ORIGINAL ARTICLE

Antigenemia and cytokine expression in rotavirus gastroenteritis in children

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Background: Antigenemia is commonly found in children with rotavirus infection, although its clinical significance is undetermined. The aim of this study was to evaluate the association of antigenemia with clinical manifestations and cytokine profiles in children infected by rotavirus.
Methods: In total, 68 children hospitalized with rotavirus gastroenteritis were enrolled. Serum samples were collected for detection of antigenemia and viremia. Clinical, laboratory and demographic data were analyzed. Proinflammatory, Th1 and Th2 cytokines were evaluated by bead-based flow cytometry.
Results: Antigenemia and viremia were found in 45.6% ($n = 31$) and 5.9% ($n = 4$) of the 68 rotavirus-infected children, respectively. The mean age of the antigenemia group was significantly greater than that of the non-antigenemia group (43.5 vs. 27.3 months; $p = 0.034$). The antigenemia group had a significantly shorter length of hospitalization (4.8 vs. 5.8 days; $p = 0.0354$) in comparison with the non-antigenemia group, and antigenemia was inversely associated with the length of hospitalization ($\beta = 0.31$, $p = 0.021$). A significantly higher tumor necrosis factor (TNF)-β level was found in the patients with antigenemia than those without (236.7 vs. 29.2 pg/mL, $p = 0.026$). The severity of disease and the rate of extra-intestinal manifestations did not differ between the groups. Viremia was associated with a higher fever ($p = 0.012$).
Conclusions: Antigenemia was positively correlated with shorter hospital stay in children with rotavirus infection. Enhanced innate and T-cell-mediated immunity evidenced by up-regulation of TNF-β was found in patients with antigenemia.
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Introduction

Viral acute gastroenteritis (AGE) is one of the leading infectious diseases worldwide. Rotavirus is detected in 30—50% of children hospitalized for diarrhea and results in an estimated 611,000 annual deaths globally.[1,2] Previous studies have addressed whether rotavirus infection is confined to the intestine.[3-5] Rotavirus genome isolated from the cerebrospinal fluid and serum, and the detection of live virus in the blood of infected children, has indicated that rotavirus can escape from the gastrointestinal tract into the circulatory system and even enter other organs.[5-10]

Antigenemia is commonly detected in rotavirus-infected children.[11-14] In the acute phase of infection, the detection of serum rotavirus antigen ranges from 43—90%. Rotavirus antigen levels usually peak 1 to 3 days after symptom onset and are undetectable beyond 1 week.[11,15] Antigenemia level is reported to be directly associated with antigen levels in stools, and inversely related to the titer of specific anti-rotavirus antibodies in the serum.[10,14] Cytokines mediate the inflammatory process and may influence the pathogenesis of rotavirus.[16,17] There are few studies addressing the cytokine responses in rotavirus-infected children.[18,19] The role the rotavirus antigenemia plays in viral pathogenesis and its influence on cytokine expression and host immunity are still undetermined. The aim of our study was to investigate the association of antigenemia with clinical manifestations and cytokine profiles in rotavirus-infected children.

Materials and methods

Patient selection and specimen collection

Previously healthy patients with ages ranging from 3 months to 18 years who were admitted to Chang Gung Children's Hospital with AGE were randomly enrolled in our study from October 2007 to September 2008. This study was approved by the Institutional Review Board of Chang Gung Memorial Hospital (96-0857B).

Fecal samples for bacterial culture (*Salmonella*, *Shigella*, *Campylobacter* and *Pseudomonas*) were obtained. Adenovirus antigen detection by rapid enzyme immunoassay (EIA) analysis and norovirus RNA detection by reverse transcriptase polymerase chain reaction (RT-PCR) of fecal samples were also performed. Rotavirus infection was confirmed by the detection of viral antigen in fecal samples using a commercial enzyme-linked immunosorbent assay (ELISA, R-Biopharm, Darmstadt, Germany). Patients with mixed infections with other viruses or who were coinfected with bacteria were excluded from the study. Serum and stool specimens were collected after informed consent was obtained from parents or guardians. Blood samples were collected within 3 days after admission for further analysis.

