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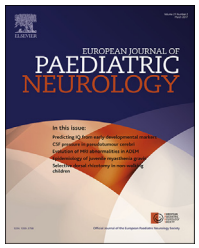

Original article

NSP4 antibody levels in rotavirus gastroenteritis patients with seizures

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ABSTRACT

Background: Rotavirus nonstructural protein 4 (NSP4) has been suggested as a pathogen of rotavirus-associated seizures. We investigated pre-existing serum antibodies against NSP4 and VP6 (the most highly immunogenic rotavirus protein) in patients with rotavirus gastroenteritis and its correlation with the occurrence of seizures.

Methods: With an enzyme-linked immunosorbent assay, IgG and IgA titers against NSP4 (genotype [A] and [B]) and VP6 were measured in acute-phase sera of 202 children aged 0.5–6.0 years with rotavirus gastroenteritis. The clinical characteristics and antibody levels were compared between patients with (seizure group) and without seizures (non-seizure group).

Results: The non-seizure and seizure groups comprised 173 and 29 patients, respectively. Age, sex, hospital stay, presence of fever, white blood cell counts, C-reactive protein, vaccine status, IgG/IgA titers for VP6, and IgA titers for both NSP4s did not differ between the groups. The seizure group showed a lower level of IgG against NSP4 [A] (184.5 *vs.* 163.0 U/mL; $P = 0.03$) and NSP4 [B] (269.0 *vs.* 196.0 U/mL; $P = 0.02$). Delayed sampling time from the onset of gastroenteritis symptoms (3 vs. 2 days; $P = 0.02$) and lower serum sodium level (133.4 vs. 136.3 mEq/L; $P < 0.01$) were observed in the seizure group. Even after adjusting these factors, anti-NSP4 [A] IgG (OR 2.56 per 100 U/mL increment; 95% CI, 1.20–5.26, $P = 0.01$) and anti-NSP4 [B] IgG (OR 1.51 per 100 U/mL-increment; 95% CI, 1.04–2.22, $P = 0.03$) were independently associated with protection against seizures.

Conclusions: Serum anti-NSP4 IgG might protect rotavirus-associated seizures.

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1. Introduction

Although rotavirus primarily causes gastroenteritis, a wide spectrum of neurological manifestations has been recognized.[1,2] The most common neurological manifestation of rotavirus infection is benign seizures following diarrhea, which occurs in 2.1%–7.7% of pediatric patients with rotavirus gastroenteritis.[3,4] However, little about the underlying pathophysiology of rotavirus-associated seizures is known.

Rotavirus nonstructural protein 4 (NSP4) has been suggested as a causative agent of the neurological manifestations of rotavirus.[5,6] NSP4 is crucial for rotavirus pathogenesis and acts as an enterotoxin, inducing secretory diarrhea by increasing the intracellular calcium concentration.[7,8] NSP4 triggers the release of neurotoxic materials including nitric oxide and reactive oxygen species.[9] NSP4 is widely distributed in cell bodies and dendrites of rotavirus-infected neurons.[10] NSP4 has been shown to have inherent membrane-destabilizing properties.[11] However, no study to date has demonstrated a direct role for NSP4 in rotavirus-associated seizures.

Serum antibodies to NSP4 protect mice from diarrhea after challenge with both NSP4 and virulent rotavirus.[12,13] A study that analyzed sera from children with acute diarrhea showed a positive correlation between pre-existing serum antibodies against NSP4 and protection from rotavirus diarrhea.[14] These findings not only suggested a role for serum antibodies against NSP4 in protection from rotavirus diarrhea, but also emphasized the importance of NSP4 in the pathogenesis of diarrhea. Thus, we hypothesized that the role of NSP4 in the pathogenicity of rotavirus-associated seizures can be evaluated by measuring levels of serum antibodies to NSP4. If patients with rotavirus-associated seizures have lower pre-existing levels of antibodies to NSP4 than do patients with rotavirus infection but without seizures, we can suggest that antibodies to NSP4 play a role in seizure protection. This would also suggest a possible role for NSP4 in the pathogenicity of rotavirus-associated seizures. However, whether antibodies to NSP4 are type-specific or cross-reactive has not been elucidated. Among the six genotypes ([A]–[F]) of the NSP4 gene, genotypes [A] and [B] are the most common among rotavirus strains infecting humans.[15] To evaluate our hypothesis, we measured levels of IgG and IgA against NSP4 proteins of genotype [A] and [B] in acute-phase sera of patients with rotavirus gastroenteritis and compared the titers in terms of the occurrence of seizures. However, it is possible that anti-NSP4 antibodies reflect merely a recent infection. Antibodies to rotavirus inner capsid VP6 protein have been considered a marker of recent rotavirus infection because VP6 protein is the most immunogenic and is highly conserved among all group A rotaviruses.[16] Thus, we also measured and compared the levels of IgG and IgA against VP6 between the two groups to confirm that serum NSP4 antibodies themselves mediate protection against seizures.

