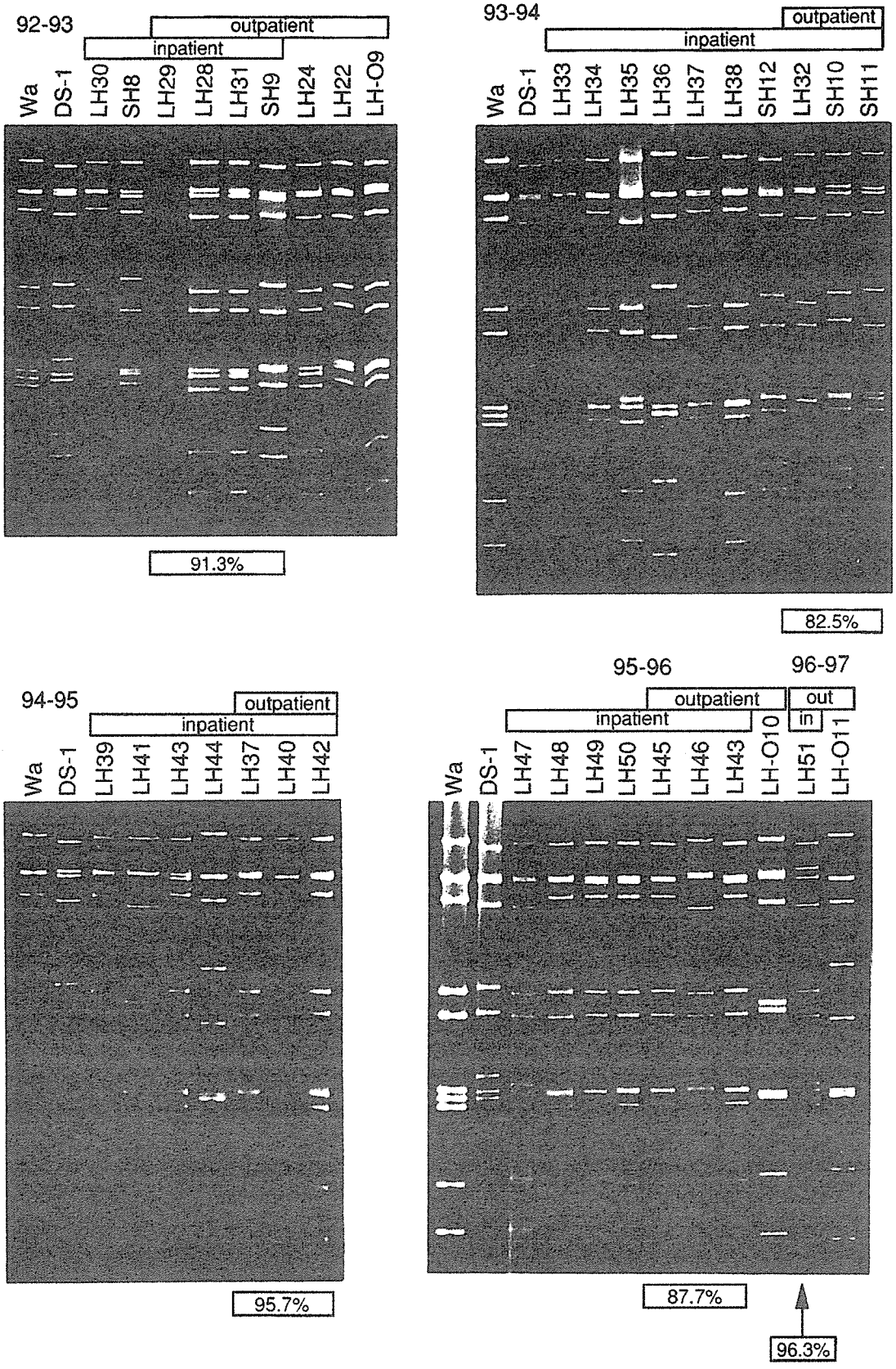
92-93
outpatient
inpatient
Wa
DS-1
LH30
SH8
LH29
LH28
LH31
SH9
LH24
LH22
LH-O9
91.3%
93-94
outpatient
inpatient
Wa
DS-1
LH33
LH34
LH35
LH36
LH37
LH38
SH12
LH32
SH10
SH11
82.5%
94-95
outpatient
inpatient
Wa
DS-1
LH39
LH41
LH43
LH44
LH37
LH40
LH42
95.7%
95-96
96-97
outpatient
out
inpatient
in
Wa
DS-1
LH47
LH48
LH49
LH50
LH45
LH46
LH43
LH-O10
LH51
LH-O11
87.7%
96.3%

Results

The initial screening by latex agglutination assays identified a total of 1906 rotavirus-positive stool specimens, of which 1018 were obtained from inpatients and 888 from outpatients. Because of either a limited amount of stool specimens available for further analysis or an insufficient amount of rotavirus particles present in stool specimens, only those rotaviruses that were present in 763 stool specimens (488 from inpatients and 275 from outpatients) were classified into 77 strains after repeated PAGE on the basis of possessing distinct electropherotypes. Except for the 96–97 season, for which the initial four months were included in this study, there was always co-circulation of several strains in every epidemic season, as evidenced by the presence of multiple electropherotypes (Fig. 1). The number of co-circulating strains was more or less similar for both the inpatient and the outpatient groups during each rotavirus season (Fig. 1). Table 1 gives the number of rotavirus-positive specimens that contained each of the distinct electropherotypes shown in Fig. 1, according to the status of the two patient groups. In both the inpatient and outpatient groups, a single strain was dominant in six epidemic seasons (88–89, 89–90, 90–91, 92–93, 94–95, 96–97), whereas two strains were co-dominant in the remaining five seasons (86–87, 87–88, 91–92, 93–94, 95–96). No strain persisted as dominant or co-dominant during any two successive seasons (Fig. 1). While long RNA patterns dominated in most seasons, short RNA patterns prevailed during the 90–91 and the 93–94 seasons (Fig. 1). In all seasons, the same strains dominated or co-dominated among the outpatient and inpatient groups. A few less-frequent strains were exclusively found in either the outpatient or the inpatient group. Furthermore, strains that were detected from both the inpatient and outpatient groups accounted for the great majority of the number of rotavirus-positive specimens, ranging from 80% in the 86–87 season to 96% in the 96–97 season (Fig. 1). This agrees with the premise that the background community population from which both outpatients and inpatients derived was universally exposed to rotavirus strains in a given season.

To examine whether such supposedly-universal exposure of rotavirus strains to the background population at risk would result in a distorted distribution between the inpatient and the outpatient groups, the chi-square test was performed for each epidemic season, with infrequently circulating strains being grouped into one category. As the P values in Table 1 indicate, there was no statistically significant difference in the distribution of dominant, co-dominant, or infrequent strains in any of the 11 seasons. Even during the 87–88 and the 90–91 seasons, in which the distribution of rotavirus strains was slightly different between the inpatient and the outpatient groups, it did not differ to a statistically significant degree ($p = 0.08$ and 0.09, respectively). Thus, the chi-square test result does not reject the null

Fig. 1. The electropherotypes of all strains detected from the inpatient and outpatient groups during each rotavirus season. Strains that were detected from both the inpatient and outpatient groups accounted for the great majority of the rotavirus-positive specimens detected in each rotavirus season, as indicated by the percentages at the bottom of each panel

Table 1. Distribution of rotavirus strains among inpatients and outpatients with acute diarrhea, according to the epidemic season of rotavirus, Honjo City, Akita, Japan, 1986–1997

Season	Inpatients	Outpatients	*P*-value
86–87	22[a] **LH1**, 6 **LH4**, 8 LH3, 1 LH2, 1 LH5, 1 LH6	7 **LH1**, 6 **LH4**, 2 LH6, 1 LH9	0.23[b]
87–88	21 **LH9**, 15 **LH13**, 3 LH10, 2 SH2, 1 LH11, 1 LH12, 1 LH7, 1 LH8, 1 SH1	18 **LH13**, 8 **LH9**, 4 LH8, 1 LHO1	0.08[c]
88–89	16 **LH14**, 4 LH15, 2 LH13, 2 LH16, 1 LH8, 1 SH3, 1 SH4	20 **LH14**, 5 LH15, 5 SH3, 2 LHO4, 2 LH13, 1 LHO2, 1 LHO3	0.77[c]
89–90	18 **LH17**, 6 SH5	13 **LH17**, 3 SH5, 2 LHO6, 1 LHO5, 1 LHO7, 1 LHO8	0.34[c]
90–91	34 **SH6**, 1 LH18, 1 LH19, 1 LH20, 1 LH21, 1 LH22	19 **SH6**, 6 LH18, 2 LH20	0.09[c]
91–92	14 **LH22**, 12 **LH24**, 5 LH28, 3 LH26, 2 LH18, 1 LH23, 1 LH25, 1 LH27, 1 SH7	10 **LH24**, 4 **LH22**, 2 LH28 2 SHO1, 1 LH18, 1 LH25, 1 LH26, 1 SHO2	0.31[c]
92–93	34 **LH31**, 8 LH28, 5 LH29, 2 SH8, 1 LH30, 1 SH9	22 **LH31**, 7 LH28, 4 LH29, 3 SHO3, 2 SH9, 1 LH22, 1 LH24, 1 LHO9	0.20[c]
93–94	42 **LH32**, 17 **SH10**, 8 LH35 5 LH34, 4 SH11, 1 LH33, 1 LH36, 1 LH37, 1 LH38, 1 SH12	9 **LH32**, 8 **SH10**, 5 SH11	0.33[c]
94–95	72 **LH37**, 4 LH40, 2 LH39, 1 LH41, 1 LH42, 1 LH43, 1 LH44	30 **LH37**, 3 LH40, 1 LH42	1.00[b]
95–96	20 **LH45**, 12 **LH43**, 5 LH46 3 LH50, 1 LH47, 1 LH48, 1 LH49	7 **LH45**, 5 **LH43**, 1 LH46 1 LHO10	0.68[b]
96–97	16 **LH51**	10 **LH51**, 1 LHO11	0.41[b]

