Evaluation of Serum Antibody Responses against the Rotavirus Nonstructural Protein NSP4 in Children after Rhesus Rotavirus Tetravalent Vaccination or Natural Infection

Esmeralda Vizzi, Eva Calviño, Rosabel González, Irene Pérez-Schael, Max Ciarlet, Gagandeep Kang, Mary K. Estes, Ferdinando Liprandi, and Juan E. Ludert

The immune response elicited by the rotavirus nonstructural protein NSP4 and its potential role in protection against rotavirus disease are not well understood. We investigated the serological response to NSP4 and its correlation with disease protection in sera from 110 children suffering acute diarrhea, associated or not with rotavirus, and from 26 children who were recipients of the rhesus rotavirus tetravalent (RRV-TV) vaccine. We used, as antigens in an enzyme-linked immunosorbent assay (ELISA), affinity-purified recombinant NSP4 (residues 85 to 175) from strains SA11, Wa, and RRV (genotypes A, B, and C, respectively) fused to glutathione S-transferase. Seroconversion to NSP4 was observed in 54% (42/78) of the children who suffered from natural rotavirus infection and in 8% (2/26) of the RRV-TV vaccine recipients. Our findings indicate that NSP4 evokes significantly (P < 0.05) higher seroconversion rates after natural infection than after RRV-TV vaccination. The serum antibody levels to NSP4 were modest (titers of ≤200) in most of the infected and vaccinated children. A heterotypic NSP4 response was detected in 48% of the naturally rotavirus-infected children with a detectable response to NSP4. Following natural infection or RRV-TV vaccination, NSP4 was significantly less immunogenic than the VP6 protein when these responses were independently measured by ELISA. A significant (P < 0.05) proportion of children who did not develop diarrhea associated with rotavirus had antibodies to NSP4 in acute-phase serum, suggesting that serum antibodies against NSP4 might correlate with protection from rotavirus diarrhea. In addition, previous exposures to rotavirus did not affect the NSP4 seroconversion rate.

Rotaviruses are the most important cause of severe diarrhea in infants and young children worldwide. Great efforts are being made to develop an effective vaccine that could reduce significantly the severity of the episodes of diarrhea in children. However, the immune mechanisms induced by natural rotavirus infection or immunization that lead to protection remain partially understood. Previous studies in animal models and humans, which investigated the effects of antivotavirus serum antibodies in protection from rotavirus disease or infection, have often yielded conflicting results about the correlates of protection against rotavirus disease (10, 13, 16, 23, 30).

Whether neutralizing antibody responses to outer capsid proteins VP4 and VP7 play a critical role in protective immunity against rotavirus-associated diarrhea remains controversial. Early studies focused on serum antibody responses to different G (VP7) serotypes, as measured by neutralization assays, and suggested that serotype cross-reactive immunity plays an important role in protection, but this has been difficult to demonstrate in humans (7, 13, 16, 29). Nonneutralizing antibodies against the inner capsid protein VP6 have also been shown to protect mice against disease after DNA vaccination or virus-like particle administration (3, 6, 22). The role of nonstructural proteins in the induction of protective immunity has not been extensively studied in rotavirus infections, but it has recently emerged from studies of infections by flavivirus and hepatitis C virus (9, 12).