2. Materials and methods

2.1. Subjects and serum samples

We retrospectively evaluated patients' data and included those who met the following inclusion criteria: 1) children 0.5–6.0 years of age who were diagnosed with rotavirus gastroenteritis in Gyeongsang National University Hospital from January 1999 to December 2012 and 2) children for whom acute-phase serum samples were available. Diagnosis of rotavirus infection was based on detection in stool samples by enzyme-linked immunoassay (ELISA). Acute-phase serum samples were defined as samples obtained within 5 days of the onset of diarrhea and which had been stored at −70 °C in the National Biobank of Korea. The patients were divided into two groups: those with seizures (seizure group) and those without seizures (non-seizure group). Patients with pre-existing neurologic conditions were excluded from the seizure group. Clinical data obtained included age, sex, time of sampling after the onset of diarrhea, maximal body temperature during illness, length of hospital stay, rotavirus vaccination, and laboratory findings. The laboratory results were obtained at the time of admission. The Institutional Review Board of Gyengsang National University Hospital (GNUH 2012-11-005-004) approved this retrospective study.

2.2. IgA and IgG ELISA

The ELISA antigens used in this study were histidine-tagged recombinant (r) NSP4 proteins (residues 52–175) from strains SA11 and Wa (genotypes [A] and [B], respectively) and histidine-tagged rVP6 protein (residues 1–397) from strain SA11, which are commercially available (Cusabio Biotech Co., Wuhan, China). For antibody assays, 96-well microtiter plates with high binding capacity (EIA plate, Costar 3590; Corning, NY, USA) were coated overnight at 4 °C with NSP4s (2.5 μg/mL for IgG, 0.25 μg/mL for IgA) or VP6 (2.5 μg/mL for IgG, 0.25 μg/mL for IgA) in 50 μL of 50-mM carbonate-bicarbonate buffer (pH 9.6; Bioshop, Burlington, ON, Canada). To establish a standard curve, serial two-fold dilutions of 1:10 to 1:10,240 diluted pooled reference sera from rotavirus-infected patients were added to duplicate wells coated with either NSP4 or VP6. The relation between reciprocal number of the dilution and optical density (OD) at 490 nm was approximated by a 4-parameter hyperbolic curve (SOFTmax PRO ver.3; Molecular Devices Co., Sunnyvale, CA). If the R^2 of that curve fit was less than 0.997, another standard serum aliquot was thawed and the entire procedure repeated. Based on the constants of the equation, antibody units were assigned to the standard as the reciprocal of the dilution giving an $OD_{490} = 1$. Through this method, the concentration of each antibody in the reference pooled sera was assigned as follows: IgG against NSP4 [A] (300 U/mL), NSP4 [B] (400 U/mL), and VP6 (400 U/mL), and IgA against NSP4 [A] (200 U/mL), NSP4 [B] (300 U/mL), and VP6 (400 U/mL). To initiate the assay for patient serum samples to be tested, plates were blocked with 150 μL/well of blocking buffer containing 3% bovine serum albumin in PBS with Tween (PBST) (BioShop) for 2 h at 37 °C. Patient sera were added to duplicate wells containing NSP4 or VP6 protein, and then the plates were incubated for 1 h at 37 °C. After four washes with buffer (PBST), peroxide-conjugated goat anti-human IgG (Bethyl Laboratories Inc., Montgomery, TX, USA) and IgA (Bethyl Laboratories Inc.) were added to the wells, followed by incubation at 37 °C for 1 h. The plates were washed with PBST, and o-nitrophenyl phosphate was added to the wells (Sigma–Aldrich). The reaction was stopped by