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

Esmeralda Vizzi,1* Eva Calvinó,1 Rosabel González,2 Irene Pérez-Schael,2 Max Ciarlet,3† Gagandeep Kang,3‡ Mary K. Estes,3 Ferdinando Liprandi,1 and Juan E. Ludert1

Laboratorio de Biología de Virus, Centro de Microbiología y Biología Celular, IVIC, Caracas, Venezuela; Instituto de Biomedicina, MSDS, UCV, Caracas, Venezuela; and Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas

Received 9 May 2005/Returned for modification 11 July 2005/Accepted 14 August 2005

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 antirotavirus 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 modulates 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 rhesus 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:

1. 2-, 3-, and 4-month-old children who received three doses at high titer (10^10 PFU of each component) (n = 11) or three doses at low titer (10^7 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);
2. 78 children, not previously vaccinated at the onset of diarrhea. 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 collected from 3 to 4 weeks after the episode. Acute diarrhea was defined as 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 rotavirus-negative diarrhea episodes. The diarrheic children were patients who attended the Ciudad Hospitalaria “Dr. Enrique Tejera” (CHITE) in Carabobo State, Venezuela (27).

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 of 751 bp were generated by PCR and subsequently cloned into the pCRII-TOPO vector (Invitrogen Corp., San Diego, CA). The nucleotide regions encoding amino acids 85 to 175 of each NSP4 genotype were reamplified using the RRV strain. A PCR product of 478 or 479 bp, depending on the primer set, was amplified to obtain the recombinant plasmid pMA104, containing the soluble proteins of the desired genotype between the nucleotides (nt) 294 to 314.

The strategy used for the generation of the recombinant plasmids included the following steps:
1. Phusion polymerase (New England Biolabs) was used to amplify the gene 10 cDNA sequences.
2. The amplified cDNA sequences were cloned into the pCRII-TOPO vector (Invitrogen Corp., San Diego, CA).
3. The resulting plasmids were transformed into E. coli BL21 (DE3) cells using the heat shock method.
4. The transformed cells were grown in LB medium containing 100 mg/ml of ampicillin at 37°C. Protein expression was induced by adding 0.1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG). After 2.5 h of incubation at 37°C, the cells were harvested and subjected to centrifugation at 8,000 rpm for 20 min. The pellets were resuspended in PBS containing 5% glycerol and stored at −80°C for further use.

Expression and purification of rNSP4 proteins in E. coli. Recombinant clones expressing each of the rNSP4(85–175) peptides as fusion proteins with the glutathione S-transferase (GST) protein, or wild-type GST alone in parallel, were generated in BL21 (DE3) cells grown in LB broth supplemented with 100 μg/ml of ampicillin at 37°C. Protein expression was induced with 0.75 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2.5 h of incubation at 37°C, the cells were harvested and subjected to centrifugation at 10,000 rpm for 30 min. The pellets were lysed by sonication in lysis buffer containing 50 mM Tris-Cl, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, and 1 mM PMSF. The soluble proteins were separated from the insoluble fractions by centrifugation at 10,000 rpm for 30 min. The supernatants were analyzed by SDS-PAGE for the presence of recombinant proteins.

The purified proteins were analyzed by SDS-PAGE and Western blotting. Preparations of 10 μg of purified GST and rNSP4(85–175) proteins from rotavirus simian SA11 (genotype A), human Wa (genotype B), and simian RRV (genotype C) strains were further characterized by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie blue staining. For Western blotting analysis, the fractionated proteins were transferred onto nitrocellulose (Amer sham Pharmacia Biotech, UK Ltd., Buckinghamshire, England) and probed using GST-specific antibodies produced in a goat (Amer sham Pharmacia Bio tech, 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-indoly phosphate, and p-nitroblue tetrazolium chloride was used to detect the blotting reaction.

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. The plates were incubated at 4°C with 2% bovine hemoglobin (Sigma Chemical Co., St. Louis, MO) in 0.05 M sodium carbonate buffer, pH 9.6, at 4°C. After washing with PBS, the heterobifunctional cross-linker sulfo-N-succinimidyl 4-(N-maleimidophenyl)-butyrate (SSMBP) (Pierce, Rockford, IL) was added by diluting the stock solution (20 mg/ml in PBS) to 0.1 M 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 μM reduced glutathione in 0.1% 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. Affinity-purified rNSP4(85–175) proteins (3 μg/ml for well) from simian rotavirus SA11 (genotype A), human Wa (genotype B), or simian RRV (genotype C) strains in PBS were added to alternate rows of glutathione-coated plates (100 μl/well) and incubated at room temperature for 2 h. GST protein alone was added to the first row and used to blank each serum test. The plates were washed six times with PBS-T and blocked with 200 μl/well of 10% nonfat dry milk in PBS for 1 h. After six washes with PBS-T, paired sera from each patient diluted in the blocking solution were tested in the same plate (serial twofold dilutions starting at 1:50) against each of the coated GST and rNSP4(85–175) proteins. Two positive internal controls, a goat anti-GST serum (Amer sham Pharmacia Biotech, UK Ltd., Buckinghamshire, England) and the anti-GST MAB B4-2/55, were included on each plate, serially diluted, to validate the assay and to ensure the reproducibility of the results. After incubation at room temperature for 1 h, the plates were washed sequentially with PBS-T and a 1:4,000-1:8,000 dilution of rabbit polyclonal antibody to NSP4 (Pierce, Rockford, IL) was added by diluting the stock solution in PBS-T to 0.1% (0.1 mM of cross-linker in PBS) and incubating this mixture for 1 h at room temperature. After six washes with PBS-T, the plates were washed 6 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 antigen was defined as the reciprocal of the highest dilution giving a net OD value greater than the calculated cutoff, 0.1. Seroconversion 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-VP6 antibodies. Antibodies to rotavirus capsid protein VP6 were determined by ELISA using a purified MAb against VP6, 4B2D2 (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 at least 2-fold the value corresponding to the well with mock-infected cells. The end-point antibody titer of each sample was expressed as the reciprocal of the highest dilution that had a corrected OD 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 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 B4-2/55 monoclonal antibody as detection antibodies. Lane 1, SA11-GST; lane 2, Wa-GST; lane 3, RRV-GST fusion proteins; lane 4 GST protein; MW, prestained protein molecular mass ladder (GIBCO BRL).
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 directed toward more than one type of NSP4 antigen whose titers were ≤100. Thus, the seroconversion rate to rNSP4 after vaccination was significantly lower \((P < 0.05)\) than that after natural infection (8 versus 54%). Furthermore, it was noticed that two other vaccinated children responded with very low antibody titers (titer of 50) against rNSP4 of genotype A (SA11), but they did not reach the ≥3-fold-rise seroconversion criterion defined above (data not shown) and were not counted. Placebo recipients showed seroconversion rates to rNSP4 comparable to those of the children with non-rotavirus-associated diarrhea (Table 1).