NSP4, a rotavirus nonstructural glycoprotein, plays a role in rotavirus morphogenesis (1) and is the viral enterotoxin capable of inducing secretory diarrhea in infant mice (2). Sequence analyses of the rotavirus enterotoxin NSP4 from humans and animals have revealed the existence of six (A to F) distinct NSP4 genotypes. Although both human and animal rotavirus strains can be grouped in the same NSP4 genotype, known human NSP4 sequences belong to NSP4 genotypes A, B, and C (8, 20). Passively acquired antibodies to NSP4 have been demonstrated to reduce both the incidence and severity of diarrhea in infant mouse pups challenged with virulent rotavirus (2), suggesting that the immune response to NSP4 could modulate rotavirus diarrhea in humans. However, the exact role of NSP4 in protection from rotavirus disease in humans has not been fully investigated. Studies with a limited number of subjects have revealed variable levels of immunogenicity of NSP4 after natural infection or vaccination, probably due to the use of the different assays or antigens employed (17, 25, 26, 33). Moreover, the response to NSP4 appears to be heterotypic, meaning that antibodies to NSP4 recognize one or more of the known human NSP4 genotypes (25, 33). It is unknown if
the immune response against NSP4 plays a role in protection from diarrhea. The aim of the present study was to determine the total serum antibody responses to NSP4 in children following rhino rotavirus tetravalent (RRV-TV) vaccination or natural rotavirus infection, and to evaluate whether the NSP4 immune response correlates with protection against rotavirus diarrhea.

MATERIALS AND METHODS

Subjects and serum samples. The study population comprised the following: (i) 2-, 3-, and 4-month-old children who received three doses at high titer (105 PFU of each component) (n = 11) or three doses at low titer (10^4 PFU) (n = 15) of RRV-TV and children who received placebo (n = 15) enrolled during an earlier phase II study conducted in 1991 in Caracas, Venezuela (11); (ii) 78 children, not previously vaccinated (average age, 10.1 months; range, 1 to 59 months), with acute watery diarrhea due to natural rotavirus infection; and (iii) 32 children (average age, 15.2 months; range, 1 to 60 months) suffering from rotavirus-negative diarrhea episodes. The diarrheic children were patients who attended the Ciudad Hospitalaria “Dr. Enrique Tejera” (CHITE) in Carabobo State, Venezuela (27).

Serum samples of RRV-TV- or placebo-vaccinated children with no acute diarrhea episode were collected before the first dose and 1 month after the first and third doses, and those from infants with diarrhea were obtained during the episodes within the first week after the onset of diarrhea. A convalescent-phase serum sample was obtained from each child 3 to 4 weeks after the episode. Acute diarrhea was considered with the occurrence of three or more bowel movements within a 24-h period, with a decrease in stool consistency. Stool samples from diarrheic infants were obtained within 72 h after the onset of symptoms and were previously examined for the presence of rotavirus antigen by enzyme-linked immunosorbent assay (ELISA) (18). Both stool and serum samples were kept frozen (−20°C) until tested.

Construction of plasmids for recombinant NSP4 (rNSP4) protein production in E. coli. Reverse transcriptase was used to generate gene 10 cDNAs, encoding the NSP4 of simian SA11 (genotype A), human Wa (genotype B), and simian RRV (genotype C) strains as described elsewhere (8). Full-length amplified DNA fragments were generated by PCR and subsequently cloned into the prokaryotic expression vector pGEX-2TK (Amersham Pharmacia Biotech, UK Ltd., Buckinghamshire, England) and a monoclonal antibody (Mab), B4-2/55 (24), specific to NSP4 (kindly supplied by H. B. Greenberg, Stanford University Medical School, Palo Alto, CA). A colorimetric standard method using alkaline phosphatase-conjugated antibodies, 5-bromo-4-chloro-3-indolyl phosphate, and p-nitroblue tetrazolium chloride was used to detect the blotting reactions.

