 \mu\text{l}^{-1}$ (74 % neutrophils); platelet count, $39.1 \times 10^4 \mu\text{l}^{-1}$; AST, 78 IU l⁻¹; ALT, 29 IU l⁻¹; urea, 48.7 mg dl⁻¹; creatinine, 1.6 mg dl⁻¹; blood glucose, 217 mg dl⁻¹; NH₃, 152 µg dl⁻¹; pH, 7.10; PaO₂, 90 mmHg; HCO₃, 4.7 mEq l⁻¹; BE, -22.7 mEq l⁻¹; and Na, 152 mEq l⁻¹. CSF findings demonstrated a mild pleocytosis (21 cells µl⁻¹) and a mild protein elevation (168 mg dl⁻¹). Cerebral magnetic resonance imaging with diffusion-weighted sequence demonstrated bilateral areas of increased signal in the subcortical white matter. An electroencephalograph showed a generalized high-voltage slow wave pattern (1.5 Hz). The boy was diagnosed with acute encephalitis and treated with steroid pulse therapy, immunoglobulin therapy, plasmapheresis, and anticonvulsants. After 50 days of hospitalization, he was discharged with severe psychomotor retardation.

Case 3

A previously healthy 11-month-old Japanese boy presented with a 3 day history of vomiting, diarrhoea and fever, followed by intermittent convulsive seizures and progressive disturbance of consciousness on day 3. On admission, his GCS score was 4 (E2, V1, M1), and his vital signs were as follows: temperature, 39.0 °C; a weak pulse over the carotid artery, 160 min⁻¹; and respiratory rate, 54 min⁻¹ with retractions. Skin turgor was decreased. Initial laboratory examination revealed the following: haemoglobin, 6.8 g dl⁻¹; leukocyte count, $3.8 \times 10^4 \text{ cells } \mu\text{l}^{-1}$ (52 % neutrophils); and platelet count, $113 \times 10^4 \mu\text{l}^{-1}$; AST, 565 IU l⁻¹; ALT, 439 IU l⁻¹; urea, 68.1 mg dl⁻¹; creatinine, 2.7 mg dl⁻¹; and blood glucose, 31 mg dl⁻¹. There was a mild pleocytosis (18 cells µl⁻¹) in his CSF, and brain computed tomography (CT) was normal. Despite intensive care, his clinical status deteriorated further, and he suffered

Table 1. Clinical characteristics of the five cases with severe rotavirus infection

Case no.	Age/sex	Disease	Prognosis	Day of admission	Cardiorespiratory distress	Neurological symptoms	Abnormal CSF finding	Abnormal brain imaging	High fever
1	18 months/F	Encephalopathy	Died	2	—	+	—	+	+
2	12 months/M	Encephalitis	Severe sequelae	4	—	+	+	+	+
3	11 months/M	SUD	Died	3	+	+	+	—	+
4	3 years/F	SUD	Died	2	+	+	+	—	+
5	14 months/M	SUD	Died	2	Unclear	—	+	—	+

SUD, sudden unexpected death; M/F, male/female.

a cardiopulmonary arrest. Immediate resuscitation was unsuccessful, and he was pronounced dead an hour after admission.

Case 4

A previously healthy 3-year-old Japanese girl presented with a 2 day history of vomiting and diarrhoea that progressed to intermittent episodes of convulsive seizures and abnormal behaviour (strange voice) with a progressive disturbance of consciousness on day 2. On arrival, her GCS score was 3 (E1, V1, M1), there was no spontaneous breathing, and her blood pressure was unobtainable. Initial laboratory examination revealed the following: haemoglobin, 15.8 g dl⁻¹; leukocyte count, 12.2 × 10⁴ μl⁻¹; platelet count, 11.3 × 10⁴ μl⁻¹; AST, 61 IU l⁻¹; ALT, 28 IU l⁻¹; urea, 97.8 mg dl⁻¹; creatinine, 3.8 mg dl⁻¹; and blood glucose, 2 mg dl⁻¹. There was a mild pleocytosis (9 cells μl⁻¹) in her CSF, and brain CT was normal. Immediate resuscitation was unsuccessful, and she was pronounced dead an hour after admission.