Detection of rotavirus antigens and RNA in serum specimens

Undiluted serum specimens (50 μL) were tested for the presence of rotavirus antigen in serum using RotaClone (Meridian Bioscience, Inc., Cincinnati, OH, USA), a commercial EIA approved for detection of rotavirus antigen in stool. A modified optical density (OD) cutoff value of 0.3 read by a spectrophotometer VICTOR™ X3 (J&H Technology Co. Ltd USA) at a wavelength of 450 nm was used for serum rotavirus antigen detection, as described in a previous report.[11] An absorbance of 0.3 or greater was defined as rotavirus antigenemia. The rotavirus RNAs were extracted from the serum specimens by using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacture's instructions. Reverse transcription of rotavirus double-strand RNA was carried out using Superscript III Reverse Transcriptase (Invitrogen Corporation, Carlsbad, CA, USA). The Beg9 and VP7-1' primers selected for the partial sequence of the *VP7* gene were based on previous studies.[20,21] The PCR condition for Beg9/ VP7-1' was: initial incubation at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds, 50°C for 30 seconds and 72°C for 1 minute, and a final extension at 72°C for 10 minutes. Electrophoresis of the PCR product was conducted on 2% agarose gels and visualized under ultraviolet light (Gel Documentation System, Vilber Lourmat, France).

Th1 and Th2 cytokine profiling

By using Th1/Th2 FlowCytomix (Bender Medsystem GmbH, Vienna, Austria), 11 cytokines were measured. Th1 cytokines consisted of interleukin (IL)-2, interferon (IFN)-γ, IL-12, and tumor necrosis factor (TNF)-β. Th2 cytokines consisted of IL-4, IL-5 and IL-10 (also assigned to T-regulatory cytokines). The proinflammatory cytokines IL-1, IL-6, IL-8 and TNF-α were also evaluated simultaneously. All serum samples collected were stored at −20°C before testing. The beads plus antibody mixture was incubated with serially diluted standard or serum samples. The analytes (cytokines) in the serum specimens bound to the antibodies linked to the fluorescent beads. A biotin-conjugated second antibody mixture was added and the second specific antibodies and bound to the analytes (cytokines) captured by the first antibodies. The mixture was incubated at room temperature for 2 hours on a microplate shaker (500 rpm). Streptavidin—phycoerythrin (PE) was added and incubated for 1 hour. Streptavidin—PE bound to the biotin conjugate and emitted fluorescent signals that were detected by flow cytometry. The fluorescent signals were read and further calculated by FloCytomix Pro Software (eBioscience, Vienna, Austria), and correlated cytokine levels were obtained.

Clinical features and demographic data collection

The clinical features were obtained by detailed history taking. The items included were age, gender, daily frequency of diarrhea, duration of diarrhea, daily frequency of vomiting, duration of vomiting, fever severity score (0 point: body temperature <37°C; 1 point: body temperature 37.1—38.4°C; 2 points: body temperature 38.5—38.9°C; 3 points: body temperature ≧39°C),[22] fever duration, blood in stools, length of hospital stay,

hypoglycemia (blood sugar $\leqq 60$ mg/dL), abnormal electrolytes (normal range: serum sodium 135—145 mmol/L; serum potassium: 3.5—5.6 mmol/L for 1—6 months; serum potassium: 3.5—6.1 mmol/L for 6 months to 1 year; serum potassium: 3.3—4.6 mmol/L for >1 year; serum chloride: 98—106 mmol/L), upper respiratory tract symptoms, abdominal pain, leukocytosis (leukocyte count >10,000/mm^3), C-reactive protein (CRP, normal range: <5 mg/L), and disease severity, using the 0—20 point scoring system proposed by Vesikari.[22] Extra-intestinal manifestations were also noted if present. Upper respiratory tract infection (URI) symptoms included cough, rhinorrhea, sore throat and nasal obstruction, which were not relevant to recognized atopic diseases or identifiable etiologies presented during the acute phase of rotavirus illness.

Statistical analysis

The chi-squared test or Fisher's exact test was applied to dichotomous variable analysis, and the unpaired-sample student's t-test was used for continuous variable analysis. The p values were two-tailed and those <0.05 were considered to indicate statistical significance. Multiple linear regression analysis was used to investigate the association of clinical confounders and the length of hospitalization. The confounders included antigenemia, age, fever severity, duration of fever, frequency of vomiting, duration of vomiting, frequency of diarrhea, duration of diarrhea and severity of dehydration. The SPSS for Windows (version 16; SPSS, Chicago, IL, USA) was used for statistical analyses.