[a]The number of rotavirus-positive stool specimens that possessed a distinct electropherotype. In this case, electropherotype LH1

[b]By Fisher's exact test

[c]By Chi-square test

hypothesis that there is no difference in the distribution of strains between the two different severity groups, thereby implying that the majority of rotavirus strains are similar in their inherent ability to cause the disease in infected children.

In order to evaluate the severity of individual strains that dominated or co-dominated in each of the 11 rotavirus seasons, case control studies were performed in which it was examined by calculating an odds ratio whether exposure to each of the dominant or co-dominant strains tended to result in hospitalization rather than in medical visit. As shown in Table 2, all dominant or co-dominant strains observed over the 11 rotavirus seasons except LH13 in the 87–88 season

Table 2. Virulence of individual strains as evaluated by crude odds ratios of hospitalization in case control studies in which exposure to the given strain was analyzed by hospitalization as outcome during the epidemic season of rotavirus, Honjo City, Akita, Japan, 1986–1997

Season	Strain	Odds ratio of hospitalization (95% CI)
86–87	LH1	1.66 (0.51–5.38)
	LH4	0.30 (0.08–1.15)
87–88	LH9	2.41 (0.90–6.51)
	LH13	0.35 (0.14–0.90)
88–89	LH14	1.16 (0.42–3.20)
89–90	LH17	1.85 (0.52–6.62)
90–91	SH6	2.86 (0.82–10.0)
91–92	LH22	2.42 (0.69–8.57)
	LH24	0.51 (0.18–1.51)
92–93	LH31	1.73 (0.74–4.03)
93–94	LH32	1.56 (0.60–4.04)
	SH10	0.46 (0.17–1.29)
94–95	LH37	0.96 (0.28–3.30)
95–96	LH45	0.87 (0.26–2.91)
	LH43	0.70 (0.19–2.51)
96–97	LH51	undefined

failed to place infected individuals at an increased risk for hospitalization at a statistically significant level. However, individuals infected with the LH13 strain were statistically significantly less likely to be hospitalized ($p = 0.027$).

Table 3. Association between G-serotype of rotaviruses and hospitalization in patients with acute diarrhea, Honjo City, Akita, Japan, 1986–1997

G-serotypes	Inpatients			Outpatients			OR[b] (95% CI)	OR[c] (95% CI)
	No.	(%)	(%)[a]	No.	(%)	(%)[a]		
G1	375	76.8	76.2	207	75.3	74.8	1.0	1.0
G2	71	14.6	14.9	47	17.1	18.3	0.83 (0.56–1.3)	0.77 (0.45–1.3)
G3	10	2.1	2.4	4	1.5	1.3	1.4 (0.46–5.1)	2.2 (0.68–8.2)
G4	26	5.3	5.5	10	3.6	3.6	1.4 (0.70–3.2)	2.0 (0.85–5.1)
NT	6	1.2	1.1	7	2.6	2.0	0.47 (0.15–1.4)	0.46 (0.14–1.5)
Long	417	85.5	85.1	227	82.5	81.4	1.0	
Short	71	14.6	14.9	48	17.5	18.6	0.81 (0.54–1.2)	0.71 (0.41–1.2)
Total	488	100	100	275	100	100		

[a]Proportion standardized for rotavirus season

[b]Crude odds ratio of hospitalization (reference = G1 or long RNA pattern)

[c]Odds ratio of hospitalization adjusted for rotavirus season. *CI* Confidence interval; *NT* not typed

To examine whether another viral attribute, the G-serotype, was associated with the virulence of rotavirus, analysis was performed on 756 of the 763 electropherotyped specimens for which the G-serotype was determined. Overall, the serotype G1 was the most prevalent (76.3%), followed by G2 (15.5%), G4 (4.7%), and G3 (1.8%). Irrespective of being standardized for rotavirus season, the relative frequencies of the G-serotype were similar between the inpatient and the outpatient groups (Table 3). This similarity was further confirmed by multiple logistic regression analysis, which showed that the G-serotype was not significantly associated with hospitalization (Table 3). When serotype G1 was used as a reference, the adjusted odds ratio of hospitalization was 2.2 for G3 and 2.0 for G4, but was not significant, as reflected by the wide 95% confidence intervals, due partly to the small number of subjects.

With two exceptional rotavirus specimens that possessed a long RNA pattern and serotype G2, all strains ($n = 116$) having a short RNA pattern belonged to serotype G2, whereas all strains having a long RNA pattern belonged to any one of G1 ($n = 582$), G4 ($n = 36$), and G3 ($n = 14$), or an untypeable G-serotype ($n = 10$). Irrespective of being standardized for season, the relative frequency of each RNA pattern was similar between the two groups (Table 3). In multiple logistic regression analysis, a short RNA pattern was not significantly associated with hospitalization, with an odds ratio of 0.71 relative to a long pattern, after adjustment for season (Table 3).

Discussion

The question of whether some strains cause severer gastroenteritis than others has its highest epidemiologic importance when it is concerned with the strains that prevail in each epidemic season, because such dominant strains are most likely to make a visible impact on the annual incidence of rotavirus-associated medical visits and hospitalizations. Infrequently circulating, minor strains with increased pathogenicity may cause severe disease. However, they are less likely to affect the overall increase in the number of hospitalizations because such minor strains, by definition, do not account for the majority of rotavirus gastroenteritis cases in the population. For this reason, this study focused on dominant or co-dominant strains as the primary target of analysis.

As for parameters that characterize rotavirus in detail, we selected electropherotype as the primary identifier of rotavirus strains. The reasons are twofold: First, each rotavirus strain shows a single distinct electropherotype upon PAGE, thereby making it practically feasible to define a strain by its electropherotype. Second, the virulence of rotavirus has not been unambiguously assigned to any single gene or any particular set of genes, thereby making it difficult to determine which gene(s) or gene product(s) to look at in association with disease severity. In animal models, for example, virulence segregated with the VP4 gene when reassortant viruses derived from parental strains that varied in their virulence for newborn mice were studied [16]. In the gnotobiotic porcine model, however, the genes encoding VP3, VP4, VP7, and NSP4 of a virulent porcine rotavirus each

played an independent role in virulence, and substitution of any one of these genes by the cognate gene from a human rotavirus that did not cause diarrhea in piglets resulted in an asymptomatic infection in piglets [7]. We therefore thought that it would be the best and the most practical to explore the association of disease severity and viral strains, not at the level of any single characteristic of rotavirus, but at the level of individual strains, which are most precisely defined by electropherotype upon PAGE.

Because this study extended over a 10-year period, it included 16 dominant or co-dominant strains, each of which circulated in one of 11 rotavirus epidemic seasons. It is particularly noteworthy that there was one dominant and another co-dominant short-RNA strain in different epidemic seasons, both of which were serotype G2, as is almost always the case. Thus, we were able to test each of these dominant G2 short RNA strains separately.

One distinctive feature of rotavirus epidemiology is the fact that multiple strains concurrently circulate in the same epidemic season. It is therefore logical to ask whether the dominant strain causes severer disease than other strains. In this regard, this study provided a suitable opportunity to address such a question.

Although a large number of strains from over a long period of time were examined, we were unable to find a significantly distorted distribution of dominant, co-dominant, or minor strains between severely affected children (inpatients) and children with milder gastroenteritis (outpatients). Furthermore, when dominant or co-dominant strains were individually tested in case control studies, it was also found that there was no statistically significantly increased risk of hospitalization. However, it was found that infection with LH13, one of the co-dominant strains in the 87–88 season resulted in a reduced risk of hospitalization. It may merit mention that LH13, which had serotype G1 appeared different only in genome segment 9 (the VP7 gene) from LH9, the other co-dominant strain in the same season, which had serotype G4 (Fig. 1) [11]. Whether this serotypic difference played any role was not clear, however. We therefore conclude that human rotavirus strains that prevail in each epidemic season have similar virulence in general, while an uneven distribution of strains between severe and less severe cases may still happen.