Case 5

A previously healthy 14-month-old Japanese boy presented with vomiting and fever, and he was treated with oral hydration. A day later, he was transferred to our hospital with poor reactivity and asthenia. On arrival, he was in cardiopulmonary arrest, and his initial laboratory results were as follows: haemoglobin, 10.3 g dl⁻¹; leukocyte count, 1.0 × 10⁴ μl⁻¹; platelet count, 50.9 × 10⁴ μl⁻¹; AST, 2047 IU l⁻¹; ALT, 892 IU l⁻¹; urea, 34.7 mg dl⁻¹; creatinine, 0.2 mg dl⁻¹; blood glucose, 214 mg dl⁻¹; pH, 6.97; HCO₃, 15.7 mEq l⁻¹; and Na, 128 mEq l⁻¹. There was a mild pleocytosis (26 cells μl⁻¹) in his CSF, and brain CT was normal. Cardiopulmonary resuscitation was attempted for 1 h, before ventilator support was discontinued and he was pronounced dead.

Investigations

On admission, serum, CSF, and stool samples were collected from all patients and stored at -70 °C until

virological analysis. As a control group, the cytokine concentrations in the blood of 39 children admitted with mild rotavirus gastroenteritis, without extraintestinal symptoms, were also measured. The patients' guardians consented to their participation in this study. This study was approved by the review boards of Fujita Health University (no. 11-235). Statistical analyses were conducted using StatView version 5.0 software (SAS Institute). The Mann-Whitney *U*-test was used for comparisons of serum cytokine levels between the cases with severe rotavirus infection and the control cases. The Wilcoxon signed-rank test was used for comparisons of CSF and serum cytokine levels in the cases with severe rotavirus infection. *P*-values <0.05 were considered statistically significant.

The results of the virological investigations in the five cases with severe rotavirus infection are summarized in Table 2. Rotavirus antigen was measured in the serum, stool and CSF of the severe cases using an ELISA that detects the VP6 antigen of the virus, as previously described (Sugata *et al.*, 2008). ELISA failed to detect the rotavirus antigen in the CSF of any case, but did detect it in the stool and serum of four of the severe cases. RNA was extracted from the serum, CSF and 10 % (w/w) faecal specimens in PBS (pH 7.2) of the severe cases using a QIAamp Viral RNA Mini kit (Qiagen). Rotavirus RNA was detected by RT-PCR using two sets of VP6 gene primers commonly reactive to group A rotavirus strains, as previously described (Taniguchi *et al.*, 1992). RT-PCR for detecting VP6 RNA was positive for the stools from all five severe cases, as well as for the serum and CSF of three and two cases, respectively. To determine the rotavirus G (VP7) and P (VP4) genotypes, RNA extracted from faecal specimens was subjected to semi-nested RT-PCR using different sets of G- and P-type-specific primers (Taniguchi *et al.*, 1992; Wu *et al.*, 1994). In PCR genotyping, rotavirus strains from two and one cases were found to be G3P[8] and G1P[8], respectively. The concentrations of IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α were determined using flow-cytometric bead array according to the manufacturer's protocol (Becton Dickinson) (Morgan *et al.*, 2004). The normal serum concentration ranges of these molecules in healthy volunteers are as follows: IL-1β,

Table 2. Rotavirus detection and cytokine concentrations in the five cases with severe rotavirus infection on admission

Case no.	Lab detection of rotavirus antigen/RNA			Cytokine concns in serum/CSF (pg ml ⁻¹)						G and P genotype
	Stool	Serum	CSF	IL-1β	IL-6	IL-8	IL-10	IL-12p70	TNF-α	
1	+/+	+/+	ND/-	0/0	41/185	208/4458	14/0	6.7/0	0/0	G1P[8]
2	+/+	+/+	ND/+	283/0	7408/950	54 763/7513	220/10.1	0/0	13.9/1.9	G3P[8]
3	+/+	+/-	-/-	0/0	616/24	511/185	228/1.3	0/1.3	0/1.4	G3P[8]
4	+/+	+/+	-/-	0/0	179/72	160/544	190/1.2	0/0	0/0	UD
5	-/+	-/-	ND/+	0/0	48/11	49/135	9.1/1.5	7.8/0	0/0	UD

SUD, sudden unexpected death; ND, not done; UD, undetermined.