ELISA to detect antibodies against rNSP4. For NSP4 antibody assays, 96-well flat-bottomed microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA) were coated with reduced glutathione as previously described (21) with some modifications. Briefly, the plates were incubated overnight at 4°C with 2% bovine hemoglobin (Sigma Chemical Co., St. Louis, MO) in 0.05 M sodium carbonate, pH 9.6, at 4°C. After 6 washes with PBS, the heterobifunctional cross-linker sulfoN-succinimidyl 4-(3-aminopropyl)butyrate (SSMBP) (Pierce, Rockford, IL) was added by incubating with the coating solution for 1 h. After washing, the plates were incubated with 0.1 mM of cross-linker in PBS and incubating this mixture for 1 h at room temperature. After six washes with PBS, a freshly prepared solution of 10 mM reduced glutathione in degassed 10 mM sodium phosphate, 0.15 M sodium chloride, 1 mM EDTA, pH 6.7, was added and the mixture was incubated overnight at 4°C. The glutathione-coated plates were then washed 6 times with PBS containing 0.05% Tween 20 (PBS-T) and were used directly. Antibody reaction was performed in each plate by adding 100 μl of patient serum, diluted 1:25, to each well of the coated GST and rNSP4 (85–175) proteins. Two positive internal controls, a goat anti-GST serum (Amersham Pharmacia Biotech, UK Ltd., Buchinghamshire, England) and a monoclonal antibody (Mab), B4-2/55, were included on each plate, serum diluted 1:100 in PBS-T, and were used directly. Affinity-purified GST and rNSP4 (85–175) proteins were immobilized on the plates by adding 100 μl of patient serum to wells containing 100 μl of reduced glutathione as previously described (21) with some modifications. Briefly, the plates were incubated overnight with 2% bovine hemoglobin (Sigma Chemical Co., St. Louis, MO) in 0.05 M sodium carbonate, pH 9.6, at 4°C. After 6 washes with PBS, the heterobifunctional cross-linker sulfoN-succinimidyl 4-(3-aminopropyl)butyrate (SSMBP) (Pierce, Rockford, IL) was added by incubating with the coating solution for 1 h. After washing, the plates were incubated with 0.1 mM of cross-linker in PBS and incubating this mixture for 1 h at room temperature. After six washes with PBS, a freshly prepared solution of 10 mM reduced glutathione in degassed 10 mM sodium phosphate, 0.15 M sodium chloride, 1 mM EDTA, pH 6.7, was added and the mixture was incubated overnight at 4°C. The glutathione-coated plates were then washed 6 times with PBS containing 0.05% Tween 20 (PBS-T) and were used directly. Antibody reaction was performed in each plate by adding 100 μl of patient serum, diluted 1:25, to each well of the coated GST and rNSP4 (85–175) proteins. Two positive internal controls, a goat anti-GST serum (Amersham Pharmacia Biotech, UK Ltd., Buchinghamshire, England) and the anti-NSP4 MAb B4-2/55, were included on each plate, serum diluted 1:100, to validate the assay and to ensure the reproducibility of the results. After incubation at room temperature for 1 h, the plates were washed six times with PBS-T and were probed with the goat anti-human IgG antibody. This secondary antibody was goat anti-mouse IgG horseradish peroxidase (rabbit anti-human polyclonal immunoglobulins [Igs] diluted 1:8,000; or rabbit anti-goat IgG [Sigma Chemical Co., St. Louis, MO]) or goat anti-mouse IgA, -G, and -M [Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD] diluted 1:4,000 was added. After incubation for 1 h at room temperature, the plates were washed six times with PBS-T and the reaction was developed with 3,3',5,5'-tetramethylbenzidine substrate system (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD), stopping the reaction after 10 min with 1.0 M phosphoric acid. The absorbance was measured within 15 min at 450 nm on an ELISA plate reader (Sunrise, TECAN, Austria GmbH). The net optical density (OD) value of each test for the NSP4 antigen was obtained by subtracting the OD of serum reacting with GST protein from the OD of serum reacting with each rNSP4 (85–175) protein, to allow determination of the proportion of signal due to antibodies to NSP4 alone. The antibody titer for each antibody was defined as the reciprocal of the highest dilution giving a net OD value greater than the calculated cutoff, 0.1. Serocconversion was defined as a ≥3-fold rise in antibody titer between consecutive serum samples from the same individual. This seroconversion criterion was defined after repeated testing of a subset of positive and negative human serum samples, to compensate for the moderate background values found when serum samples from humans were tested.