Several earlier studies have tried to find an association between disease severity and several characteristics of rotaviruses, such as subgroup, G-serotype, P-serotype and electropherotype (RNA pattern), but the results from these preceding studies were inconclusive. One study claimed that it examined the association between electropherotype of rotavirus strains and disease severity [2]. In that study, however, electropherotype was defined as the collection of similar but not necessarily identical RNA migration patterns on PAGE, as clearly indicated by the fact that strains with different G-serotypes were assigned to a single electropherotype. It is extremely rare, if not nonexistent, that two strains with identical electropherotype have different G serotypes [14].

In a previous study involving 118 Swedish patients with diarrhea, fever was more frequently observed for subgroup I (short RNA pattern) rotaviruses, whereas diarrhea and vomiting were severer for subgroup II (long RNA pattern) [21]. Bern et al. [1] reported that serotypes G2 and G3 were associated with significantly

severer dehydration than other G-serotypes among 718 Bangladeshi children. However, the authors did not choose to stress its clinical significance because the difference was statistically significant but very subtle. Cascio et al. [2] reported that a strain with G2P1B[4] was associated with severer gastroenteritis among 401 Italian children. Mota-Hernandez et al. [12] showed that the severity of gastroenteritis and the frequency of dehydration and hypovolemic shock were significantly higher for strains with serotype G3 and an untypeable P type than for G3P1A[8] and G1P1A[8] in Mexico. Cascio et al. [2] and Mota-Hernandez et al. [12] speculated that introduction of a rare and new G- or P-serotype into the community resulted in the occurrence of severer diarrhea. Recent outbreaks of gastroenteritis associated with G2 rotavirus among adults in three different locations in the United States of America are good examples of weakened herd immunity [5]. However, it may be a separate issue whether a rare serotype influences the severity of gastroenteritis during early childhood, which is usually caused by the first encounter with rotavirus [23]. In contrast, Fruhwirth et al. [3] reported that there was no significant difference in the distribution of G- or P-types among outpatients, inpatients, and nosocomially infected patients in Austria. A similar conclusion was also drawn by a recent study in Mexico, in which Polanco-Marin et al. [17] showed that severity of diarrhea was not significantly associated with either particular serotype or subgroup.

Thus, there are papers in the literature that have shown a positive association of a particular G-serotype or a particular combination of G-and P-serotypes with severer forms of gastroenteritis, whereas other papers did not support such observations. The specific reasons for the different conclusions drawn from our study and some other studies [1, 2, 12] are hard to give, but it deserves mention that our study analyzed the largest number of rotavirus specimens at the level of individual strains for the multiple rotavirus seasons spanning a 10-year period. Thus, our study may be less subject to haphazard variables or yet unknown confounding factors emerging from season to season.

There are, however, limitations in our study that deserve clarification and brief discussion. First, the necessity for hospitalization was taken as the sole outcome measure of disease severity, and it may be less precise and more liable to misclassification than scoring systems that have been used to quantify disease severity in rotavirus vaccine trials. The rationale behind this is that the ultimate goal of a rotavirus vaccine in developed countries is not to prevent mild illness or infection, but to prevent children from severe dehydrating diarrhea leading to hospitalization [10]. Moreover, it is the direct medical cost of hospitalization that heavily affects the economic consequences of rotavirus gastroenteritis [20].

Second, no clinical data were collected for patients who were hospitalized or who were treated only at the outpatient department. Thus, no further stratification is possible regarding the host factors, including age. As discussed earlier, weakened herd immunity was speculated to be one possible cause of gastroenteritis outbreaks due to serotype G2 rotavirus [5]. Unfortunately, we are not able to address this issue in this study. However, such stratification, in general, would be more meaningful in cases in which statistically significant differences were observed, because it

would then require determination of whether a distorted distribution of strains is ascribed to the virus strain or the host factors.

Third, we included only a fraction of children who were infected with rotavirus strains since it is thought that there were a large number of children who did not seek medical intervention because of the mildness of the symptoms.

Fourth, the results obtained for LH9 and LH13 (Table 1) approached a statistically significant level, and the odds ratio for LH13 actually reached a significance level (Table 2). We therefore admit that less virulent or more virulent strains may exist, which this study failed to detect due to the lack of statistical power.

Despite these limitations, our results suggest that the virulence or disease-causing potential of human rotaviruses that dominantly circulate in each epidemic season does not substantially differ in the majority of strains.

Acknowledgements

The authors thank Shigeki Sasaki for his work concerning polyacrylamide gel electrophoresis in the early stage of this study.

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Journal of Gastroenterology and Hepatology (2006) **21**, 521–530 DOI: 10.1111/j.1440-1746.2005.03977.x

GASTROENTEROLOGY

Rotavirus double-stranded RNA induces apoptosis and diminishes wound repair in rat intestinal epithelial cells

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Abstract

Background: Recent studies have shown that toll-like receptor 3 (TLR3) recognizes double-stranded RNA (dsRNA). Rotaviruses, having a dsRNA genome, infect intestinal epithelial cells (IEC) and cause acute gastroenteritis in young children. The aim of the present study was to clarify the pathophysiological function of rotavirus dsRNA in IEC.

Methods: Expression of TLR3 mRNA or protein in IEC cell lines (IEC-6, HT-29, Caco-2) was assessed by reverse transcription polymerase chain reaction (RT–PCR), Western blot analysis or immunohistochemistry. Induction of cytokines (TNF-α, interferon-β, interleukin-6) mRNA and activation of signal proteins (ERK1/2 MAPK and IκB-α) in IEC after stimulation with rotavirus dsRNA were assessed by RT–PCR or Western blot analysis. IEC-6 cells were wounded and cell migration into wound areas after stimulation with rotavirus dsRNA (1–25 μg/mL) was assessed. Induction of apoptosis after stimulation with rotavirus dsRNA was also assessed.

Results: Expression of TLR3 mRNA and TLR3 protein was detected in IEC. Expression of TLR3 mRNA in IEC-6 tended to be up-regulated by exposure to IFN-γ. Induction of cytokine mRNA and activation of the signal proteins were detected after stimulation with rotavirus dsRNA. Apoptosis was induced and epithelial migration into the wound area was dose-dependently diminished (44.1–94.4%, $P < 0.01$) by exposure to rotavirus dsRNA. Diminishment of wound repair was suppressed by anti-TLR3 antibody or caspase inhibitor.

Conclusion: Rotavirus dsRNA induces severe apoptosis and diminishes wound repair in IEC through TLR3, which might be involved in the pathogenesis of rotavirus-induced enteritis.

Key words: dsRNA, intestinal epithelial cells, rotavirus, TLR3, toll-like receptor.

INTRODUCTION

Toll-like receptors (TLR) are innate immune-recognition receptors that recognize molecular patterns common to bacterial and viral antigens.[1–4] Ten TLR and some of their ligands have been identified to date.[4] In these TLR, TLR3 recognizes double-stranded (ds) RNA,[5,6] which is found in the genome of some RNA viruses, though ligands of other TLR are mainly associated with bacterial components, that is, TLR2 recognizes lipoproteins, peptidoglycan and lipoteichoic acid from Gram-positive bacteria;[7,8] TLR4 recognizes lipopolysaccharide from Gram-negative bacteria;[1–4] TLR5 recognizes flagellin from bacterial flagella;[9] and TLR9 recognizes deoxycytosine-deoxyguanosine motifs from bacterial DNA.[10] Intestinal epithelial cells (IEC) are constantly exposed to microbial and viral products and serve as an essential barrier from these antigens. Recent studies have shown that IEC also express TLR and respond to microbial components,[11–18] although other studies have shown negative results.[19–21] In contrast, it has not been precisely investigated whether IEC

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Accepted for publication 23 February 2005.

express TLR3 and respond to ligands of TLR3 like viral dsRNA.

Rotavirus is a member of the *Reoviridae* genus and has 11 segments of dsRNA.[22] Rotavirus is the single most important etiological agent of severe diarrhea in young children and leads to an estimated 600 000–870 000 deaths a year in developing countries.[22,23] Rotavirus infects enterocytes on the tip of the intestinal villi through a host receptor that is suggested to be a complex of several cell molecules likely to be immersed in plasma membrane lipid microdomains.[22] In contrast, a previous study showed that reovirus, having the dsRNA genome[24] or dsRNA itself, induced apoptosis in virus-infected cells and perhaps virus-uninfected cells,[25] and our current study also showed that polyinosine-polycytidylic acid (poly [I : C]), a synthetic dsRNA analog, induces apoptosis in IEC (data in submission). It is of note that IEC initially have no contact with the rotavirus dsRNA, because genomic dsRNA is surrounded by three concentric layers of protein in the mature rotavirus. It is only later, after the virus has successfully infected IEC and its genome has been replicated within the cells, that epithelial cells have exposure to rotavirus dsRNA derived from broken up infected cells. However, the pathophysiological function of rotavirus dsRNA in IEC remains unclear. The present study was conducted to clarify the pathophysiological function of rotavirus dsRNA in IEC, especially through the TLR3 pathway.

