MAJOR ARTICLE

Rotavirus Nonstructural Protein NSP4 Induces Heterotypic Antibody Responses during Natural Infection in Children

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Seroconversion of immunoglobulin A (IgA) and immunoglobulin G (IgG) (⩾4-fold rise) to rotavirus nonstructural protein 4 (NSP4) was determined, by use of enzyme-linked immunosorbent assay with fusion proteins glutathione *S*–transferase (GST)–NSP4 from strains SA11 (A), 116E (B), and RRV (C), in 40 children with acute rotavirus gastroenteritis and in 30 with the same disease due to other pathogens. The IgG seroconversion rates in the rotavirus group were 67.5%, 70%, and 60% when recombinant (r) NSP4A, -B, and -C, respectively, were used as antigen in the assay, and, for rotavirus-uninfected children, rates were 10%, 13%, and 7%. IgA seroconversion occurred in 57%, 70%, and 50%, respectively, of children with rotavirus gastroenteritis; in rotavirus-uninfected children, 1 child each seroconverted to the different rNSP4s. Among 9 children infected with strain NSP4A, 7, 6, and 5 children showed IgG seroconversion, and, among 18 infected with NSP4A, -B, and -C, 16, 17, and 15, respectively, showed IgG seroconversion. Between NSP4A-infected and NSP4B-infected children, IgA responses were similar to IgG responses. In conclusion, significant NSP4-specific antibody response occurs in natural rotavirus infection, and the antibody response appears to be broad and heterotypic in nature.

Rotavirus accounts for ~800,000 child deaths worldwide annually [1]. An improved understanding of immunity against specific rotavirus proteins will accelerate development and evaluation of new candidate vaccines. This is particularly important now, because of the withdrawal of a licensed reassortant rotavirus vaccine due to a reported increased risk of intussusception [2, 3]. Whether the neutralizing proteins VP4 and VP7 play an important role in induction of protective immunity against rotavirus-associated diarrhea remains controversial. Extensive field trials of rhesus rotavirus–based quadrivalent vaccine, conducted in different populations and in various parts of the world, have demonstrated, in the target population, a high efficacy of the vaccine against severe diarrhea caused by G serotypes of epidemiologic importance (G1–G4), and these trials have thus provided strong support for a serotype-specific immunity [4–8]. In clinical trials, however, antibodies to the neutralizing antigens did not correlate well with clinical protection [9, 10]. In recent studies, the nonneutralizing protein VP6 was shown to protect mice against rotavirus infection after active or passive immunizations [11–13].

Nonstructural proteins of Japanese encephalitis virus, cytomegalovirus, and hepatitis C virus have been shown to induce protective immunity [14–16]. The involvement of rotavirus nonstructural protein 4 (NSP4) in

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rotavirus pathogenesis was first shown by use of rotavirus reassortants in a gnotobiotic piglet model [17]. After that, this protein was identified as an enterotoxin on the basis of its ability to induce diarrhea in infant mice when challenged with infectious virus [18–21]. In neonatal mice, antibodies to NSP4 reduced severity of diarrhea caused by infection with either simian rotavirus or a highly virulent murine rotavirus [18, 19]. Furthermore, a plant-based multicomponent vaccine containing NSP4 was found to protect mice from enteric disease [22]. Several in vitro studies have also suggested that NSP4 plays a role in rotavirus pathogenesis [23–25].

Antibody response to NSP4 in humans has been assessed in a few studies with small numbers of subjects [26, 27]. An IgG, but not an IgA, antibody response was observed after natural infection [27]. Because there is a possibility that NSP4-induced immunity may be involved in protection against rotavirus diarrhea, it is important to clearly establish whether such immunity develops during natural infection in children and to relate immune response to the characteristics of the infecting rotavirus strains.

In this study, we evaluate IgG and IgA antibody responses to NSP4 in children with rotavirus and nonrotavirus acute gastroenteritis and dehydration; we used various glutathione *S*–transferase (GST)–NSP4 fusion proteins as antigens in an ELISA. We also compared the immune response to NSP4 genotypes of infecting strains.

