

Lack of Maternal Antibodies to P Serotypes May Predispose Neonates to Infections with Unusual Rotavirus Strains

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Rotavirus (RV) strains infecting newborns often have unique neutralization antigens (P serotypes) on their outer capsids that are distinct from those found on RV strains that cause diarrhea in older children. We examined the hypothesis that unusual RV strains preferentially infect newborns because the newborns lack maternal neutralizing antibodies to these strains. To test this hypothesis, sera and saliva samples collected from neonates infected with 116E-like (P[11]G9) strains in the maternity ward of the All India Institute of Medical Sciences (AIIMS) hospital in New Delhi were tested for neutralizing antibodies against common RV strains and those infecting newborns and these titers were compared with those of newborns who did not become infected (controls). The infected neonates had significantly lower levels of cord blood neutralizing antibodies to 116E than the controls, suggesting that immunity to neonatal RV infection is acquired transplacentally through maternal antibodies. Further, this study confirmed the immunogenicity of the AIIMS neonatal strain 116E, a vaccine candidate, in its ability to evoke a potent RV-specific immunoglobulin A and neutralizing antibody response in serum and saliva among the infected babies. Our findings have important implications for the development of an effective RV vaccine. In India, where G9 strains are common in the community, the use of 116E as a vaccine, together with the rhesus tetravalent vaccine, may provide a broader protection against all the circulating RV serotypes, including serotype G9, which is not represented in the current rhesus RV tetravalent vaccine (G1-G4).

The asymptomatic nature of rotavirus (RV) infection in neonates has raised many questions and provided numerous insights into the pathogenesis of RV diarrhea in older children (15). Early observations that newborns infected with RV were protected against subsequent RV diarrhea provided evidence for natural immunity and laid the groundwork for the development of RV vaccines based on live attenuated strains (1a, 2). Similarly, the observation that primary RV infections in children aged 3 to 24 months were associated with diarrhea whereas infections in neonates were usually asymptomatic suggested that understanding the difference between these infections might yield insights into an approach for preventing RV diarrhea.

Initial attempts to explain the asymptomatic nature of neonatal infections were based on the hypothesis that "neonatal" RV strains were naturally avirulent and distinct from RVs that caused diarrhea in older infants and children. This hypothesis was supported by the early discovery that RV strains isolated independently from newborns on four continents contained a distinct VP4 protein (P2A[6]) (8, 12). This protein, which is important in virus neutralization and virulence, was distinct from the VP4 proteins present in RV strains that caused diarrhea in older children (P1A[8] and P1B[4]) (12). Furthermore, in limited global surveys, RV strains with a distinct P serotype, P2A[6], were rarely found in older children with diarrhea. Two neonatal RV vaccines have been developed based on the potential avirulence of these neonatal (i.e., P2A[6]) strains (3, 22).

Recently, we characterized RVs in diarrheal samples from Indian children and found an unusual diversity in strains (21). In contrast with earlier studies, P2A[6] strains were common among children with diarrhea, contradicting the earlier observation that these strains were naturally avirulent and found only in neonates. This observation suggested the need to examine other factors, including the levels of maternal neutralizing antibodies, which may be important in preventing symptomatic diarrhea in neonates. The present study took advantage of a long-term investigation of nosocomial RV infection among neonates born in the maternity unit at the All India Institute of Medical Sciences (AIIMS) in New Delhi that has been previously described (1a, 6, 16, 20). The purpose of this study was to compare the levels of cord blood neutralizing antibodies of the infected and noninfected neonates against the four common RV serotypes as well as the AIIMS prototype neonatal strain, 116E, to determine whether the differences between the preexisting levels of neutralizing antibodies in the infected and noninfected neonates play an important role in prevention of neonatal RV infection. Further, we also assessed the breadth of immune response elicited by 116E-like strains by measuring the RV-specific immunoglobulin A (IgA) and neutralizing antibody response in serum and saliva samples of the infected and noninfected babies against common and neonatal RV strains.

MATERIALS AND METHODS

Subjects and study design. The study was conducted between June 1992 and March 1993 at the AIIMS, an urban hospital in New Delhi, where we conducted earlier studies on the epidemiology, immunity, and outcome of neonatal RV infections (1a, 6, 10, 16, 20). Healthy neonates with no major congenital abnormalities who were born in the maternity unit of the hospital and who lived within a 7-km radius of the AIIMS were recruited into the study. The study was

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explained to the mothers, whose verbal consent was obtained before the babies were enrolled.