MATERIALS AND METHODS

Materials

IEC-6 cells were purchased from ATCC (Manassas, VA, USA) and were used at the 17th–21st passage.[26] Both HT-29 cells and Caco-2 cells were also purchased from ATCC. We used simian rhesus rotavirus strain RRV in the present study. A cell culture-adapted RRV strain was grown in MA104 cells and virus particles were purified as described before.[27] Antibodies to p44/42 MAP kinase (ERK1/2 MAPK), threonine and tyrosine-phosphorylated p44/42 MAPK, IκB-α, and serine-phosphorylated IκB-α were obtained from Cell Signaling (Beverly, MA, USA).

Assessment of TLR3 expression in intestinal epithelial cells

Expression of TLR3 mRNA in IEC cell lines (IEC-6, HT-29 and Caco-2) was assessed by reverse transcription polymerase chain reaction (RT–PCR). Total RNA was extracted from IEC with TRIzol reagent (Invitrogen, San Diego, CA, USA) and 2 μg of RNA was applied for reverse transcription reaction by incubating at 42°C for 50 min with oligo dT primers (Invitrogen). Converted DNA was amplified by PCR (35 cycles) with TAKARA Taq (TAKARA, Otsu, Japan) using the specific primers to rat TLR3 (annealing temp 52°C), glyceraldehydes-3-phosphate dehydrogenase (annealing temp 57°C) (Table 1) or human TLR3 (annealing temp 53°C), GAPDH (annealing temp 57°C)[6,17] (Table 2). The primers for rat TLR3 and rat GAPDH were originally designed by Iizuka M *et al.* Sequencing analysis of the PCR products using these primers confirmed that each primer effectively amplifies the targeting gene (data in submission).

Expression of TLR3 protein in IEC cell lines was assessed by Western blot analysis and immunohistochemical methods. Western blot analysis was carried out as described in the section of 'Assessment of activation of signaling proteins' using the anti-TLR3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), which reacts to both human and rat TLR3 protein, and the HRP-conjugated secondary antibody (Dako, Copenhagen, Denmark). In immunohistochemistry, we used the same anti-TLR3 antibody (Santa Cruz Biotechnology) for the first antibody, and fluorescein-conjugated antigoat immunoglobulin (Santa Cruz Biotechnology) was applied as the second antibody. Cultured cells on sterile glass cover slips were fixed in 2% paraformaldehyde for 10 min. After washing with phosphate-buffered saline (PBS, three times, 5 min each), non-specific binding was blocked with normal bovine serum (1:10 in PBS) for 60 min at room temperature. After removal of the blocking solution, specimens were incubated in a moist chamber with anti-TLR3 polyclonal antibody (1:100 in PBS with 1.5% normal bovine serum) for 2 h at room temperature. Then, specimens were incubated with fluorescein-conjugated antigoat immunoglobulin for 1 h at room temperature. After the incubation, samples were

Table 1 Oligonucleotide sequences for rat primers

TLR3	Sense	5′-GCAACAACAACA TAGCCAAC-3′
	Antisense	5′-CCTTCA GGAAATTAACGGGAC-3′
TNF-α[28]	Sense	5′-TCACAGAGCAATGACTCCAA-3′
	Antisense	5′-TTGCCACTTCATACCAGGAGAA-3′
IFN-β[29]	Sense	5′-ATGGCCAACACGTGGACCCT-3′
	Antisense	5′-TCAGTTCTGGAAGTTTCTAT-3′
IL-6[28]	Sense	5′-TTGGGATATCAGGTTTCTGGATGG-3′
	Antisense	5′-GCCAGAGTCATTCAGAGCAATACTG-3′
GAPDH	Sense	5′-AATGCATCCTGCACCACCAACTGC-3′
	Antisense	5′-GGAGGCCATGTAGGCCATGAGGTC-3′

Table 2 Oligonucleotide sequences for human primers

TLR3[6]	Sense	5′-CTCAGAAGATTACCAGCCGCC-3′
	Antisense	5′-CCATTATGAGACAGATCTAATG-3′
TNF-α[17]	Sense	5′-GAGTGACAAGCCTGTAGCCCATGTTGTAGCA-3′
	Antisense	5′-GCAATGATCCCAAAGTAGACCTGCCCAGACT-3′
IFN-β[30]	Sense	5′-GAACTTTGACATCCCTGAGGAGATTAAGCAGC-3′
	Antisense	5′-GTTCCTTAGGATTTCCACTCTGACTATGGTCC-3′
IL-6[31]	Sense	5′-CCACACAGACAGCCACTCACCTC-3′
	Antisense	5′-CTGGCTTGTTCCTCACTACTCTC-3′
GAPDH[17]	Sense	5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′
	Antisense	5′-CATGTGGGCCATGATGGTCCACCAC-3′

washed three times in PBS and mounted (Crystal/Mount; Biomeda, Foster City, CA, USA). Nuclear staining was also carried out in IEC-6 cells with 1 μg/mL of 4′, 6-diamino-2-phenylindole (DAPI) (Roche, Mannheim, Germany).

Quantitative real-time RT–PCR of TLR3 after stimulating IEC-6 cells with IFN-γ

Expression of TLR3 mRNA in IEC-6 cells after 0, 2, 8 and 24 h of stimulation with rat IFN-γ (R & D, Minneapolis, MN, USA) was quantified by quantitative real-time RT–PCR. After reverse transcription of 2 μg of total cellular RNA, PCR on a LightCycler (Roche, Basel, Switzerland) was carried out according to the manufacturer's instructions using a LightCycler DNA Master SYBR Green I Kit (Roche). The primers shown in Table 1 were used for amplification of TLR3 and GAPDH cDNA. The amounts of target RNA (cDNA) were determined from standard curves for each experimental sample. The experiments were carried out twice. In order to obtain a normalized target value, the target amount was divided by GAPDH amount. In addition, expression of TLR3 protein or actin protein in 15 μg of IEC-6 cells after 0, 2, 8 and 24 h of stimulation with rat IFN-γ was quantified by Western blot analysis using anti-TLR3 polyclonal antibody (Santa Cruz) or antiactin antibody (Sigma, Saint Louis, MO, USA) and HRP-conjugated secondary antibody (Dako). Western blot analysis was carried out as described in the section of 'Assessment of activation of signaling proteins'.

Assessment of induction of cytokines by stimulation of IEC with rotavirus dsRNA

Expression of cytokines (tumor necrosis factor-α [TNF-α], interferon β [IFN-β], interleukin-6 [IL-6]) mRNA by IEC-6 and HT-29 cells was assessed by RT–PCR. Confluent IEC cell lines were stimulated for 2 h with 25 μg/mL of rotavirus dsRNA. Total RNA was extracted from IEC cell lines before and after the stimulation. Then 2 μg of the extracted RNA was applied for reverse transcription reaction, and PCR (35 cycles) was carried out with the specific primers to rat TNF-α (annealing temp 57°C), IFN-β (annealing temp 50°C), IL-6 (annealing temp 55°C)[28,29] (Table 1) or human TNF-α (annealing temp 57°C), IFN-β (annealing temp 60°C), IL-6 (annealing temp 58°C) (Table 2).[17,30,31]

Assessment of activation of signaling proteins

Activation of signaling proteins in IEC-6 cells after stimulation with rotavirus dsRNA was assessed by Western blotting. Confluent IEC-6 monolayers grown on six well dishes, maintained for 24 h in serum-deprived Dulbecco's modified Eagle's medium were stimulated for 15, 30 and 60 min with 25 μg/mL of rotavirus dsRNA. Cells were rinsed in a cold phosphate-free medium (Invitrogen, Carlsbad, CA, USA) and placed on ice in 300 μL of lysis buffer per well (1% Triton X-100, 150 mmol NaCl, 20 mmol Tris-HCl, pH 7.5, 2 mmol ethylene diamine tetra acetic acid, containing 10 μg/mL leupeptin, 10 mg/mL aprotinin, 1 mmol sodium fluoride, 1 mmol sodium orthovanadate and 2 mmol phenyl methyl sulfonyl fluoride). Lysates were centrifuged (12 000 × g, 15 min at 4°C) and the protein concentration in each supernatant was determined by colorimetric Bradford protein assay (Bio-Rad, Hercules, CA, USA). Proteins (per lane, 15 μg) from the resulting supernatant were heated (90°C, 3 min), subjected to sodium dodecyl sulfate polycrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Millipore, Yonezawa, Japan) followed by immunoblotting with specific polyclonal antibodies to p44/42 MAPK, threonine and tyrosine-phosphorylated p44/42 MAPK, IκB-α, or serine-phosphorylated IκB-α. Positive bands were visualized with a secondary HRP-conjugated antibody (Dako) and the ECL-system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Wound assays

Wound assays were carried out with IEC-6 cells as previously described[32,33] using a modification of the method described by Sato and Rifkin.[34,35] Confluent monolayers of IEC-6 cells (17th–21th passage) in 30 mm plastic dishes were wounded with a razor blade. Generally, two wounds approximately 20–25 mm across the dish were made and separated by 1 cm. Cells

were washed with a fresh serum-deprived medium, and the wounded monolayers were further cultured for 24 h in fresh serum-deprived medium in the presence and absence of various concentrations of rotavirus dsRNA (1, 10, 25 μg/mL). We also carried out wound assay using polyinosine-polycytidylic acid (poly [I : C], 600 μg/mL, 1200 μg/mL, Calbiochem, San Diego, CA, USA) and the results were compared to that with rotavirus dsRNA. Migration of IEC-6 cells was assessed in a blinded fashion by determination of the number of migrated cells found across the wound border in a standardized wound area. Wound areas were standardized by taking photomicrographs at 100-fold magnification using an inverted microscope (Olympus IX70) and DP Controller (Olympus, Tokyo, Japan). Experiments were carried out in triplicate and several wound areas per plate were used to quantify migrated cells. Data are expressed as mean value + SD.