Results

Demographic data and association of antigenemia with clinical presentation

From October 2007 to September 2008, 106 hospitalized pediatric patients were included in our study. Eight had positive results from stool culture for bacteria, 12 had positive norovirus infection, seven had mixed viral infections, four had mixed bacterial and viral infections and seven had incomplete clinical or laboratory data. These patients were excluded from the study.

Finally, a total of 68 patients diagnosed with rotavirus gastroenteritis who had not been vaccinated against rotavirus were enrolled. There were 44 boys and 24 girls; their ages ranged from 5 months to 14 years (mean age 34.7 months, median age 25.5 months). An additional five patients admitted to hospital for causes other than AGE were enrolled as negative controls. Among the five negative control patients, there were two boys and three girls with ages ranging from 6 to 23 months (mean age 14.2 months, median age 13.0 months). Rotavirus antigen was detected in the blood of 31/68 (45.6%) patients, and was not found in the other 37 patients or the negative control group. Serum rotavirus RNA was positive in four patients. Two of the patients with viremia had antigenemia.

The patients were divided into antigenemia ($n = 31$) and non-antigenemia ($n = 37$) groups. The demographic and clinical comparison between the two groups is shown in Table 1. The mean age of the antigenemia group was greater than that of the non-antigenemia group (43.5 ± 36.0 vs. 27.3 ± 25.3 months, $p = 0.034$). Mean hospital stay was significantly shorter ($p = 0.035$) in the antigenemia group (4.9 ± 1.4 days) than in the non-antigenemia group (5.8 ± 2.1 days). The associations between clinical confounders and the length of hospital stay were assessed using a multiple regression model. We found that antigenemia was inversely associated with the length of hospital stay (standardized $\beta = -0.31$, $p = 0.021$) while the association of age or other clinical variables and length of hospital stay was unremarkable. The severity of vomiting, diarrhea, fever and abdominal pain did not differ significantly between the two groups. The mean disease severity, based on the Vesikari scoring system, was 13.0 for the antigenemia group and 13.3 for the non-antigenemia group ($p = 0.671$). Children with viremia had higher fever severity score compared with those without ($p = 0.012$). The other clinical presentations of children with viremia and those without viremia were similar (see Table 1).

Laboratory data

Leukocytosis was detected in 12/31 (38.7%) patients in the antigenemia group and 16/37 (43.2%) patients in the non-antigenemia group ($p = 0.705$). CRP level was elevated in 17/31 patients with and 23/37 patients without antigenemia (54.8% vs. 62.2%; $p = 0.541$). The majority of CRP levels in both groups were mildly elevated, without significant difference in mean value (the mean values were 19 mg/L in the antigenemia group and 26 mg/L in the non-antigenemia group; $p = 0.456$). Other than leukocyte count and CRP level, the detection rate of blood in the stools, hypoglycemia and electrolyte imbalance were similar in both groups.

Extra-intestinal manifestation in rotavirus gastroenteritis

Upper respiratory tract symptoms were the major extra-intestinal symptoms in our patients, and they were found in 25.8% of the antigenemia group and 40.5% of the non-antigenemia group ($p = 0.201$). A 14-month-old girl, who presented with pancreatitis (serum amylase 2449 U/L and lipase 2021 U/L) during hospitalization, was included in the non-antigenemia group.

Cytokine profiles

Levels of the Th1 cytokines (IFN-γ, IL-2, IL-12 and TNF-β) were higher in the antigenemia group than in the non-antigenemia group, except for IFN-γ. The TNF-β level was significantly higher in the non-antigenemia group (236.7 vs. 29.2 pg/mL, $p = 0.026$). There was no significant difference between the two groups when considering proinflammatory cytokines (IL-1, IL-6, IL-8 and TNF-α) and Th2 cytokines (IL-4, IL-5 and IL-10), see Table 2.