SUBJECTS, MATERIALS, AND METHODS

Subjects and serum samples. The group of study subjects comprised 70 children with acute watery diarrhea and dehydration who were admitted at All India Institute of Medical Sciences (AIIMS), New Delhi, India. Their average age ($\pm$SD) was 9.8 ($\pm$4.69) months. Stool samples and acute-phase serum samples were obtained within 72 h of the onset of diarrhea. Stools were tested for rotavirus by ELISA, as described elsewhere [28]. Convalescent-phase serum samples were collected 2–3 weeks later. Both stool samples and serum samples were stored at −20°C, until analyzed.

Rotavirus strains. The human 116E rotavirus strain used for cloning NSP4 was originally isolated, in the neonatal unit of AIIMS, from feces of a newborn with asymptomatic rotavirus infection and was adapted to grow in cell culture by serial passage in MA104 cells [29]. Other rotavirus strains (SA11 and RRV) used in the study were obtained from the Centers for Disease Control and Prevention.

Reverse transcription–polymerase chain reaction (RT-PCR) and NSP4 typing assay. Rotavirus double-stranded RNA was extracted from either stools or infected cell cultures and was used for determination of G, P, and NSP4 genotype of infecting rotavirus strains. G and P types were determined by RT-PCR, by use of primers specific for G1–4 and G9 (G type) or for P4, P6, P8, and P11 (P type) [28, 30]. An RT-PCR method was also used to determine NSP4 genotype. Oligonucleotide primers specific for NSP4 genes were synthesized complementary to the 3′ ends of both viral RNA strands. A consensus primer (ConF4) was used to reverse transcribe NSP4 RNA, and 3 type-specific primers—NSP4-TA, NSP4-TB, and NSP4-TC—corresponding to genotypes A, B, and C were used as the pool of primers in the PCR amplification for typing assay. Positions and sequences of these primers (5′–3′) were as follows: ConF4 (nt 120–140), GATCCTGGAATGGCGTATTTT; NSP4-TA (nt 710–729), TCAGCACCGGACGTTAATGG; NSP4-TB (nt 475–495), CGAAGGAATTCAATCAGAAAA; NSP4-TC (nt 251–272), CTGCATTGTGTCAATTTTTAAC. In brief, 1–5 μL of dsRNA was mixed with 2 μL of ConF4 primer (25 μM) and was denatured for 5 min at 97°C. RT was done for 45 min at 42°C in 50 μL final reaction volume. The cDNA was then subjected to 30 PCR amplification cycles.

Construction of recombinant expression vectors and purification of rNSP4 proteins. The truncated regions of NSP4 that correspond to amino acid residues 81–175 (SA11), 83–175 (116E), and 78–175 (RRV) were generated by RT-PCR with respective primer pairs and was cloned into the *Sma*I/*Not*I cut pGEX-5X1 expression vector (Amersham Pharmacia Biotech) [31]. GST protein and GST-SA11 $NSP4_{83–175}$, GST-116E $NSP4_{81–175}$, and GST-RRV $NSP4_{78–175}$ fusion proteins were expressed and purified by a method described elsewhere by Ma et al. [32]. Cultures of DH5 α transformed with recombinant/nonrecombinant pGEX-5X-1 were induced with 0.1 m*M* isopropyl thio-β-D-galactoside (IPTG), and, after 2 h, cells were harvested and pellets were resuspended in a 1/50 volume of PBS. After sonication, Triton X-100 was added to the cell lysate, at a final concentration of 1%, and cell debris was removed by centrifugation at 8200 g at 4°C. Four milliliters of 50% glutathione–Sepharose 4B was added to 100 mL of sonicate, and the resultant mixture was incubated with gentle agitation for 1 h at room temperature. After the mixture was washed extensively with PBS, proteins were eluted with an equal volume of 10 m*M* reduced glutathione in 50 m*M* Tris-HCl (pH 8.0). Protein concentrations were determined by use of a standard protein assay kit. rNSP4s were confirmed by nucleotide sequencing of the insert, by use of a T7 sequencing kit (Amersham Pharmacia Biotech) [32] and also by immunoblotting with specific serum samples.