Collection and screening of specimens. Neonates were enrolled at birth, and their stool specimens were collected by trained health workers daily for the first month of life either in the hospital or during home visits after discharge. The stool specimens were refrigerated at 4°C immediately after collection, transported to the laboratory at the AIIMS, and stored at -70°C for further analysis. All specimens collected during the first 7 days and every other specimen collected during the rest of the month were screened for RV by an enzyme-linked immunosorbent assay (ELISA). Neonates were classified as infected if RV was detected in their stool specimens for one or more days during the first week of life and were classified as "controls" if none of the first seven daily specimens were positive. Since it has been shown that duration of hospital stay is a risk factor for neonatal RV infection, infected neonates and controls were matched for length of hospital stay (17). Neonates infected with RV after the first week were still considered controls but were noted to be "misclassified."

Saliva samples were collected on day 1 of life and 4 weeks later with calibrated swabs by a method described previously (16). Cord blood was obtained at birth, and infant sera were obtained after 4 weeks. These specimens were all collected during hospital visits and stored at -70°C until processed.

Antigen detection and strain characterization. RV-positive stool specimens were electropherotyped to determine if they represented a common strain. RV double-stranded RNA was extracted from 10% stool suspensions by a glass powder method, followed by separation of the double-stranded RNA segments by discontinuous polyacrylamide gel electrophoresis, and was visualized by silver stain (20). The P and G genotypes of RV strains were determined by reverse transcription-PCR and classified according to the recently revised classification system (7, 11, 13).

Assays for antibodies. (i) **ELISA.** Saliva specimens from RV-infected and noninfected babies were tested for total IgA by ELISA (18). Briefly, polystyrene microtiter plates (Dynatech Labs, Chantilly, Va.) were coated with rabbit anti-human IgA (Sigma Chemical Co., St. Louis, Mo.) at 1 µg/ml in carbonate buffer, pH 9.6, overnight at 4°C. Control wells contained only carbonate buffer. Three fivefold dilutions of the saliva samples were added to the test and control wells in duplicate, starting at a 1:25 dilution. For each run, a standard curve with purified human colostral IgA (Sigma) at concentrations of 800 to 1.56 ng/ml was determined. The plates were incubated for 2 h at 37°C and washed before the addition of peroxidase-conjugated anti-human IgA. The optimal dilution of peroxidase-conjugated goat anti-human IgA (Sigma) was determined by check-board titration with 1 µg of purified human colostral IgA/ml. After incubation for 1 h at 37°C, the plates were washed, followed by the addition of chromogen *o*-phenylenediamine (Sigma) and H₂O₂. The reaction was stopped after a 15-min incubation, and the absorbance was read at 492 nm. The total IgA determinations were made only from the linear portion of the standard curve. After the total IgA contents of the saliva samples were determined, they were assayed for RV-specific IgA. Pooled breast milk samples that contained high titers of RV-specific IgA were used as a positive control.

RV-specific IgA in serum and saliva was measured by ELISA with SA11 as the antigen (16). Serial twofold dilutions of serum and saliva specimens were tested, starting at a 1:50 dilution for serum and a 1:25 dilution for saliva. Starting dilutions were determined based on the background nonspecific absorbance observed at lower dilutions of the specimens. Briefly, polystyrene microtiter plates (Dynatech) were coated overnight at 4°C with 50 µg of either SA11 antigen or control antigen/ml, prepared from uninfected MA-104 cells. The plates were washed, and test samples were added in duplicate to both control wells and the antigen-coated wells. After incubation for 2 h at 37°C, the plates were washed and incubated for 1 h after the addition of the optimal dilution of peroxidase-conjugated anti-human IgA. This was followed by the addition of chromogen *o*-phenylenediamine (Sigma) and H₂O₂. The reaction was stopped after 15 min, and the absorbance was measured at 492 nm. Specimens with a fourfold increase in titers were considered positive.