ELISA to detect anti-vNp6 antibodies. Antibodies to rotavirus capsid protein VP6 were determined by ELISA using a purified MAb against VP6, 4B12D2 (19), to coat the plates. Tissue culture-passaged rotavirus OSU and mock-infected MA104 cell lysates, pretreated with 1.0 mM EDTA, were used as antigens in alternate columns to test serially diluted human sera starting at 1:25. A positive...
control human serum was included in each plate to verify the performance of the assay and to ensure the reproducibility of the results. A sample was considered positive at a given dilution if the OD value of the well coated with OSU was 2-fold the value corresponding to the well with mock-infected cells. The endpoint antibody titer of each sample was expressed as the reciprocal of the highest dilution that had a corrected $A_{450}$ value (OD value in OSU-coated well / OD value in mock-infected cell antigen-coated well) greater than the calculated cutoff value of 0.16. This value was chosen because it was greater than 2 standard deviations above the mean of the background values obtained testing 10 negative serum samples. Seroconversion was defined as a 4-fold rise in antibody titer between consecutive serum samples from the same individual.

**Statistical analysis.** Data were analyzed by EpiInfo software (version 3.2.2; CDC, Atlanta, GA) for the comparisons of the seroconversion rates between selected groups by 2 by 2 tables with $\chi^2$ test or Fisher's exact test (two-tailed) when the size sample was less than 5. McNemar's test was used to evaluate correlated proportions on the same group of subjects. Pearson's correlation coefficients ($r$ values) were calculated to analyze the correlation between anti-body titers to NSP4 or VP6 and protection from diarrhea. Student's test was applied to logarithmically transformed (base 10) titers (convalescent-phase serum titer / acute-phase serum titer) for comparisons of antibody response in children with rotavirus-associated diarrhea. Statistical significance was assessed at $P < 0.05$ for all the analyses in this study.

**RESULTS**

**Cloning, expression, and production of the rNSP4$_{(85-175)}$ proteins.** Recombinant GST-NSP4$_{(85-175)}$ fusion proteins from the rotavirus SA11, Wa, and RRV strains, representing NSP4 genotypes A, B, and C, respectively, and GST protein alone were expressed in *E. coli* BL21(DE3) cells. Approximately 25 mg of the recombinant proteins was produced per liter of bacterial culture with each of the clones. When visualized by PAGE, the molecular masses of the rNSP4$_{(85-175)}$ and GST proteins were approximately 34 kDa and 26 kDa, respectively (Fig. 1A). The Western blotting analysis with anti-GST goat serum antibodies and anti-NSP4 MAb B4-2/55 confirmed the identity of the preparations. Anti-GST serum antibodies bound to all rNSP4$_{(85-175)}$ and to GST proteins, while the anti-NSP4 MAb B4-2/55 reacted specifically with 34-kDa rNSP4$_{(85-175)}$ proteins and not with 26-kDa GST (Fig. 1B).

**Serum antibody response to rNSP4 in unvaccinated children with diarrhea.** As shown in Table 1, the seroconversion rate to rNSP4 was significantly higher ($P < 0.05$) in children with rotavirus-associated diarrhea than in children with non-rotavirus-associated diarrhea (54 versus 6%). Reactivity to more than one NSP4 genotype was detected in 20 of the 42 (48%) children infected with rotavirus who responded to rNSP4, and the most common NSP4 antibody response was directed against the genotype B (Wa) antigen (100%). The NSP4 antibody titers were $>200$ in most of the children who responded against one or more antigens of NSP4 (ranging from 50% to 62%, depending on the NSP4 antigen examined) (Fig. 2). No significant difference ($P > 0.05$) was observed in seroconversion rates to rNSP4 among rotavirus-infected children of different ages (data not shown).