SDS-PAGE and Western blot analysis of recombinant NSP4s. Uninduced and induced culture lysates of the recombinant clones and affinity-purified GST-NSP4s and GST proteins were fractionated by electrophoresis on a 10% SDS-polyacrylamide gel. Proteins were visualized by staining with Coomassie brilliant blue R-250. For immunoblotting, 2–5 μg each of purified GST, GST-NSP4 fusion proteins, and tissue culture lysate (SA11

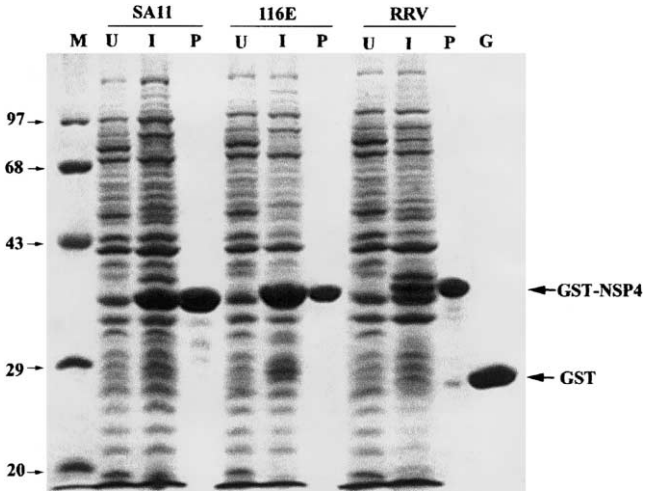

Figure 1. SDS-PAGE analysis of SA11 GST-nonstructural protein 4 $(NSP4)_{83–175}$ *(lanes 2–4),* 116E GST-$NSP4_{81–175}$ *(lanes 5–7),* and RRV GST-$NSP4_{78–175}$ *(lanes 8–10)* fusion proteins from uninduced (U) and induced (I) *Escherichia coli* culture lysates and purified fusion protein (P) and purified GST (G), from *E. coli* extracts. Positions of GST-NSP4 and GST proteins are noted with arrows. M, molecular weight markers.

infected or mock infected) were fractionated on a 10% SDS-polyacrylamide gel and then were transferred to polyvinylidene fluoride membrane (Millipore). The membranes were probed with either anti-GST antibody or known anti-rotavirus human serum samples (1:500), and, correspondingly, alkaline phosphatase–conjugated anti-rabbit or anti-human IgG/IgA was used to develop the respective blots. To visualize the antibodies bound to the proteins, 5-bromo-4-chloro-3-inodlyl phospahte and nitroblue tetrazolium were used as enzyme substrates [32, 33].

IgA and IgG ELISA. For NSP4 antibody assays, 96-well microtiter plates with high binding capacity (Costar) were coated overnight at 4°C with either GST-NSP4 (1 μg/well) or GST (0.7 μg/well), in 100 μL of 0.1 *M* carbonate-bicarbonate buffer (pH 9.6). To generate a standard curve, serial 2-fold dilutions of 1:100–1:12,800 (i.e., 100–0.78 U/mL) of known anti-rotavirus human serum samples were added to duplicate wells coated with either GST-NSP4 or GST. The concentration of NSP4 IgG or IgA antibody in the reference serum samples was arbitrarily assigned a value of 10,000 U/mL. Patient serum samples that were to be tested were also added to duplicate wells containing GST-NSP4 (SA11, 116E, or RRV) or GST protein. Paired serum from each patient was tested in the same plate at a 1:100 or higher dilution. All dilutions of antibodies and conjugates were made in PBS-Tween containing 1% skim milk. After addition of serum, plates were incubated for 2–3 h at room temperature. For IgG ELISA, horseradish peroxidase–conjugated anti-human IgG (Jackson ImmunoResearch laboratories) was added; for IgA ELISA, biotin-conjugated anti-human IgA (Jackson ImmunoResearch laboratories) and horseradish peroxidase–conjugated avidin-biotin (Vector Laboratories) were added, according to the manufacturers' protocols or as described elsewhere [34]. Color was developed with *o*-phenylenediamine substrate (Sigma), and optical density was measured as absorbance at 490 nm (A_{490}). Antibody titers (IgA and IgG) were expressed as units per milliliter of serum; values were determined from a standard line plot of the reference serum samples, after subtraction of the average A_{490} values of duplicate wells coated with GST from those with NSP4-GST. In each plate, both a known positive and negative control serum were included, and the assay was regarded to be valid only when negative sample was clearly negative and positive sample was within 2-fold of its assigned value [34]. Seroconversion was defined as a $\geq$4-fold rise in antibody titer between acute and convalescent serum samples. Titers of IgG and IgA antibody, to whole rotavirus, were determined by ELISA, as described by McNeal et al. [34]. Tissue culture lysate from either SA11-infected cells or mock-infected cells was used as coating antigen. Antibody titers were expressed as units per mil-