Detection of apoptotic cells after stimulation of IEC-6 cells with rotavirus dsRNA

IEC-6 cells were stimulated with 10 μg/mL or 25 μg/mL of rotavirus dsRNA and 24 h later, cells were washed three times with PBS. Apoptotic cells, with condensed nuclei and fragmented DNA, were assessed using DAPI and gel electrophoresis. Namely, nuclear staining was carried out with 1 μg/mL of DAPI (Roche, Mannheim, Germany) in methanol for 15 min followed by washing with methanol. Then, apoptotic cells were directly observed using fluorescence microscopy. In contrast, DNA was extracted from IEC-6 cells after stimulation with rotavirus dsRNA using phenol chloroform extraction method. After incubation for 1 h with RNase A (Wako, Tokyo, Japan) at 37°C, extracted DNA was electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide and exposure to UV light.

Effects of TLR3 antibody on wound repair of IEC-6 cells stimulated with rotavirus dsRNA

We calculated the proportion of apoptotic cells after stimulating IEC-6 cells with 25 μg/mL of rotavirus dsRNA in the presence of anti-TLR3 antibody and compared it with that with only 25 μg/mL of rotavirus dsRNA or control. Wound assays were also carried out by stimulation of IEC-6 cells with 10 μg/mL or 25 μg/m of rotavirus dsRNA in the presence of anti-TLR3 antibody (2.5 μg/mL). The numbers of migrated cells were counted and compared with those with only 10 μg/mL or 25 μg/mL of rotavirus dsRNA.

Effects of caspase inhibitor on wound repair in IEC-6 cells stimulated with rotavirus dsRNA

Wound assays were carried out by stimulation of IEC-6 cells with 10 μg/mL of rotavirus dsRNA in the presence of 40 μmol of caspase inhibitor (Caspase inhibitor Z-VAD-FMK, Cell Signaling). Numbers of migrated cells were counted and the results were compared with that with only 10 μg/mL of rotavirus dsRNA.

Statistics

Statistical analysis was carried out using the unpaired *t*-test, Mann–Whitney *U*-test (wound assays, quantitative real-time RT–PCR) or McNemar χ^2 test (proportion of apoptotic cells), and a two-tailed probability of 0.05 was considered statistically significant.

RESULTS

IEC cell lines express both TLR3 mRNA and TLR3 protein

Expression of TLR3 mRNA was detected by RT–PCR in three IEC cell lines (IEC-6, HT-29 and Caco-2) (Fig. 1a). Western blot analysis using the anti-TLR3 antibody showed that TLR protein was also expressed in three IEC cell lines (Fig. 1b). Results of immunohistochemistry of IEC-6 (1), HT-29 (3), Caco2 (4) using anti-TLR3 antibody and DAPI staining of IEC-6 (2) are shown in Figure 1c. These figures showed that TLR3 protein was expressed mainly in the cytoplasm of the three IEC cell lines.

Expression of TLR3 mRNA by IEC-6 cells tends to be enhanced by stimulation with IFN-γ

Expression of TLR3 mRNA in IEC-6 cells tended to be time dependently up-regulated by exposure to IFN-γ (Fig. 2a). The mean relative amount for the TLR3 mRNA level in IEC-6 cells was enhanced by 2.3-fold at 24 h after stimulation with IFN-γ compared with the mean relative amount for the TLR3 mRNA level in IEC-6 cells before the stimulation. Expression of TLR3 protein was not clearly increased by exposure to IFN-γ, but TLR3 protein appeared to be slightly increased 24 h after stimulating with IFN-γ (Fig. 2b).

Rotavirus dsRNA induces expression of cytokine mRNA by IEC

TNF-α, IFN-β and IL-6 mRNA were not expressed by IEC-6 cells before stimulation with rotavirus dsRNA. Expression of each cytokine mRNA by IEC-6 cells was clearly induced after stimulation with rotavirus dsRNA (Fig. 3). In contrast, TNF-α mRNA was not expressed, but IFN-β and IL-6 mRNA were slightly expressed by HT-29 cells before stimulation with rotavirus dsRNA. Expression of TNF-α mRNA was induced and expression of IFN-β and IL-6 mRNA were enhanced by HT-29 cells after stimulation with rotavirus dsRNA (Fig. 3).

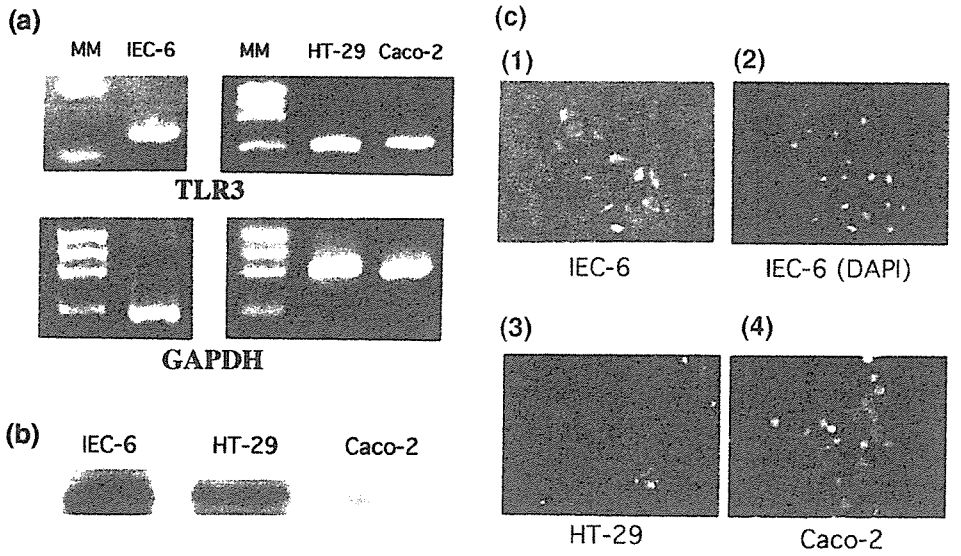

Figure 1 Intestinal epithelial cells (IEC) cell lines express both toll-like receptor 3 (TLR3) mRNA and TLR3 protein. (a) Results of reverse transcription polymerase chain reaction for TLR3 in IEC-6 cells, HT-29 cells and Caco-2 cells. MM, molecular size marker. (b) Results of Western blot analysis of IEC-6 cells, HT-29 cells and Caco2 cells using the anti-TLR3 antibody. (c) Results of immunohistochemistry of (1) IEC-6 cells, (3) HT-29 cells, and (4) Caco2 cells using anti-TLR3 antibody, and (2) 6-diamino-2-phenylindole staining of IEC-6 cells (original magnification, ×400).

Rotavirus dsRNA activates signal proteins of ERK1/2 MAPK and IκB-α IEC-6 cells

Phosphorylation of ERK1/2 MAPK protein was clearly observed after stimulation of IEC-6 cells with rotavirus dsRNA, especially 15 min after the stimulation (Fig. 4a). Phosphorylation of IκB-α protein in IEC-6 cells was also observed 15 min after stimulation with rotavirus dsRNA (Fig. 4b).

Rotavirus dsRNA significantly diminishes wound repair in IEC-6 cells

Rotavirus dsRNA reduced epithelial cell migration into a wound area in a concentration-dependent manner (Fig. 5a). The number of migrated cells decreased by 44.1% ($P < 0.01$), 85.1% ($P < 0.01$) or 94.4% ($P < 0.01$) after exposure to 1 μg/mL, 10 μg/mL or 25 μg/mL, respectively, of rotavirus dsRNA compared with that with no stimulation (control). The number of migrated cells also decreased by 40.2% ($P < 0.01$) or 62.0% ($P < 0.01$) after exposure to 600 μg/mL, 1200 μg/mL of high dose poly (I : C) compared with that with no stimulation. However, as shown in Figure 5b, small doses of rotavirus dsRNA more strongly diminished epithelial migration compared with high doses of poly (I : C) ($P < 0.01$).

Rotavirus dsRNA strongly induces apoptosis in IEC-6 cells

DAPI staining clearly showed a significant increase of apoptotic cells with condensed nuclei after stimulation of IEC-6 cells with 25 μg/mL of rotavirus dsRNA (Fig. 6a). The DNA ladder, which shows apoptotic DNA fragments, was also observed after exposure of IEC-6 cells to rotavirus dsRNA (Fig. 6b). The DNA ladder was not observed in the DNA extracted from IEC-6 cells without stimulation with rotavirus dsRNA (control).