(ii) **Fluorescent focus neutralization reduction assay.** Cord blood, convalescent sera, and saliva specimens were tested for neutralizing antibodies against RV strains Wa (P1A[8]G1), DS1 (P1B[4]G2), SA11 (P2[2]G3), ST3 (P2A[6]G4), and 116E (P8[11]G9) by the fluorescent focus reduction neutralization assay described by Coulson et al. (5). Virus strains were diluted to give 2×10^2 fluorescent focus units/100 µl of inoculum for use in neutralization assays. All sera were tested for neutralizing antibodies at dilutions of 1:200 and 1:1,000, and saliva samples were tested in twofold serial dilutions, starting at 1:50. Briefly, MA-104 cells were grown to confluence in 96-well cell culture plates (Costar Inc., Cambridge, Mass.). RV strains pretreated with 5 to 10 µg of pancreatic trypsin (catalog no. T-2271; Sigma)/ml were mixed with equal volumes of serum or saliva samples and incubated at 37°C for 60 min. The mixture was then inoculated into duplicate wells of MA-104 cells. After 15 to 18 h of incubation at 37°C in a 5% CO₂ atmosphere, the medium was aspirated and the cells were fixed with pre-chilled 80% acetone and stained by indirect immunofluorescence with guinea pig hyperimmune serum to RV (1) and fluorescein isothiocyanate-conjugated anti-guinea pig antibody (catalog no. F-9887; Sigma). The fluorescent foci were counted in each well, mean values of duplicate wells for each dilution were calculated, and the neutralizing titer was expressed as the reciprocal of the sample dilution giving a 50% or greater reduction in the number of fluorescent cells. A neutralization titer of <200 was considered to be negative for serum

samples, and a titer of <50 was considered negative for saliva samples. A neutralizing antibody response in saliva was defined as a fourfold or greater increase in neutralizing antibody titer to the specific virus strain. In sera, a neutralizing antibody response was defined as a rise in titer from $\leq 1:200$ to $\geq 1:1,000$.

Statistical analysis. We compared the levels of neutralizing antibodies against the neonatal strain (116E) and other common RV reference strains in the cord blood of newborns who were infected to the levels in the cord blood of controls. Since we found some mixed and nontypeable infections among the infected group, the infected newborns were subdivided based on the P and G genotypes of the infecting strain and were compared with controls by the chi-square test or Fisher's exact test (two-tailed).

RESULTS

Between June 1992 and March 1993, 30 neonatally infected infants and 20 noninfected controls born at the AIIMS who fulfilled the study inclusion criteria and from whom sufficient serum and saliva samples were obtained were selected for the study. The mean durations of hospital stay were similar for infected neonates (range, 4 to 6 days; mean, 5 days) and controls (range, 3 to 8 days; mean, 5 days). Only one control who first excreted virus on day 20 was considered misclassified.

Strain characterization. By polyacrylamide gel electrophoresis analysis, 80% ($n = 24$) of the RVs in infected neonates had similar long RNA electropherotypes, and the remaining specimens contained insufficient RNA to type. The strains were P and G genotyped by reverse transcription-PCR; 18 specimens (68%) were P[11]G9 strains, 3 were P[6]G9, 5 had mixed infections with genotypes G9 and G1, and 4 could not be typed. For purposes of analysis, the neonates were grouped as having a P[11]G9 infection ($n = 18$) alone or other neonatal RV infections ($n = 12$).

Neutralizing antibodies in cord blood. We hypothesized that newborns are predisposed to RV infections with unusual strains because they have low levels of maternal antibody to these strains. Since the cord blood obtained from the neonate is a reflection of the mother's antibody levels, we compared the titers of neutralizing antibody against strain 116E (P8[11]G9), the predominant asymptomatic neonatal strain at the AIIMS, and against four reference serotypes of RV strains, Wa, DS1, SA11, and ST3, in the cord blood of infected neonates and controls (Table 1). While neutralizing antibodies against all five RV G-serotype strains tested were identified in the neonates' cord blood, significantly fewer newborns infected with P[11]G9 strains than controls had cord blood antibodies against the homologous strain, 116E (39% versus 75%; $P < 0.03$). In contrast, newborns infected with other strains had high titers against 116E that did not differ significantly from the noninfected controls. Of note, most mothers had neutralizing antibodies to strain ST3 (P2A[6]G4), a classic neonatal P2A[6] strain.