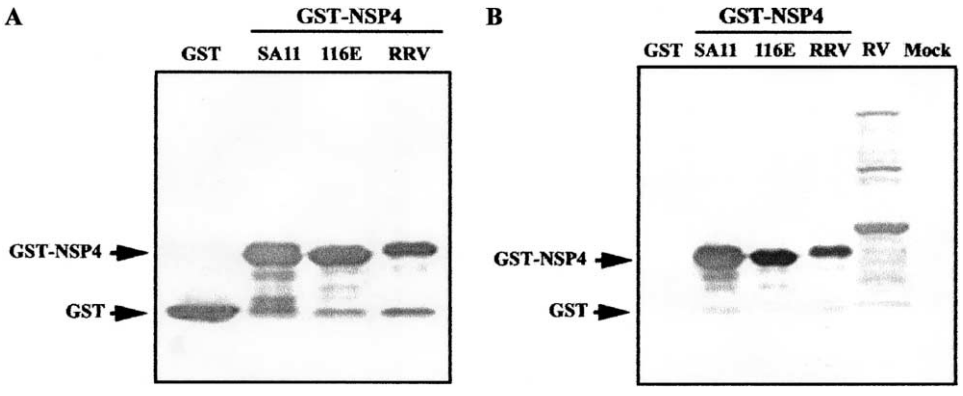

Figure 2. Confirmation of SA11 GST-nonstructural protein 4 $(NSP4)_{83–175}$, 116E GST-$NSP4_{81–175}$, and RRV GST-$NSP4_{78–175}$ fusion proteins and GST protein, purified from isopropyl thio-β-D-galactoside–induced *Escherichia coli* cell lysates, by immunoblotting with anti-GST rabbit serum samples *(A)* and anti-rotavirus human serum samples *(B).* Reactivity with SA11-infected (RV) and mock-infected (mock) tissue culture lysates is shown in rightmost lanes in *B.*