**Serum antibody response to rNSP4 in RRV-TV-vaccinated children.** To measure the antibody response attributed solely to vaccination, 26 children who received the RRV-TV vaccine...
TABLE 1. Seroconversion rates to rNSP4_{(85–175)} and VP6 in different groups of children

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of subjects</th>
<th>No. (%) of subjects with total serum antibody response toa:</th>
<th>NSP4 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>VP6 NSP4</td>
<td>A  B  C</td>
</tr>
<tr>
<td>Not vaccinatedd</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotavirus-associated diarrhea</td>
<td>78</td>
<td>56 (72) 42 (54)</td>
<td>16 (38) 42 (100) 13 (31)</td>
</tr>
<tr>
<td>Non-rotavirus-associated diarrhea</td>
<td>32</td>
<td>4 (13) 2 (6)</td>
<td>2 (100) 2 (100) 1 (50)</td>
</tr>
<tr>
<td>RRV-TV vaccinatedd</td>
<td>26</td>
<td>8 (31) 2 (8)</td>
<td>2 (100) 1 (50) 1 (50)</td>
</tr>
<tr>
<td>Placebo recipientsd</td>
<td>15</td>
<td>2 (13) 0 (0)</td>
<td>0 (0) 0 (0) 0 (0)</td>
</tr>
</tbody>
</table>

a Data are the number (percentage) of subjects with total serum antibody response to antigen by ELISA. A seroresponse was defined as a ≥4-fold increase in titer to VP6 and as a ≥3-fold increase in titer to NSP4.

b Seroconversion rates based on study of acute- and convalescent-phase serum samples.

c Seroconversion rates after administration of three doses of RRV-TV vaccine or placebo in children 8 weeks old (mean age) not suffering from diarrhea.

d $P < 0.05$ by $\chi^2$ test for comparison of seroconversion rates to VP6 and NSP4 of children who developed rotavirus-associated diarrhea with those of children with non-rotavirus-associated diarrhea or with those of RRV-TV-vaccinated children. $P < 0.05$ by McNemar’s test for comparison of correlated proportions of seroconversion rate to VP6 with that to NSP4 in children with rotavirus-associated diarrhea and in RRV-TV-vaccinated children. $P > 0.05$ by Fisher’s exact test (two tailed) for comparison of the VP6 and NSP4 seroconversion rates of placebo recipient children with those of children with non-rotavirus-associated diarrhea.

and did not develop diarrhea during the follow-up period (8 months total) were studied. Of the 26 children, only 2 who had received three doses of vaccine at low and at high titer, respectively, developed antibodies to rNSP4 after vaccination (Table 1), and antibody responses of both children were di-tively, developed antibodies to rNSP4 after vaccination received three doses of vaccine at low and at high titer, respec-tively, to NSP4 A, B, or C antigen. The seroconversion rate to VP6 was significantly higher ($P < 0.05$) than that detected against rNSP4 (geometric mean titer [GMT] = 650 to VP6 versus GMT = 235, 240, or 290, respectively, to NSP4 A, B, or C antigen). The seroconversion rate observed to VP6 was higher than that detected to rNSP4 not only after natural infection but also after RRV-TV vaccination (Table 1).

The mean antibody titers to VP6 in children who developed a rotavirus-associated-diarrhea were significantly higher ($P < 0.05$) than those detected against NSP4 (geometric mean titer [GMT] = 650 to VP6 versus GMT = 650 to VP6 versus GMT = 235, 240, or 290, respectively, to NSP4 A, B, or C antigen). The seroconversion rate observed to VP6 was higher than that detected to rNSP4 not only after natural infection but also after RRV-TV vaccination (Table 1).

Presence of baseline antibodies against rNSP4 or VP6 as a correlate of protection. The role of serum antibodies as a marker of protection against rotavirus diarrhea was evaluated in children suffering from diarrhea, according to the occur-

TABLE 2. Preexisting antibodies to NSP4 or VP6 proteins in children with acute diarrhea associated or not with rotavirus

<table>
<thead>
<tr>
<th>Baseline antibody</th>
<th>No. (%) of subjects with specificity of antibodies toa:</th>
<th>NSP4$^b$</th>
<th>VP6$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RV infected (n = 78)</td>
<td>RV uninfected (n = 32)</td>
<td>RV infected (n = 78)</td>
</tr>
<tr>
<td>Detectable</td>
<td>0 (0)</td>
<td>6 (19)</td>
<td>14 (18)</td>
</tr>
<tr>
<td>Not detectable</td>
<td>78 (100)</td>
<td>26 (81)</td>
<td>64 (82)</td>
</tr>
</tbody>
</table>

a Data are the number (percentage) of subjects who developed diarrhea associated or not with rotavirus according to the presence or absence of total antibodies to NSP4 or VP6 in the acute-phase serum, as revealed by ELISA. Pearson’s correlation coefficients were calculated for evaluating the correlation between presence or absence of baseline serum antibodies and the protection from rotavirus diarrhea.