Discussion

In our study, the rate of antigenemia (45.6%) in the acute phase of rotavirus infection was similar to that of previous

Table 1 Comparison of clinical presentations of rotavirus-infected children with or without antigenemia

	Antigenemia (n = 31)	Non-antigenemia (n = 37)	P
Age (months)	43.5 ± 36.0	27.3 ± 25.3	0.034[†]
Gender (male/female)	10/21	23/14	0.632
Clinical presentations			
Frequency of diarrhea (times/d)	4.2 ± 2.6	4.0 ± 1.9	0.669
Duration of diarrhea (d)	4.8 ± 1.9	4.8 ± 2.4	0.974
Frequency of vomiting (times/d)	3.3 ± 3.9	2.6 ± 2.2	0.435
Duration of vomiting (d)	2.0 ± 1.3	2.3 ± 1.5	0.452
Fever severity score	2.3 ± 1.1	1.9 ± 1.4	0.164
Fever duration (d)	2.5 ± 1.4	2.3 ± 1.9	0.694
Blood in stool, *n* (%)	3 (9.7%)	4 (10.8%)	0.878
Upper respiratory tract symptoms, *n* (%)	8 (25.8%)	15 (40.5%)	0.201
Abdominal pain, *n* (%)	17 (54.8%)	18 (48.6%)	0.611
Disease severity score	13.0 ± 2.4	13.3 ± 2.2	0.671
Length of hospital stay (d)	4.9 ± 1.4	5.8 ± 2.1	0.035[†]
Laboratory results			
White blood cell count elevation	12 (38.7%)	16 (43.2%)	0.705
C-reactive protein value (mg/L)	19.1 ± 30.5	25.8 ± 40.8	0.456
Hypoglycemia, *n* (%)	14 (45.2%)	13 (35.1%)	0.400
Abnormal electrolyte, *n* (%)	5 (16.1%)	3 (8.1%)	0.307

[†] $p < 0.05$ indicates statistical significance.

studies.[11,12] Several studies have shown that viremia is common in rotavirus infection and is transient during the disease course.[14,23] Among the 68 children with rotavirus infection enrolled in our study, viremia was only found in four patients. This rate was lower than other reports.[10,23] The timing of serum sample collection was considered to be the determining factor for such a low rate. Positive serum rotavirus RNA was associated with high fever, and this finding agrees with a previous study.[23] Viremia is thought to be responsible for more extra-intestinal manifestations in rotavirus pathogenesis. Animal models that simulate the natural course of human rotavirus infection support the association of transient rotavirus viremia with

extra-intestinal manifestations.[24] No positive correlation was found between viremia and extra-intestinal manifestations in this study, however, due to the limited number of cases in which viremia was detected.

There have been increasing numbers of reports describing the presence of rotavirus outside the intestine and supporting the correlation of rotavirus and extra-intestinal involvements. One previous report demonstrated rotavirus nonstructural protein in the livers and kidneys of immunodeficient children.[25] The detection of rotavirus RNA in cerebrospinal fluid has been described.[5,6,8,26] Extra-intestinal manifestations associated with rotavirus infection have also been reported, including pancreatitis,[27,28]

Table 2 Comparison of serum cytokine profile expressions of rotavirus-infected children with or without antigenemia and control children

Serum Cytokine profile expressions (pg/ml)	Antigenemia (pg/ml)[†]	Non-antigenemia (pg/ml)[†]	Control (pg/ml)[†]	P
IL-1β	28 (0−61.0)	106.4 (9.9−187.4)	0 (0−0)	0.747
IL-2	137.1 (23.5−184.3)	78.9 (0−198.7)	0 (0−0)	0.333
IL-4	208.1 (0−667.9)	228.1 (0−759.8)	0 (0−0)	0.156
IL-5	77.5 (36.3−130.8)	66.3 (25.7−187.4)	0 (0−0)	0.440
IL-6	83 (19.9−158.5)	16.7 (6.4−33.6)	0 (0−0)	0.510
IL-8	646.9 (56−1047.7)	902.6 (34.3−1801.6)	5.29 (0−13.22)	0.370
IL-10	39.6 (10.5−107.6)	54 (3.74−149.7)	0 (0−0)	0.440
TNF-α	43.6 (0−114.6)	178.5 (10.9−351.3)	0 (0−0)	0.382
TNF-β	236.7 (27.6−342)	29.2 (0−102.3)	0 (0−0)	0.026*
IL-12p70	74.3 (0−154.9)	15.5 (0−48.2)	0 (0−0)	0.544
IFN-γ	29.4 (4.1−65.5)	43.4 (9.7−122)	0 (0−0)	0.145