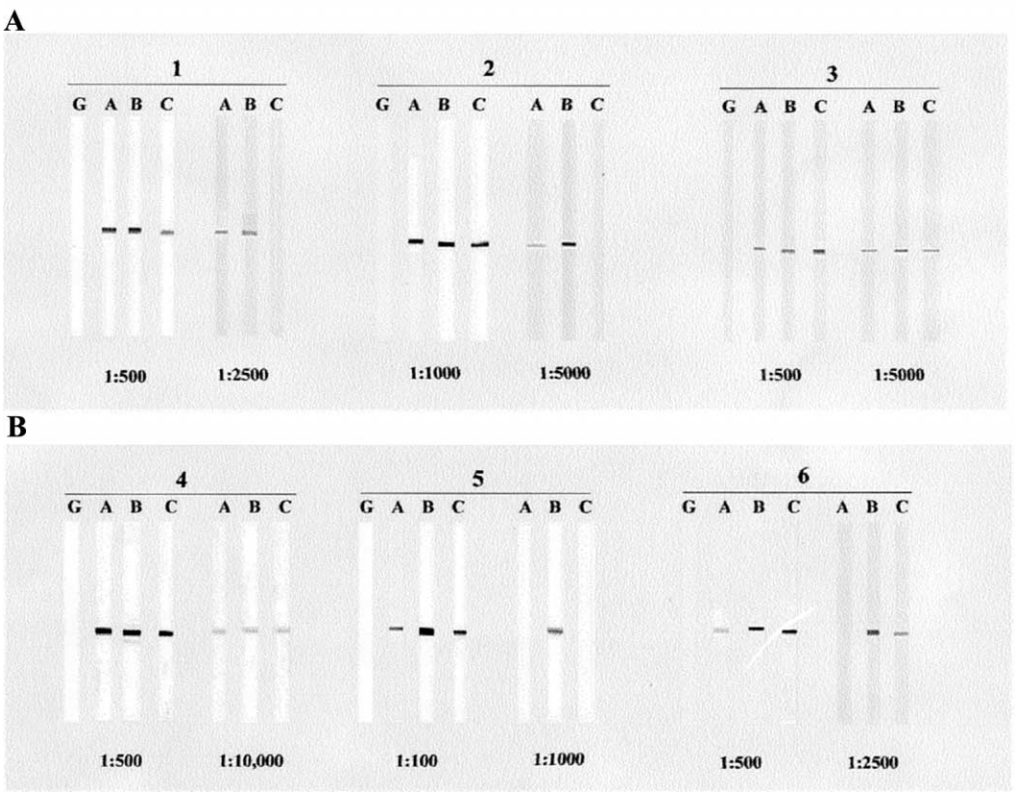

Figure 3. Immunoblotting of GST-nonstructural protein 4 (NSP4) A, NSP4B, and NSP4C, with convalescent serum samples from 6 children infected with rotavirus—3 infected with strain NSP4A *(A, lanes 1–3)* and 3 with NSP4B *(B, lanes 4–6)*—tested at 2 different dilutions *(bottom)*, against equal amounts of NSP4A (A), NSP4B (B), NSP4C (C), and GST (G) antigens.

liliter; values were determined from the standard curve [34]. The limits of detection were 156, 156, 78, and 78 units/mL for serum NSP4 IgG, NSP4 IgA, whole rotavirus IgG, and whole rotavirus IgA, respectively, and these values were used to calculate geometric mean titers when antibody titers were below the limit of detection.

Statistical analysis. Data were analyzed by Stata (version 7; Stata). Categorical variables were compared by χ^2 test. Antibody titers were log transformed, and geometric mean antibody titers of different groups were compared by analysis of variance. $P < .05$ was considered significant.

RESULTS

Cloning, expression, and purification of GST-NSP4 fusion proteins. Because expression of full-length NSP4 in *Escherichia coli* was unsuccessful, truncated proteins SA11-NSP4$_{83–175}$, 116E-NSP4$_{81–175}$, and RRV-NSP4$_{78–175}$, representing genotypes A, B, and C, respectively, were expressed in *E. coli* as GST-NSP4 fusion proteins. SDS-PAGE analysis of whole cell lysates from IPTG-uninduced and IPTG-induced culture-purified and affinity-purified GST-NSP4 fusion proteins and from GST are shown in figure 1. All of the fusion proteins (SA11, 116E, and RRV) are clearly seen as ~38-kDa bands, and the GST protein as a 26-kDa band, in the IPTG-induced culture lysate (figure 1). A high level of expression (5–15 mg/L of bacterial culture) was obtained with each of the GST-NSP4 fusion proteins.

Confirmation of rNSP4 proteins by immunoblotting. Identities of GST-NSP4 fusion proteins were confirmed by immunoblotting with anti-GST rabbit serum samples and anti-rotavirus human serum samples (figure 2). Although anti-GST serum samples bound with equal intensity to all GST-NSP4s and to the GST protein (figure 2), human serum samples (1:500 dilution) reacted specifically with 38-kDa GST-NSP4s, and not with 26-kDa GST, in the immunoblot developed with either anti-human IgG conjugate (figure 2) or anti-human IgA conjugate (result not shown). As expected, human serum samples also specifically bound to various rotavirus proteins present in rotavirus-infected tissue culture lysate (figure 2).

IgG and IgA antibody response to NSP4 in children with rotavirus and nonrotavirus gastroenteritis. The rotavirus-infected children showed significantly higher IgG and IgA seroconversion rates to each rNSP4 tested than did rotavirus-uninfected children. The IgG seroconversion rates in rotavirus-infected children were 67.5%, 70%, and 60% when rNSP4A, rNSP4B, and rNSP4C, respectively, were used as antigen in the

Table 1. Seroconversion in rotavirus-infected and rotavirus-uninfected children, determined by use of various recombinant nonstructural protein 4 (NSP4) antigens in ELISA.

Response, patient group	No. of subjects	Antigen			
		NSP4A	NSP4B	NSP4C	Whole rotavirus
IgG					
Rotavirus-infected	40	27 (67)	28 (70)	24 (60)	31 (77)
Rotavirus-uninfected	30	3 (10)	4 (13)	2 (7)	4 (13)
IgA					
Rotavirus-infected	40	23 (57)	28 (70)	20 (50)	29 (72)
Rotavirus-uninfected	30	1	1	1	3

NOTE. Data are no. (%) with response to antigen by ELISA. All comparisons between rotavirus-infected and rotavirus-uninfected groups were significant ($P < .001$).

assay, and the corresponding rates for rotavirus-uninfected children were 10%, 13%, and 7%, respectively (table 1). The rate of IgA seroconversion was 57.5%, 70%, and 50% in rotavirus-infected children when rNSP4A, rNSP4B, or rNSP4C, respectively, were tested in the ELISA. Among rotavirus-uninfected children, only 1 seroconverted to each rNSP4 (table 1). Furthermore, 34 (85%) of 40 rotavirus-infected children who were examined showed ⩾2-fold rises in IgG or IgA antibody titer to ⩾1 GST-NSP4. A high rate of IgG (77.5%) and IgA (72.5%) seroconversion to whole rotavirus was also observed in rotavirus-infected children (table 1).