TLR3 antibody suppresses the induction of apoptosis and the diminishment of wound repair in IEC-6 cells stimulated by rotavirus dsRNA

The proportion of apoptotic cells around the wound area in IEC-6 cells was significantly increased by stimulation with 25 μg/mL of rotavirus dsRNA (78.1%) compared with that with no stimulation (4.5%, $P < 0.01$). However, the proportion of apoptotic cells was significantly decreased by stimulation with 25 μg/mL of rotavirus dsRNA in the presence of 2.5 μg/mL of anti-TLR3 antibody (50.1%) compared with that with only 25 μg/mL of rotavirus dsRNA (78.1%, $P < 0.01$). DAPI-stained wound areas 24 h after stimulation with 25 μg/mL rotavirus dsRNA, 25 μg/mL rotavirus dsRNA in the presence of 2.5 μg/mL of TLR3 antibody, or no stimulation (control) are shown in Figure 7a.

In contrast, the number of epithelial cells that migrated into the wound area increased 2.45-fold by stimulation with 10 μg/mL of rotavirus dsRNA in the presence of 2.5 μg/mL of TLR3 antibody compared with that without TLR3 antibody ($P < 0.01$, Fig. 7b). The number of epithelial cells that migrated also increased 5.4-fold by stimulation with 25 μg/mL of rotavirus dsRNA in the presence of 2.5 μg/mL of TLR3

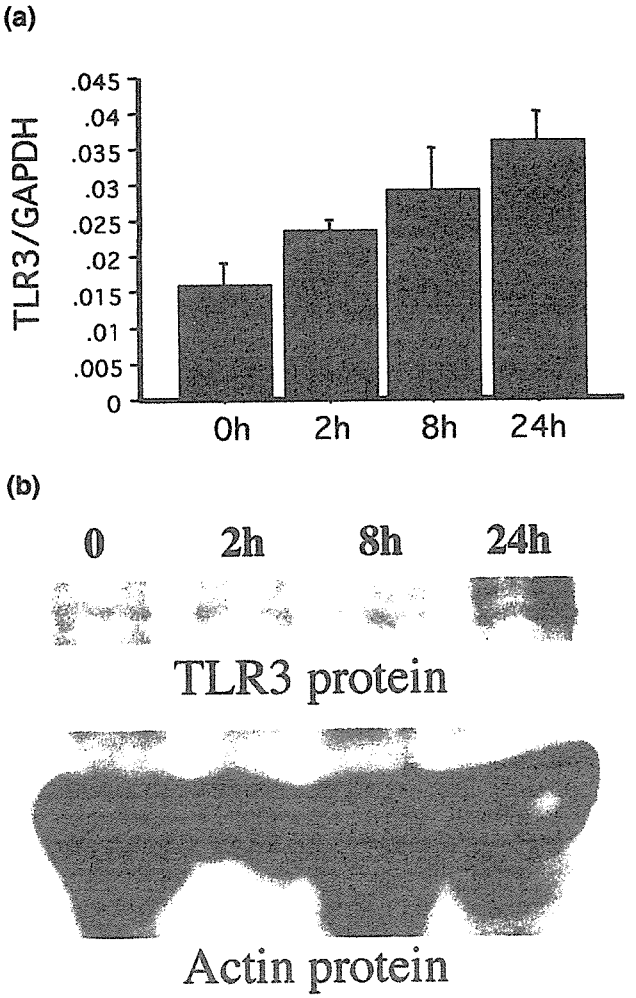

Figure 2 Quantification of toll-like receptor 3 (TLR3) mRNA and TLR3 protein in IEC-6 cells after stimulation with IFN-γ. (a) Mean relative amount for TLR3 (TLR3/GAPDH) mRNA in IEC-6 cells at 0, 2, 8, and 24 h after stimulation with 3 ng/mL of IFN-γ is shown. They were quantified by quantitative real-time reverse transcription polymerase chain reaction. IEC-6 cells were stimulated with 3 ng/ml IFN-γ. (b) Results of Western blot analysis of TLR3 protein and actin protein in IEC-6 cells 0, 2, 8, and 24 h after stimulation with IFN-γ are shown.

antibody compared with that without TLR3 antibody ($P < 0.01$, Fig. 7b).

Caspase inhibitor suppresses the diminishment of wound repair in IEC-6 cells stimulated by rotavirus dsRNA

As shown in Figure 5, the number of epithelial cells that migrated into the wound area decreased by 85.1% by exposure to 10 μg/mL of rotavirus dsRNA compared with that with no stimulation (control). However, the number of epithelial cells that migrated into the wound area increased 2.73-fold by stimulation with 10 μg/mL of rotavirus dsRNA in the presence of 40 μmol of caspase inhibitor ($P < 0.01$, Fig. 8).

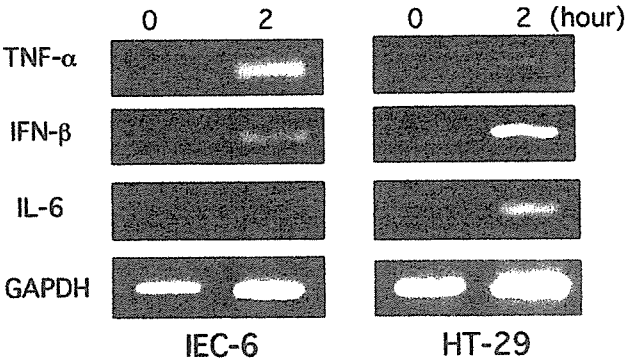

Figure 3 Rotavirus dsRNA induces expression of cytokine mRNA in IEC. (a) Expression of cytokines (tumor necrosis factor-α [TNF-α], interferon β [IFN-β], IL-6) mRNA by IEC-6 cells after stimulation with rotavirus dsRNA is shown. Confluent IEC-6 monolayers were stimulated for 2 h with rotavirus dsRNA (25 μg/mL). Expression of each cytokine mRNA was assessed by reverse transcription polymerase chain reaction.

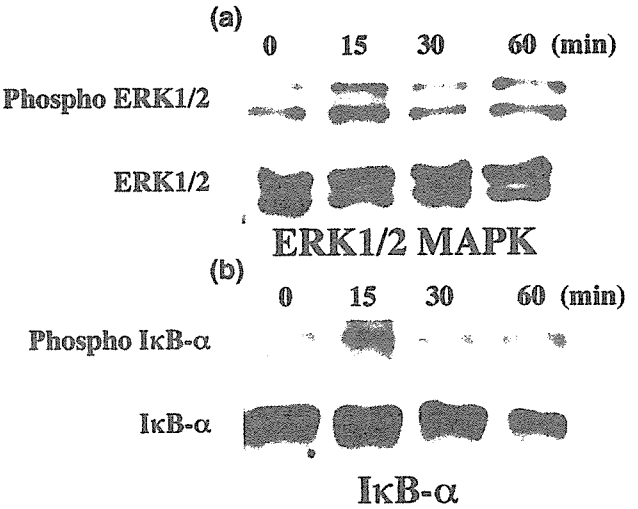

Figure 4 Rotavirus dsRNA activates signal proteins in IEC-6 cells. (a) Time course of activation of ERK1/2 MAPK in IEC-6 cells after stimulation with rotavirus dsRNA (25 μg/mL) is shown. Confluent IEC-6 monolayers grown on six well dishes maintained for 24 h in serum-deprived Dulbecco's modified Eagle's medium containing 0.1% FBS were stimulated for 15, 30 and 60 min with rotavirus dsRNA (25 μg/mL). Whole cell lysates from IEC-6 monolayers were harvested after the stimulation. Phosphorylated or total ERK1/2 MAPK was determined by Western blot analysis. (b) Time course of activation of IκB-α in the wounded IEC-6 cells after stimulation with rotavirus dsRNA (25 μg/mL) is shown.