b $P = 0.005$ and $r = 0.27$ for antibodies directed against rNSP4_{(85–175)} from SA11, $P = 0.014$ and $r = 0.23$ for antibodies directed against rNSP4_{(85–175)} from SA11, and $P = 0.014$ and $r = 0.23$ for antibodies directed against rNSP4_{(85–175)} from RRV.

c $P = 0.002$ and $r = 0.29$ for antibodies directed against VP6.

FIG. 2. Serum antibody titers against NSP4 of different genotypes from 78 children convalescing from rotavirus-associated diarrhea.
TABLE 3. Seroconversion rates to NSP4 or VP6 proteins, in children suffering from rotavirus-associated diarrhea, according to the presence or absence of baseline antibody to rotavirus

<table>
<thead>
<tr>
<th>Baseline antibody</th>
<th>No. of subjects</th>
<th>No. (%) of subjects with seroconversion to:&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NSP4</td>
</tr>
<tr>
<td>Detectable</td>
<td>14</td>
<td>7 (50)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Not detectable</td>
<td>64</td>
<td>35 (55)</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data are the number (percentage) of subjects who seroconverted to NSP4 or VP6, according to the presence or absence of total antibodies to NSP4 and/or VP6 in acute-phase serum, as revealed by ELISA.

<sup>b</sup> *P* < 0.05 by *χ*<sup>2</sup> test for the comparison among subjects who developed a sororesponse to NSP4.

<sup>c</sup> *P* < 0.05 by Fisher’s exact test for the comparison among subjects who developed a sororesponse to VP6.

dherence of an episode associated or not with rotavirus. Table 2 shows the prevalence of total serum antibodies against rNSP4 or against VP6 in the serum samples collected in the acute phase (baseline antibodies) from 78 children who developed rotavirus-associated diarrhea and 32 who suffered from diarrhoea not associated with rotavirus. A detectable baseline level of antibodies was defined by the presence of an ELISA signal (net OD) to VP6 or to rNSP4 greater than the calculated cutoff at the lower dilution of serum tested (as explained in Materials and Methods). As shown in Table 2, a positive correlation between the presence of baseline antibodies against rNSP4 and protection from rotavirus diarrhea was observed, as a significant proportion (19%) of children who did not develop diarrhoea associated with rotavirus had baseline antibodies to rNSP4, in comparison with those who developed rotavirus-associated diarrhea (*r* < 0.3, *P* < 0.05; Pearson’s correlation coefficient). Similarly, a significant correlation between the presence of baseline antibodies to VP6 and protection from rotavirus diarrhea was detected, since most of the children (53%) who did not develop diarrhea associated with rotavirus had VP6 baseline antibodies, in comparison with the lower percentage (18%) of those who developed rotavirus-associated diarrhea (*r* < 0.3, *P* < 0.05, Pearson’s correlation coefficient).

Effect of prior exposure to rotavirus infection on the immune responses to rNSP4 and VP6. To evaluate the effect that prior exposure to rotavirus infection had on the immune response to NSP4 and VP6, the presence of baseline antibodies against both proteins was compared with the seroconversion rates to rNSP4 and VP6 detected in 78 children who developed rotavirus-associated diarrhea. Detectable antibodies to either antigen constituted evidence of prior exposure to rotavirus, but because no children had detectable levels of baseline antibodies against rNSP4, all of the results were based on the detection results of baseline antibodies to VP6. As shown in Table 3, the seroconversion rates to rNSP4 were similar (*P* > 0.05) in children with or without detectable baseline antibodies (50 versus 55%), while the seroconversion rate to VP6 was significantly lower (*P* < 0.05) among those with detectable baseline antibodies than it was among children without detectable baseline antibodies (43 versus 78%). Such an immunomodulatory effect was not observed in the group of RRV-TV-vaccinated children. Rotavirus antibodies detected in sera collected before the first dose of vaccine, presumably passively acquired antibodies of maternal origin, had no effect on the seroconversion rates obtained to either VP6 or rNSP4 (data not shown).