Fig. 1. Comparison of the serum cytokine concentrations between the clinically severe cases and the clinically mild controls with rotavirus infection. Filled circles, patients with severe rotavirus infection ($n=5$); open triangles, patients with rotavirus gastroenteritis without extraintestinal symptoms ($n=39$). Bars indicate mean $\pm$ SD/SE.

0.0 ± 0.0 pg ml⁻¹; IL-6, 1.1 ± 4.2 pg ml⁻¹; IL-8, 12.3 ± 4.2 pg ml⁻¹; IL-10, 3.9 ± 4.8 pg ml⁻¹; IL-12p70, 0.0 ± 0.0 pg ml⁻¹; and TNF- α , 0.0 ± 0.0 pg ml⁻¹ (Yoshikawa *et al.*, 2011). The serum cytokine levels were compared between the severe and control rotavirus infection groups (Fig. 1). Serum IL-6 and IL-10 levels were significantly higher in the severe cases than in the control cases. No other cytokine levels were significantly different between the two groups. Then, these cytokine levels were compared between serum and CSF obtained from the five severe cases. The serum IL-10 levels were significantly higher than the CSF IL-10 levels in the severe cases, but the levels of IL-6 and IL-8 were not significantly different (Fig. 2).

Discussion

Neurological manifestations such as encephalitis, encephalopathy or seizures occur in approximately 2–3 % of patients with rotavirus gastroenteritis (Lynch *et al.*, 2001; Schumacher & Forster, 1999). Rotavirus encephalopathy is a rare disease that frequently results in severe sequelae. No routine surveillance system is available for rotavirus-related deaths in Japan; however, the recent nationwide questionnaire survey demonstrated that rotavirus-associated encephalitis/encephalopathy and sudden unexpected death are estimated to account for 44 cases (morbidity 12.1 %) and 4.9 cases, respectively, in Japan per year (Kawamura *et al.*, 2014). We encountered five

very severe cases of rotavirus infection during the 2011 season, among which one survived and four died. During that season, the number of cases admitted with rotavirus gastroenteritis did not increase, but we had an annual mortality rate higher than that estimated for rotavirus-related death (1.4 cases year⁻¹) in the Aichi Prefecture (14 % of the total population of Japan). In addition, because all of our cases occurred in previously healthy children, we analysed the severe cases in detail.

The pathophysiology of rotavirus-related deaths is poorly understood. In the 1970s, cardiac arrest due to dehydration and electrolyte imbalance was the primary cause (Kilgore *et al.*, 1995). Although it continues to occur, improved clinical management has dramatically reduced the number of deaths due to diarrhoea (Ramig, 2004). However, it is unclear whether dehydration or other pathology is the primary reason for death in rotavirus infection. Despite early treatment with oral rehydration, under the supervision of the family paediatrician at home, we observed worsening and severe dehydration due to gastrointestinal symptoms in three of the severe cases; however, it was not present in the physical findings of the other two cases. It is interesting to note that four cases had neurological symptoms or abnormal CSF findings and two cases were diagnosed with encephalopathy/encephalitis. These findings indicate that severe cases of rotavirus infection can be associated with extraintestinal involvement in the absence of physical findings of dehydration.

Fig. 2. Serum and CSF interleukin concentrations (IL-10, IL-6 and IL-8) in the five patients with severe rotavirus infection. Dotted lines link samples from the same child on the same day.

Few reports have investigated the G and P genotypes of severe rotavirus infection presenting with encephalopathy/encephalitis or sudden death. In our cases, we identified the G1P[8] and G3P[8] genotypes, which are dominant both in Japan and worldwide. No relationship between G and P genotypes and pathogenicity has been shown concisely, and no rotavirus G and P genotypes were found to be specific for central nervous system involvement (Ushijima *et al.*, 1994). It appears that host-related factors are more strongly responsible for severe or fatal disease than virus-related factors.