We assessed the effect that prior exposure to rotavirus infection has on immune response during natural rotavirus infection. For this analysis, detectable IgG and IgA antibody to both NSP4 and whole rotavirus, in the baseline serum, constituted evidence of prior exposure to rotavirus. Within the group of 40 children with rotavirus gastroenteritis, the seroconversion rate to NSP4 was significantly lower among those with prior exposure to rotavirus than it was among children without detectable baseline antibody ($P = .005$). The seroconversion rate to whole rotavirus was similar in children with and children without detectable baseline antibody (table 2).

NSP4 antibody response in relation to NSP4 genotype of infecting strain. Among 40 rotavirus-infected children, NSP4 genotype was determined in samples from 27 (9 genotype A [8G2 and 1G1] and 18 genotype B [6G9 and 5G1]). Of 9 children infected with NSP4A strains, 5 showed IgG seroconversion to all GST-NSP4 antigens tested in the ELISA; 1 child seroconverted to NSP4A and NSP4B but did not seroconvert to NSP4C, and 1 other child seroconverted only to NSP4A. In children infected with NSP4B strains, 15 of 18 showed seroconversion to rNSP4A, rNSP4B, and rNSP4C. One child showed seroconversion only when NSP4B was used in the ELISA (table 3). The findings, with regard to IgA response and in relation to NSP4 type of the infecting strain, were similar to those for IgG antibody (table 3).

We also assessed the magnitude of antibody response in relation to NSP4 genotype of the infecting strain. The geometric mean IgG or IgA antibody titers of the 9 children infected with NSP4A strains, compared with those of 18 children infected with NSP4B strains, were not significantly different when any of the 3 rNSP4s were used in the ELISA (table 4).

Convalescent serum samples from 6 children (NSP4 seroconverted), 3 infected with NSP4A and 3 with NSP4B strains, were also tested in immunoblots with GST-NSP4A, GST-NSP4B, and GST-NSP4C antigens in equal amounts. All 6 samples reacted strongly to NSP4A, NSP4B, and NSP4C antigens when tested at lower dilutions of serum (figure 3). Some variability in the reactivity of serum samples with different rNSP4s was observed when serum samples were tested at higher dilutions (figure 3).

DISCUSSION

The main findings from this study are that natural rotavirus infection results in a significant NSP4-specific IgG and IgA antibody response in children and that the immune response is essentially similar in the presence of infection with strains of different NSP4 genotypes. In a study by Johansen et al. [27], IgG antibodies to NSP4 were found in convalescent serum samples

Table 2. Seroconversion rates in children with acute rotavirus gastroenteritis, with or without detectable antibodies to either whole rotavirus or rotavirus nonstructural protein 4 (NSP4), at baseline.

Baseline antibody	No. of subjects	NSP4 (⩾1 antigen)	Whole rotavirus
Detectable	22	12 (55)[a]	16 (73)[b]
Not detectable	18	17 (94)	15 (83)

NOTE. Data are no. (%) of children who seroconverted to antigen.

[a] $P = .005$.

[b] $P = .424$.

Table 3. Antibody response to nonstructural protein 4 (NSP4), according to NSP4 genotype of infecting rotavirus.

Response, genotype	No. of subjects	rNSP4A	rNSP4B	rNSP4C	All 3
IgG					
A	9	7	6	5	5
B	18	16	17	15	15
IgA					
A	9	6	6	5	5
B	18	11	14	11	10

NOTE. Data are no. with seroconversion to recombinant (r) antigen in ELISA.

of all 10 children examined. Baseline antibody levels, and therefore seroconversion rates, were not reported in that study.

Unlike in the present study, Johansen et al. [27] did not detect IgA antibodies to either NSP4 or VP6 in the limited number of rotavirus-infected children they examined, by use of SA11 NSP4 or VP6 antigen expressed in insect cells, in the ELISA [27]. The inconsistency between the studies may be related to differences in antigens used or other assay conditions.