DISCUSSION

Since NSP4 was reported to function as a viral enterotoxin (2), efforts are being made to understand its role in rotavirus pathogenesis and immunity. Little information is available about the contribution of NSP4 or any other rotavirus non-structural protein to the induction of a protective immune response after natural infection or vaccination (14, 15, 26, 32, 33). Several techniques have been applied to quantify the level of NSP4 antibody response in children (15, 17, 25, 26, 33). However, the results have been variable, probably due to differences in sample size, antigens used, and the nature and sensitivity of the applied assays. Because divergence in NSP4 amino acid sequence might also have affected the detection rate of the NSP4 antibody responses in those previous studies (15, 17, 26), we decided to include in our assay rNSP4 antigens representative of the three known human rotavirus NSP4 genotypes (A, B, and C). The use of GST fusion proteins captured by immobilizing glutathione on ELISA microtiter plates probably allows antigen preservation in a more native conformation than does a simple direct adsorption to the plastic surface (21). In addition, it enables the testing of large numbers of specimens and quantification in parallel of the immune responses against all three types of NSP4 antigens.

So far, an evaluation of the NSP4 seroconversion rates in children after rotavirus natural infection and vaccination using the same assay to detect antibodies has not been carried out. In this report, we compared the NSP4 and VP6 antibody responses in children with rotavirus-associated diarrhea and in children following RRV-TV immunization. The seroconversion rates to NSP4 observed after natural infection and after vaccination were both comparable to those described in previous studies (25, 33). Rotavirus natural infection was observed to elicit a sororesponse against NSP4 in 54% of the children, a rate similar to that reported by Ray et al. (25) in a study by ELISA including 40 rotavirus-infected children conducted in New Delhi, India. In our study, the seroconversion rate to NSP4 after RRV-TV vaccination was significantly lower (*P* < 0.05) than those observed after natural infection. Low seroconversion rates to NSP4 after vaccination were also found by Yuan et al. (33) in a study based on the use of an immunocytochemical staining assay which included 12 RRV-TV-vaccinated Finnish children.

In the present report, the seroconversion rates to NSP4 were significantly lower than those detected to VP6 in diarrheic children infected with rotavirus and in RRV-TV vaccine recipients. This result contrasts with that obtained by Ray et al. (25), who described a seroconversion rate to NSP4 as high as the one obtained for the whole virus in children with rotavirus-associated diarrhea. Differences in the design of the assays used may in part account for this variation. On the other hand, Yuan et al. (33) found percentages of sororesponse to NSP4 lower than those to VP6 after RRV-TV vaccination, in agreement with our data. Taken together, our data indicate that RRV-TV vaccine can induce serum antibodies to NSP4 and...
in the induction of a protective response. Other host effectors, such as rotavirus-specific B, CD4⁺, and CD8⁺ T cells, can mediate antiviral activity in humans, and the relative importance of each appears to be dependent on the immunogen and route of administration, as has been demonstrated in the adult mouse model (31). Further studies are needed to assess if NSP4 antibodies are directly involved in disease protection after natural infection or RRV-TV vaccination.

In contrast to the results obtained by Ray et al. (25), our results suggest that prior exposures to rotavirus limit the response to VP6, but not to NSP4, during natural rotavirus infection. Preexisting antibodies might affect the induction of an immune response by suppressing or enhancing the secondary antibody response, depending on the level and specificity of such antibodies, as suggested by other authors (33). Moreover, such responses might depend on the nature or immunogenic features of the antigen. NSP4 and VP6 may induce different humoral (immunologic memory) or cellular (immunosuppressor) immune mechanisms during a first infection, which would result in dissimilar secondary immune responses.