DISCUSSION

TLR family members recognize pathogen-associated molecular patterns of bacterial components and they were first noted to be expressed by immune cells.[1-4] Recent studies have shown that intestinal epithelial cells also express TLR and can respond to bacterial components.[11-18] In contrast, it has been shown that TLR are

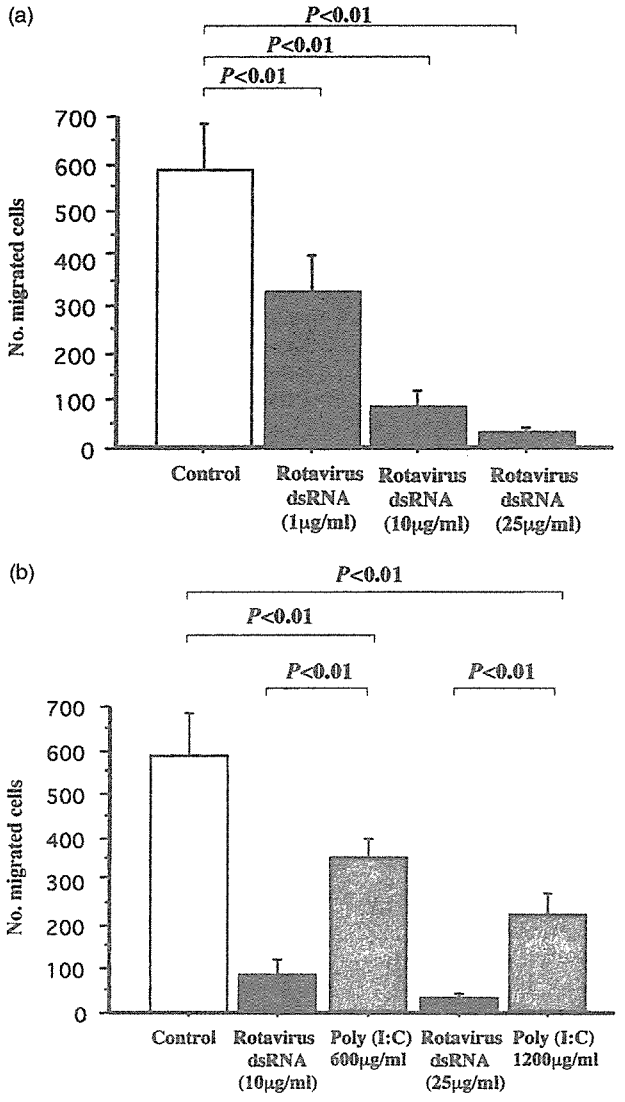

Figure 5 Rotavirus dsRNA diminishes wound repair in IEC-6 cells in a concentration dependent manner. (a) The numbers of migrated epithelial cells (mean + SD) into wound areas after stimulating with various concentrations of rotavirus dsRNA are shown. Confluent monolayers maintained for 24 h in serum-deprived Dulbecco's modified Eagle's medium (DMEM) were wounded and cultured for 24 h after replacement of medium with fresh serum-deprived DMEM in the presence of various concentrations of rotavirus dsRNA (1, 10, 25 μg/mL) or no reagent (control). (b) The numbers of migrated epithelial cells (mean + SD) into wound areas after stimulating with 10 or 25 μg/mL of rotavirus dsRNA, 600 or 1200 μg/mL of poly (I : C), or control are shown. The numbers of migrated epithelial cells after stimulating with 10 μg/mL of rotavirus dsRNA were compared with the numbers of migrated epithelial cells after stimulating with 600 μg/mL of poly (I : C), the numbers of migrated epithelial cells after stimulating with 25 μg/mL of rotavirus dsRNA were compared with the numbers of migrated epithelial cells after stimulating with 1200 μg/mL of poly (I : C).

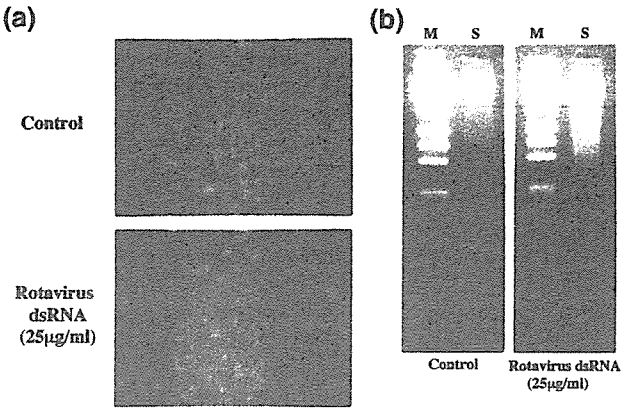

Figure 6 Rotavirus dsRNA induces apoptosis in IEC-6 cells. (a) The monolayers of IEC-6 after stimulating with rotavirus dsRNA were stained with 6-diamino-2-phenylindole. Confluent monolayers maintained for 24 h in serum-deprived Dulbecco's modified Eagle's medium (DMEM) were cultured for 24 h after replacement of medium with fresh serum-deprived DMEM in the presence of rotavirus dsRNA (25 μg/mL) or no reagent (control). (b) Gel electrophoresis of DNA extracted from IEC-6 cells 24 h after stimulation with rotavirus dsRNA (25 μg/mL) or no reagent (control) is shown. M, molecular size marker; S, sample (IEC-6).

also implicated in the recognition of viral antigens, that is, TLR3 recognizes viral dsRNA[5,6] and TLR7 recognizes viral single-stranded (ss) RNA.[36,37] Rotaviruses, having a dsRNA genome, are the leading cause of severe dehydrating diarrhea in infants and young children.[22,23] Regarding the TLR3-mediated function against actual viral infections, only a few reports have been published to date.[5,38] Among these reports, it is of note that Edelmann *et al.* showed that the absence of TLR3 does not alter either viral pathogenesis or impair the host's generation of adaptive antiviral responses.[38] However, little is known regarding the pathophysiological functions of rotavirus dsRNA through TLR3.

Rotaviruses have a very specific cell tropism and infect enterocytes only on the tip of the intestinal villi through host receptors.[22] In addition, several mechanisms such as enterotoxigenic effects,[23] involvement of the enteric nervous system[23] and cytoskeleton disorganization[39] have been suggested to be involved in rotavirus infection-induced enterocyte injury. However, the precise pathophysiological mechanism by which rotavirus infection induces severe diarrhea remains unclear.[39,40] In this context, the present study might have answered this question by showing that rotavirus dsRNA induces severe apoptosis and diminishment of wound repair in IEC. We see that this biological function of rotavirus dsRNA was conferred through TLR3 on the basis of the findings that induction of apoptosis and diminishment of wound repair were suppressed by the anti-TLR3 antibody. The current study showed that synthetic dsRNA poly (I : C) also induced activation of signal proteins including ERK1/2 MAPK and IκB-α, expression of cytokines, cell apoptosis, and diminished wound repair in IEC (data in submission). However, it is of interest that the function of rotavirus dsRNA

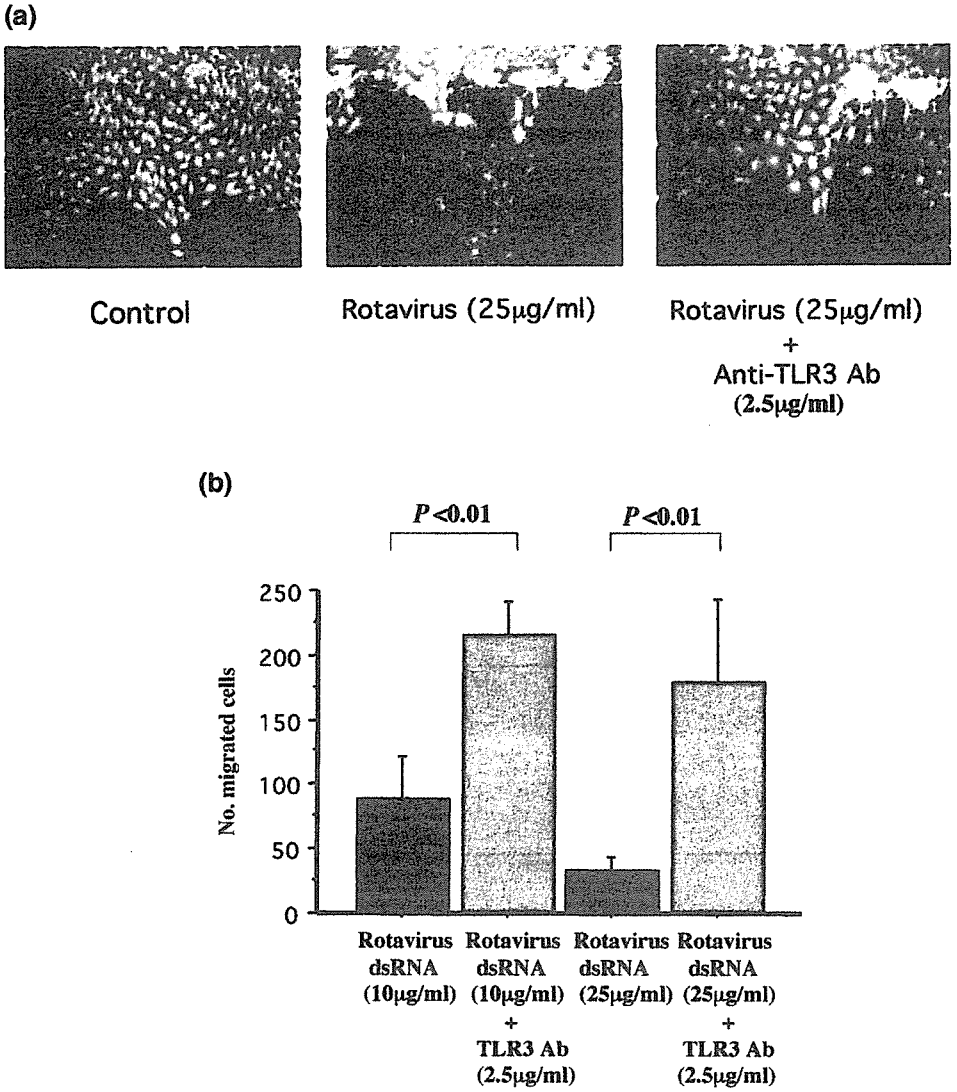

Figure 7 Toll-like receptor 3 (TLR3) antibody suppresses the induction of apoptosis and the diminishment of wound repair in IEC-6 cells stimulated by rotavirus dsRNA. (a) DAPI-stained wound areas 24 h after stimulation with 25 μg/mL of rotavirus dsRNA, 25 μg/m of rotavirus dsRNA in the presence of anti-TLR3 antibody, or no reagent (control) are shown. (b) Confluent monolayers of IEC-6 cells were wounded. The numbers of migrated cells (mean + SD) into wound areas after stimulation with 10 μg/mL or 25 μg/mL of rotavirus dsRNA in the presence of anti-TLR3 antibody are shown. They are compared with the numbers of migrated cells (mean + SD) into wound areas after stimulation with 10 μg/mL or 25 μg/mL of rotavirus dsRNA without anti-TLR3 antibody. (right pointing arrow →), Edge of wound line.