Total IgA in saliva. Of the 50 babies tested for total IgA in saliva, none had a detectable amount of IgA on day 1. In the infected group, the average total IgA in fourth-week saliva specimens was 27 µg/ml (range, undetectable to 50 µg/ml). None of the 4-week-old babies in the control group had any detectable salivary IgA. The saliva samples were not corrected for the total IgA antibody because we did not find any statistically significant difference on comparing the optical density readings of total IgA and RV-specific IgA in saliva (1:25 dilution) of each patient by paired *t* test analysis ($P = 0.015$). Of note, only one 4-week-old baby that was misclassified as a control had a total IgA of ~30 µg/ml.

Immune responses in neonates. Titers of IgA and type-specific neutralizing antibodies to RV in serum and saliva samples of infected neonates versus those in noninfected neonates were measured by ELISA and fluorescent focus neu-

TABLE 1. Type-specific cord blood neutralizing antibodies among neonates infected with RV and noninfected controls

RV strain ^a	% (no.) of neonates with cord blood antibody titer ≥ 200			<i>P</i> value ^b for group infected with:	
	Infected (<i>n</i> = 30) with:		Noninfected (<i>n</i> = 20)	P[11]G9	Other
	P[11]G9 (<i>n</i> = 18)	Other (<i>n</i> = 12)			
116E (P8[11]G9)	39 (7)	83 (10)	75 (15)	0.03	0.68
Wa (P1A[8]G1)	83 (15)	75 (9)	70 (14)	0.45	1.0
DS1 (P1B[4]G2)	72 (13)	59 (7)	85 (17)	0.44	0.12
SA11 (P[2]G3)	61 (11)	83 (10)	70 (14)	0.57	0.68
ST3 (P2A[6]G4)	72 (13)	50 (6)	60 (12)	0.43	0.59

^a Strain which the neutralizing antibody is directed against.

^b *P* value obtained by Mantel-Haenszel's test or Fisher's exact test.

tralization assay (Table 2). Infected neonates showed a significant increase in IgA and neutralizing antibodies to strain 116E in both serum and saliva compared with noninfected controls. None of the controls, with the exception of one misclassified infant with a late infection, had any rise in antibody titer. In the infected group, 81% of the babies developed a fourfold rise in RV-specific IgA antibodies (range of reciprocal titers, 100 to 400; geometric mean titer, 169) in saliva, while 56% mounted serum IgA responses (range of reciprocal titers, 200 to 1,600; geometric mean titer, 511). Neither cord blood nor saliva samples collected on day 1 had detectable levels of IgA to RV. The finding that 44% of the babies did not develop RV-specific serum IgA antibodies was not unusual and corroborated other studies (9, 18). Neonates infected with 116E-like strains mounted a homotypic neutralizing antibody response in saliva (56%) and sera (37%) but failed to produce a heterotypic response to the other reference strains except Wa. Neonates infected with other RV strains developed salivary (62%) and serum (50%) IgA responses and neutralizing antibodies to both 116E (37%) and Wa (25%).

TABLE 2. IgA and type-specific neutralizing antibody response to RV strains in serum and saliva samples of infected and noninfected neonates

Body fluid	RV antibody	% (no.) of neonates responding			<i>P</i> value ^b
		Infected (<i>n</i> = 24 ^a) with:		Noninfected (<i>n</i> = 20)	
		P[11]G9 (<i>n</i> = 16)	Other (<i>n</i> = 8)		
Saliva	IgA	81 (13)	62 (5)	5 (1 ^d)	<0.001
	NA ^c				
	116E	56 (9)	37 (3)	0	<0.001
	Wa	19 (3)	25 (2)	0	<0.08
Serum	IgA	56 (9)	50 (4)	0	<0.001
	NA				
	116E	37 (6)	12 (1)	0	<0.05
	Wa	0 (0)	0 (0)	0	NS

^a Six infected children were eliminated from the study due to insufficient volumes of sera for completion of the assays.

^b Rotavirus-infected and noninfected groups were compared by Fisher's exact test. NS, not significant.