A few children whose stools were negative for rotavirus but who showed antibody response to NSP4 and to whole rotavirus were, in all probability, rotavirus infected. Stool negativity may have resulted from less-than-maximal sensitivity of the detection kit used. Alternatively, the observed antibody response may be due to anamnestic response to rotavirus proteins in those children.

The less frequent seroconversion to NSP4, and not to whole rotavirus, in children with prior rotavirus exposure is of interest. Prior exposure (preexisting antibodies) to rotavirus may limit replication of virus in the intestinal cells, and this is more likely to affect immune responses to nonstructural proteins.

Sequence analysis of rotavirus NSP4 from human and animal strains has revealed the presence of 4 distinct NSP4 alleles (A, B, C, and D) or genetic groups (I, II, III, and IV); the majority of human strains were classified as either genogroup I (genotype A) or genogroup II (genotype B) [35]. It has been postulated that antigenic differences between NSP4s from different infecting rotavirus strains may affect immune responses after infection. In a study by Richardson et al. [26], NSP4 antibody response in children with G1 rotavirus infection varied between 67% and 100%, depending on the rotavirus strain used as antigen in the assay. Furthermore, NSP4 from a G2 strain was not immunoprecipitated by any serum sample from children infected with G4 strain, but it was recognized by 67% of samples from children infected with G1 rotavirus.

Our data suggest that the NSP4 antibody response is not significantly influenced by NSP4 genotype of the infecting strains. IgG or IgA seroconversion rates or geometric mean antibody titers in convalescent serum samples of rotavirus gastroenteritis patients, whether infected with NSP4 genotype A or B strains, were similar when the same NSP4 antigen was used in the assay. Our data indicate that the NSP4 antibody response is heterotypic in nature, because the immune response did not vary among children, irrespective of whether NSP4A, NSP4B, or NSP4C was used as antigen in the assay. In a recent study, Estes et al. [19] observed heterotypic protection against rotavirus diarrhea in mice. A heterotypic immune response to NSP4 would be advantageous for vaccine development in the event that NSP4-induced antibody response turns out to be, in future studies, protective in nature. One potential limitation related to this observation is that baseline antibody levels may not completely differentiate children as having had either prior exposure or a lack of it. Therefore, a naive gnotobiotic animal system may be a suitable model to analyze true primary and secondary homotypic and heterotypic serum IgG and IgA antibody responses to NSP4 protein [36].

NSP4-specific antibody responses were also observed in a limited number of subjects immunized with rhesus rotavirus–tetravalent vaccine [27]. As with natural infection, the NSP4-specific IgG antibody response was of a lower magnitude than was that to VP6. The study involved too few subjects to be able to determine whether NSP4 antibody induced by immunization protects against subsequent rotavirus infection or against diarrhea [27]. Future vaccine trials may provide an ideal opportunity to address this issue.

In conclusion, our findings indicate that, during natural ro-

Table 4. IgG and IgA antibody titer, according to nonstructural protein 4 (NSP4) genotype of infecting rotavirus strain.

Genotype	IgG response		IgA response	
	NSP4A (n = 9)	NSP4B (n = 18)	NSP4A (n = 9)	NSP4B (n = 18)
rNSP4A	1686 (437–6503)	1633 (889–3011)	1426 (229–8889)	536 (213–1348)
rNSP4B	2168 (572–8184)	2771 (1437–5324)	1415 (287–6986)	1291 (506–3289)
rMSP4C	1541 (493–4817)	1628 (898–2981)	976 (268–3548)	626 (300–1305)

NOTE. Data are geometric mean titer (95% confidence interval) of antibody to recombinant (r) antigen in children infected with particular genotype. Comparisons were not statistically significant by analysis of variance: infecting strain NSP4A, IgG or IgA (P = .9 for each); infecting strain NSP4B IgG (P = .1) or IgA (P = .3).

tavirus infection in children, NSP4 IgG and also IgA antibody responses are induced in a majority of cases. Of potential importance for vaccine development, the magnitude of the antibody response is not significantly affected by the genotype of the infecting strain, and it appears to be heterotypic in nature. A priority now is to assess mucosal immune responses to NSP4 and to determine whether the NSP4 immune response protects against all or severe rotavirus diarrhea.

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