We detected rotavirus antigenaemia in four out of the five cases. There have been few reports describing such antigen detection in severe or fatal rotavirus infection. Blutt *et al.* (2007) reported that antigenaemia is predictive of viraemia and can be used as a marker of extraintestinal rotavirus involvement. Rotavirus antigen and RNA have been detected in endothelial cells (Cioc & Nuovo, 2002; Morrison *et al.*, 2001), the central nervous system (Nakano *et al.*, 2011), and the liver (Gilger *et al.*, 1992; Nakano *et al.*, 2011) of autopsy cases with fatal rotavirus infection. Because permission for autopsy was not granted in our fatal cases, we could not investigate the relationship between antigenaemia and extraintestinal involvement.

We were also unable to detect rotavirus antigens in any of the CSF samples in our series though RNA was detected in CSF samples of two cases, which is consistent with previous reports (Liu *et al.*, 2009; Nakagomi & Nakagomi, 2005). These reports also failed to identify rotavirus antigens in the CSF of children with rotavirus gastroenteritis and neurological manifestations that were positive for rotavirus RNA by RT-PCR. This suggests that the level of rotavirus antigen in CSF is less than that in the serum. Consequently, we suggest that the main pathogenesis of neurological complications in severe rotavirus infection

results from indirect mechanisms, such as diffuse endothelialitis (Morrison *et al.*, 2001), cytokine storm (Ioi *et al.*, 2006), and nitrogen oxides (Kawashima *et al.*, 2004), and not through the direct invasion of rotavirus into the central nervous system.

Many reports show that cytokines are important in the pathophysiology of encephalopathy and sudden death (Ioi *et al.*, 2006; Morita *et al.*, 2005; Vennemann *et al.*, 2012). Few reports have comprehensively described the cytokine profiles in severe cases with rotavirus infection, though some case reports exist (Ioi *et al.*, 2006). In our severe cases, the serum levels of two of the six cytokines measured (IL-6 and IL-10) were significantly elevated. Moreover, the CSF IL-10 concentration was significantly lower than the serum IL-10 level, whereas the IL-6 and IL-8 concentrations in CSF were not. IL-6 is considered a marker of pro-inflammatory cytokine activation, and IL-10 is an immunosuppressive and anti-inflammatory cytokine that decreases the production of IL-6 (de Waal Malefyt *et al.*, 1991). Several reports have shown higher serum IL-6 and IL-10 levels in children with rotavirus gastroenteritis than in healthy children, and patients with fever had significantly higher serum IL-6 levels (Chen *et al.*, 2012; Jiang *et al.*, 2003). We suggest that elevation of serum IL-6 probably indicates severe systemic inflammation in the blood and that serum IL-10 is induced in response to the production of IL-6. Thus, the absence of CSF IL-10 elevations may be associated with acute exacerbations without sufficient feedback time to elevate CSF IL-6. Influenza virus is another major pathogen associated with encephalopathy and sudden unexpected death, and studies have shown elevated serum IL-6 and IL-10 levels in severe influenza encephalopathy (Ichiyama *et al.*, 2003, 2004; Kawada *et al.*, 2003). Our findings suggest that IL-6 and IL-10 are important in the pathogenesis of fatal or severe rotavirus infection. More research into the precise

mechanisms of cytokine-induced aggravation of rotavirus infection is necessary. On the basis of the assumed pathophysiological relationship, we could assume that anticytokine and anti-inflammatory therapies are reasonable; however, steroid and immunoglobulin therapies were not effective in our severe cases. The establishment of clearly effective therapeutic or prevention strategies is warranted.

Since 2006, two safe and effective rotavirus vaccines have been licensed in over 100 countries worldwide, Rotarix (GlaxoSmithKline) and RotaTeq (Merck). In Japan, Rotarix and RotaTeq were approved in November 2011 and in July 2012, respectively, and although all of our severe cases were age-eligible to receive rotavirus vaccine, none had been vaccinated prior to the implementation of routine rotavirus immunization. It is not clear whether rotavirus vaccination reduces the rates of extraintestinal complications and sudden unexpected deaths. Given the striking reduction in symptomatic rotavirus infection and hospitalizations among vaccinated children, severe or fatal cases similar to ours could have been prevented.

In conclusion, these five cases demonstrate that cytokines may mediate the pathogenesis of severe rotavirus infection with extraintestinal involvement and that very severe rotavirus infection can occur in advanced countries, despite early treatment with appropriate therapy, highlighting the need for the implementation of a routine rotavirus immunization programme.

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