^c NA, neutralizing antibody response. A neutralizing antibody response in saliva was defined as a fourfold rise in titer, and in sera it was defined as a rise from <200 to >1,000, the two dilutions used for screening. A neutralizing antibody response was not detected in any of the neonates against RV strains DS1, SA11, and ST3.

^d This baby excreted RV only on its 20th day of life.

DISCUSSION

In this study, we found that 116E-like RV strains preferentially infect neonates born to mothers with low levels of neutralizing antibodies against this strain, suggesting that immunity to neonatal infection may be conferred transplacentally by maternal neutralizing antibody. We propose that this mechanism may help explain why RV infection among neonates is often caused by strains that are not commonly encountered in the community. Supporting this theory, we found classic neonatal RV strains with genotype P[6] commonly circulating in the community, whereas the newborns were infected predominantly with 116E-like (P[11]G9) strains (21). One explanation of why the neonates were not infected with P[6] strains could be that they acquired VP4-neutralizing antibodies to these strains from their mothers, who had prior exposure to the strains in the community, as demonstrated by the cord blood neutralizing antibody titers to the neonatal strain ST3 (P2A[6]G4) in the infants' cord blood. Although 116E-like strains are not common in the community, their G type, G9, is common. In spite of this fact, the newborns were infected with P[11]G9 strains, possibly because of their low levels of maternal neutralizing antibodies to the VP4 protein (P8[11]), since the genotype P[11] is uncommon in the community and the mothers may not have been exposed to this genotype. Thus, it is possible that the level of neutralizing antibodies to VP4 and not to VP7 is critical in determining with which RV strains the neonates become infected. Unfortunately, we could not measure the levels of antibodies directed against the individual neutralizing antigens VP7 and VP4, because the fluorescent focus assay used in this study does not discriminate between VP7 and VP4 antibody responses. One way to do this would be to select reassortant RVs, with a P2A[6] or P8[11] VP4 gene in combination with a G9 VP7 gene or any other VP7 gene that is rare in India for P-type-specific immune studies. Alternatively, the expressed proteins of these VP4 and VP7 genes can be used to design specific immunoassays.

Strain 116E is under consideration as a vaccine candidate because of the protection it confers on infants against severe RV diarrhea and its ability to elicit a potent RV-specific immune response (1a, 6, 16). This study confirmed the immunogenicity of 116E-like strains in their ability to evoke strong serum and salivary IgA and neutralizing antibody responses in the infected babies. The serum IgA response was considerably lower than the salivary IgA response in the infected babies, possibly because of the asymptomatic nature of RV infection in neonates (19). The neutralizing antibody response was predominantly homotypic in both serum and saliva, and a heterotypic response to other reference RV strains, except Wa, was not observed. Our findings were not unusual and corroborated

the outcomes of other studies, in which the primary RV infection in infants usually evoked a homotypic response while the heterotypic response was most often found among older children and adults who had prior exposure to RVs (4, 9, 14). However, one of the main limitations of this study was the fact that the low volumes of cord blood and convalescent sera collected from the babies restricted the analysis of the neutralizing antibodies to two dilutions in the assay. Consequently, we were unable to determine the specific neutralization titer required for protection against infection.

Overall, this study provides evidence that the RV strains that infect neonates may evolve through a process of immune selection and suggests that maternal neutralizing antibodies are important in protecting neonates from RV infection and perhaps disease. However, the actual mechanism of protection conferred by neutralizing antibodies in the serum is still unclear. Further, our findings have important implications for the development of an effective RV vaccine. The vaccines that are currently being developed with human RV strains (89-12; cold-adapted RV strain) having common P types may not be effective in babies with residual maternal antibodies against the strains to which their mothers may have had prior exposure. The U.S. RV vaccine study group drew similar conclusions in their recent report, in which they found that high levels of transplacental maternal IgG interfered with the development of infants' neutralizing antibody responses following oral inoculation with live RV vaccines (23). On the other hand, animal RV strains having P types that are not common in the community and to which the mothers have had no prior exposure may be more appropriate for use as vaccines in young infants. Further studies are needed to examine these issues. Specifically for India, where G9 strains are common in the community, the use of 116E as a vaccine, together with the rhesus tetravalent vaccine, may provide a broader protection against all the circulating RV serotypes, including serotype G9, which is not represented in the current rhesus RV tetravalent vaccine (G1-G4).

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