diminishing wound repair in IEC is much stronger than that of poly (I : C). We suggest that rotavirus dsRNA induces apoptosis and kills epithelial cells near the wound line, and therefore only a small number of epithelial cells that survive the rotavirus dsRNA migrate into wound areas, which results in the diminishment of wound repair in IEC. Thus, we hypothesize that rotavirus might induce severe enterocyte injury and diarrhea through two different receptors, namely, the previously identified host receptor and TLR3. Recent studies showed that a MyD88 independent TRIF-mediated pathway is closely involved in the signaling pathways of TLR3[41,42] and cell apoptosis is induced through TLR3-TRIF-RIP-FADD-caspase 8 dependent pathways.[43] The present study also showed that induction of cell apoptosis was significantly suppressed by caspase inhibitor. From these observations, we suggest that rotavirus dsRNA presumably induced apoptosis in IEC through a TLR3-TRIF-RIP-FADD-caspase cascade. In contrast, induction of IFN-β by stimulation with rotavirus dsRNA might partly be involved in TLR3-mediated epithelial apoptosis, because IFN are considered as apoptosis-inducing cytokines.[44]

In the present study, induction of apoptosis and diminishment of wound repair by rotavirus dsRNA were not completely suppressed by the anti-TLR3 antibody. This was probably because of the inadequate neutralizing action of the anti-TLR3 antibody used in the present study, because this antibody has lesser reactivity to rat TLR3 compared with human TLR3. However, we can not exclude the possibility that PKR,[45] an intracellular receptor of virus dsRNA, was partly involved in the biological function of rotavirus dsRNA found in the present study. In contrast, recent studies suggested that TLR3 is also involved in the activation of the PKR pathway.[46]

The present study showed that IFN-γ up-regulates the expression of TLR3 mRNA in IEC. IFN-γ is usually produced by T cells, NK cells and macrophages in response to viral infection.[40] Jiang *et al.* reported that the serum IFN-γ level was significantly elevated 4 days after the onset of illness in children with acute rotavirus

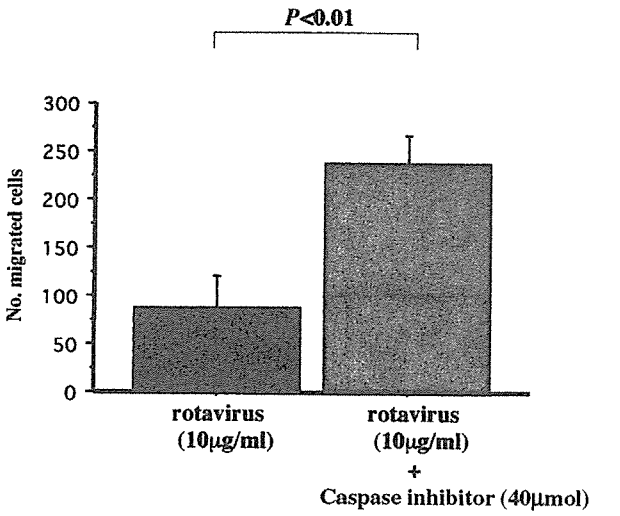

Figure 8 Caspase inhibitor suppresses the diminishment of wound repair in IEC-6 cells stimulated by rotavirus dsRNA. Confluent monolayers of IEC-6 were wounded. The numbers of migrated cells (mean + SD) into wound areas after stimulation with 10 μg/mL of rotavirus dsRNA or 10 μg/mL of rotavirus dsRNA in the presence of 40 μmol of caspase inhibitor are shown.

infection.[40] We suggest that up-regulation of TLR3 in IEC induced by IFN-γ during rotavirus infection might synergistically increase the responsibility of IEC to rotavirus dsRNA.

In summary, the present study showed that crypt-derived IEC strongly responded to rotavirus dsRNA, and severe apoptosis and diminishment of wound repair were induced. Based on these findings, we hypothesize that rotavirus might cause severe diarrhea to the hosts by inducing apoptosis in IEC through TLR3 as well as by directly infecting enterocytes through the previously identified host receptor. In contrast, we suggest that TLR3 might be a novel molecular target for the treatment of rotavirus-induced diarrhea. Further investigations are needed to elucidate this possibility.

ACKNOWLEDGMENTS

This work was supported in part by a grant from Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science. We thank Ms Takako Sasaki and Ms Yuki Watanabe for their technical support in this study.

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JOURNAL OF CLINICAL MICROBIOLOGY, Oct. 2006, p. 3499–3505 Vol. 44, No. 10
0095-1137/06/$08.00+0 doi:10.1128/JCM.01089-06

Molecular Epidemiology of Rotavirus Diarrhea among Children and Adults in Nepal: Detection of G12 Strains with P[6] or P[8] and a G11P[25] Strain

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Received 26 May 2006/Returned for modification 8 June 2006/Accepted 20 July 2006

In anticipation of a rotavirus vaccine in Nepal, this study was undertaken to determine the distribution of the G and P serotypes and electropherotypes of rotaviruses in order to examine if there is any emerging serotype or unusual strain circulating in children and adults in Nepal. Of 1,315 diarrheal stool specimens, rotavirus was detected by an enzyme-linked immunosorbent assay in 116 (17%) of 666 patients less than 5 years of age, in 18 (7%) of 260 patients 5 to 14 years of age, and in 19 (5%) of 358 patients 15 years of age and older. Approximately 75% of rotavirus diarrhea occurred in children less than 5 years of age. Approximately 70% of rotaviruses found in each of the three age groups belonged to serotype G1P[8]. Interestingly, there were 29 (20%) G12 rotaviruses carrying either P[8] or P[6] and one (0.7%) G11 rotavirus carrying an unusual P[25] genotype. RNA polyacrylamide gel electrophoresis discriminated 19 strains (electropherotypes), among which there were three codominant strains carrying G1P[8] and long RNA patterns. Five electropherotypes were discriminated among G12 rotaviruses, all of which had long RNA patterns. The fact that 20% of rotaviruses were G12 strains carrying either P[8] or P[6] and had multiple electropherotypes suggest that G12 strains are not more rare strains but that they pose an emerging challenge to current and future vaccines. The presence of multiple strains as defined by electropherotypes suggests the richness of the rotavirus gene pool in Nepal, where unusual strains may continue to emerge.

Globally, each year, 2 million children are hospitalized (32) and 700,000 children die due to rotavirus diarrhea (8). The majority of these deaths occur in developing countries, although virtually all children experience rotavirus infection by the age of 3 to 5 years irrespective of whether they live in developing countries or developed countries (7, 8). Thus, it is clear that the standards of hygiene and sanitation available and practiced in developed countries are not sufficient to prevent the spread of rotavirus infection within the community. For these reasons, efforts are currently being made at various levels to accelerate the introduction of rotavirus vaccines into the countries where they are most needed (8). Recently, two live, attenuated rotavirus vaccines have gone through large-scale safety and efficacy trials, each involving more than 60,000 children in both developing and developed countries, and both vaccines have shown encouraging results (36, 46).

The genome of rotavirus comprises 11 segments of double-stranded RNA, which are encased within a triple-layered capsid. The outermost capsid of the virion is composed of two independent neutralization antigens, VP7 and VP4, which define the G serotype and the P serotype, respectively (8). Classification based on the VP7 sequence corresponds to serological classification, so the same numbers are assigned for both genotype and serotype. However, with respect to VP4, the classification defined by molecular methods does not completely agree with the classification defined by serological assays.

It is generally believed that serotype-specific immunity plays a role in protection against disease, so the epidemiology of G and P serotypes of circulating strains forms a critical knowledge base for the development and implementation of rotavirus vaccines (16, 38). For this purpose, regional rotavirus strain surveillance networks have been established in Africa (African Rotavirus Network) and in Asia (Asian Rotavirus Surveillance Network) (3, 43). In addition, the identification of electropherotypes provides complementary information on the genomic diversity of rotavirus strains circulating in the region, because the migration pattern of 11 segments of double-stranded RNA on a polyacrylamide gel helps define an individual strain of rotavirus (13, 25, 28, 29).

Since natural infection with rotavirus does not provide complete protection against subsequent infections, older children and adults may repeatedly be infected, with some of these

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