To conclude, our findings indicate that rotavirus nonstructural protein NSP4 evokes moderate seroconversion rates after natural infection but lower rates after RRV-TV vaccination. Also in both cases, the level of response appears to be modest. A previous exposure to rotavirus does not appear to limit the NSP4 response. Finally, our results suggest that NSP4 antibodies acquired after natural infection correlate with protection from rotavirus diarrhea.

ACKNOWLEDGMENTS

This work was partially supported by Proyecto S1-200100906 and Proyecto Iniciativa Científica del Milenio (FONACIT)—Ministerio de Ciencias y Tecnología—Venezuela, research grants DK30144 and DK56338 from the National Institutes of Health, and a grant from the Wellcome Trust.

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VP6. However, the vaccination appears to be less efficient than natural infection in inducing antibodies to both proteins. The differences found in seroconversion rates to both proteins NSP4 and VP6 in naturally rotavirus-infected and RRV-TV-vaccinated children are difficult to explain. Most likely they reflect differences in the way both antigens are processed and are presented to the immune system following homologous and heterologous rotavirus infection. In addition, differences in seroconversion rate due also to the younger age of the vaccinated infants cannot be ruled out.

In this study, all the children who seroconverted to NSP4 after natural infection showed a response to NSP4 genotype B antigen, but it was also evident that many of the children recognized at least one additional NSP4 genotype, supporting the notion that antibody response to NSP4 is at least partially heterogeneous (25, 33). Both natural infection and RRV-TV vaccination generated modest or low serum antibody titers to NSP4: e.g., ≤100. Johansen et al. (17) also found modest serum IgG antibody responses against NSP4 in children following natural infection and RRV-TV vaccination (GMT of 324 and 259, respectively), responses that were significantly lower (P < 0.05) than those against VP6. Very low serum titers to NSP4 were also found after homologous (EHP) or heterologous (RRV) rotavirus infection in mice by other authors (15).

In addition, we found that prior natural exposure to rotavirus, revealed by the presence of baseline antibodies, did not result in higher antibody titers in subjects who experienced subsequent natural rotavirus diarrhea (data not shown), suggesting a lack of a priming effect. The modest titers to the NSP4 protein were obtained independently of the peptide used in the ELISA. High levels of NSP4 protein are produced after rotavirus infection and are only a general indicator for the true antigen in the ELISA may not be an exact reflection of the antigen in the immune system. It is unclear how long lasting the antibody response is (4, 28). Therefore, such modest levels of NSP4 antibody response may not be directly related to the amount of antigen produced. Instead, they may be a consequence of ineffective NSP4 epitope exposure or presentation to the immunologic system. It is unclear how long lasting the antibody response is to NSP4, but the low NSP4 antibody titers generated after acute infection suggest that they will fall below detection levels sooner than do antibodies to other proteins, such as VP6, and therefore may only be detected briefly after rotavirus infection.

The role of the antibody response against NSP4 in protection against rotavirus diarrhea is not clear. We found a positive correlation between the presence of baseline serum antibodies against NSP4 and protection from rotavirus diarrhea. This observation suggests that NSP4 antibodies may play a role in disease protection after natural infection, but we cannot exclude the possibility that such antibodies merely reflect a recent infection and are only a general indicator for the true effectors of protection from disease. A limitation of this study is that total levels of antibody binding the recombinant NSP4 antigen in the ELISA may not be an exact reflection of the antibodies involved in protection, i.e., those antibodies putatively able to neutralize the toxigenic activity of NSP4. In the same way, we observed that the presence of VP6 antibodies correlated positively with protection. Correlation between serum VP6 antibodies and protection has been observed previously (5). On the other hand, the lack of correlation between seroconversion rate to NSP4 and protection afforded by the RRV-TV vaccine suggests other mechanisms may be involved.