**Serum antibody response to rotavirus VP6.** The serum antibody response to the VP6 protein was studied, and the results are shown in Table 1. The seroconversion rate to VP6 was significantly higher \((P < 0.05)\) in children with rotavirus-associated diarrhea than in those suffering an episode of diarrhea not associated with rotavirus (72 versus 13%). Additionally, the seroconversion rate to VP6 was also significantly higher \((P < 0.05)\) in diarrheic rotavirus-infected children than in RRV-TV recipients (31%). Placebo recipients showed a seroconversion rate to VP6 comparable to that of the children suffering an episode of non-rotavirus-associated diarrhea (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 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).

**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 occurrence of baseline serum antibodies against rNSP4 or VP6 (Table 2). The correlation between presence or absence of baseline serum antibodies and the protection from rotavirus diarrhea was significantly higher \((P < 0.05)\) in children with acute diarrhea associated or not with rotavirus compared to those with non-rotavirus-associated diarrhea. The Pearson's correlation coefficients were calculated for evaluating the correlation between presence or absence of baseline serum antibodies and the protection from rotavirus diarrhea. The correlation coefficient was calculated for evaluating the correlation between presence or absence of baseline serum antibodies and the protection from rotavirus diarrhea.

---

**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>VP6</th>
<th>NSP4</th>
<th>NSP4 genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Not vaccinated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rotavirus-associated diarrhea</td>
<td>78</td>
<td>56 (72)</td>
<td>42 (54)</td>
<td>A 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>
<td></td>
</tr>
<tr>
<td>RRV-TV vaccinated</td>
<td>26</td>
<td>8 (31) 2 (8)</td>
<td>2 (100) 1 (50) 1 (50)</td>
<td></td>
</tr>
<tr>
<td>Placebo recipients</td>
<td>15</td>
<td>2 (13) 0 (0)</td>
<td>0 (0) 0 (0) 0 (0)</td>
<td></td>
</tr>
</tbody>
</table>

- 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.
- Seroconversion rates based on study of acute- and convalescent-phase serum samples.
- Seroconversion rates after administration of three doses of RRV-TV vaccine or placebo in children 8 weeks old (mean age) not suffering from diarrhea.
- \(P\) < 0.05 by \(P\) 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 rates 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.

---

**FIG. 2. Serum antibody titers against NSP4 of different genotypes from 78 children convalescing from rotavirus-associated diarrhea.**

---

**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 to (a) (b)</th>
<th>NSP4</th>
<th>VP6</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>

- 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.
- \(P\) = 0.005 and \(r = 0.27\) for antibodies directed against NSP4(85–175) from SA11, \(P = 0.014\) and \(r = 0.23\) for antibodies directed against NSP4(85–175) from Wa, and \(P = 0.014\) and \(r = 0.23\) for antibodies directed against NSP4(85–175) from RRV.
- \(P = 0.002\) and \(r = 0.29\) for antibodies directed against VP6.
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 toa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NSP4</td>
<td>VP6</td>
</tr>
<tr>
<td>Detectable</td>
<td>14</td>
<td>7 (50)b</td>
</tr>
<tr>
<td>Not detectable</td>
<td>64</td>
<td>35 (55)</td>
</tr>
<tr>
<td>Total</td>
<td>78</td>
<td>50 (78)</td>
</tr>
</tbody>
</table>

a 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.

b P < 0.05 by γ2 test for the comparison among subjects who developed a seroresponse to NSP4.

c P < 0.05 by Fisher’s exact test for the comparison among subjects who developed a seroresponse to VP6.

discernence 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 diarrhea 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 diarrhea 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 seroresponse 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 seroresponse 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...
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 heterotypic (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 (SqN11) 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 replication in cultured cells and presumably also in vivo (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 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 cells) 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 S1-2001000906 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.

REFERENCES