[†] Mean values and 25% (Q1) and 75% (Q3) quartiles are given as the mean (Q1−Q3).
*p values were obtained by comparison between the antigenemia group and the non-antigenemia group; $p < 0.05$ indicates statistical significance.

hepatic transaminase elevation,[29] pneumonia,[30] myocarditis and pneumonitis,[31] encephalitis,[32,33] meningoencephalitis,[26] cerebellitis,[34] convulsions or seizures.[8,9] There was no associated seizure or convulsion in our study population. Pancreatitis with simultaneous elevated serum transaminase was noted in one patient in our non-antigenemia group. Our findings suggest that extra-intestinal manifestations or systemic spread were not increased in children with rotavirus antigenemia, and this result was consistent with a recent study.[35]

In clinical studies, elevated levels of IL-6, IL-10, IFN-γ, IFN-α and TNF-α have been reported in children with rotavirus infection.[18,19] Sugata et al reported that IL-8 was significantly positively correlated but IL-10 was significantly negatively correlated with rotavirus antigen levels.[13] In our study, a significantly higher TNF-β level was found in the antigenemia group compared with the non-antigenemia group. IL-12 (Th1 inducer), IFN-γ and IL-2 levels were not significantly elevated. TNF-β (also known as lymphotoxin-α), released by Th1 helper cells and CD8 cytotoxic T cells, could promote macrophage activation and recruitment to the sites of infection, strengthening the cytotoxic T cell response. TNF-β shares receptors with TNF-α and they exert overlapping influences on peripheral lymphoid tissues and immune responses. TNF-β, along with the TNF superfamily, also constitutes an integral signaling network that is responsible for effective innate and adaptive immunity.[36–38] Based on profile of the cytokines in our study, we propose that upregulation of TNF-β expression and integral Th1/Th2 cytokines promoted the host immune response against rotavirus in the antigenemia group and furthermore led to a shorter hospital stay, but this needs further investigation.

Among the viral pathogens that cause AGE, rotavirus causes more severe vomiting, diarrhea, fever, and leads to a significantly higher disease score.[39] TNF-α levels have been reported to correlate with disease severity of rotavirus infection in children, and did not differ significantly between the groups in our study.[19] One previous study proposed that higher disease severity scoring exists in rotavirus-infected children with antigenemia in comparison with those without antigenemia.[35] In that study, however, lower disease severity might have occurred in asymptomatic patients who were mostly distributed in the non-antigenemia group. Our study enrolled patients who were all symptomatic; disease severity did not differ between those with and without antigenemia. Furthermore, antigenemia was recognized as a predictor of shorter hospital stay by a multiple regression model.

Previous studies reported that the production of antibodies after natural rotavirus infection could protect the host from subsequent different serotypes of rotavirus infection.[40] As the number of rotavirus infections increases, the anti-rotavirus IgA and IgG titers increase and the relative risk of subsequent rotavirus infection or rotavirus diarrhea decrease.[41] A longitudinal prospective cohort study found that naturally acquired serum anti-rotavirus IgA > 1:800 and IgG > 1:6400 could be reached and provide efficacious protection against rotavirus infection and moderate-to-severe diarrhea in children after two consecutive symptomatic or asymptomatic rotavirus infections.[42] Since rotavirus infection may be subclinical or asymptomatic and the complexity of individuals' innate and adaptive immunity background can differ, a further longitudinal prospective cohort study would be needed to determine the complicated relationships between antigenemia, host immune response and clinical presentations.

In summary, our findings indicate that antigenemia is common in symptomatic rotavirus infection and is more commonly found in older children. The children with antigenemia exhibited upregulated TNF-β production, which could enhance macrophage activation and cytotoxic T cell responses leading to acute-stage rotavirus infection being combatted. Antigenemia in rotavirus infection was associated with shorter hospital stay and was not associated with increased disease severity or the occurrence of extra-intestinal manifestations.

Acknowledgments

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