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Serotype Specificity of the Neutralizing-Antibody Response Induced by the Individual Surface Proteins of Rotavirus in Natural Infections of Young Children

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The relative contribution of the rotavirus surface proteins, VP4 and VP7, to the induction of homotypic as well as heterotypic neutralizing antibodies (NtAbs) in natural infections was studied. The NtAb titers of paired sera from 70 infants with serologically defined primary rotavirus infections were determined with a panel of rotavirus reassortants having one surface protein from a human rotavirus (serotypes G1 to G4 for VP7 and P1A and P1B for VP4) and the other surface protein from a heterologous animal rotavirus strain. A subset of 37 children were evaluated for epitope-specific antibodies to the two proteins by an epitope-blocking assay. The infants were found to seroconvert more frequently to VP4 than to VP7 by both methods, although the titers of the seroconverters were higher to VP7 than to VP4. Both proteins induced homotypic as well as heterotypic NtAbs. G1 VP7 frequently induced a response to both G1 and G3 VP7s, while G3 VP7 and P1A VP4 induced mostly homotypic responses.

Group A rotaviruses are the leading cause of severe dehydrating gastroenteritis in children under 3 years of age (29). These viruses are an important cause of infant morbidity in developed countries and of infant mortality in developing countries, where they are responsible for nearly 1 million diarrheal deaths per year (28, 29); therefore, there is considerable interest in developing an effective vaccine.

The surfaces of rotaviruses are formed by two proteins, VP4 and VP7. Antibodies to these proteins have the ability to neutralize the infectivity of the virus in vitro as well as in vivo (34, 39, 53), and the specificities of these antibodies to neutralize different rotavirus strains have been used to classify rotaviruses into various serotypes. Since both proteins induce neutralizing antibodies, the viruses can be classified based on either VP7 (G serotypes) or VP4 (P serotypes).

On the basis of VP7, 14 different serotypes have been identified among group A rotaviruses (14, 27). Ten of these serotypes infect humans, although four of them (G1 to G4) appear to account for the majority of isolates (4, 26, 63). VP4 from human rotaviruses has been classified into at least 20 genetic groups (P genotypes) by hybridization and sequence analysis (14). Eight of these P genotypes have been found in human rotaviruses, seven of which have been confirmed to represent different antigenic groups (P serotypes) as determined by neutralization with hyperimmune sera to baculovirus-expressed VP4 proteins or to reassortant rotaviruses (14, 26). Although the number of potential combinations of VP4 and VP7 proteins in human rotavirus strains is large, epidemiological studies with VP4 genotyping methods indicate that rotavirus strains with G1, G3, or G4 VP7 proteins usually have a P1A VP4 protein, while the G2 VP7 protein is usually associated with P1B VP4 (17).

Natural rotavirus infection protects against disease caused by reinfections with the same or different rotavirus serotypes (3, 58), and the level of intestinal virus-specific secretory immunoglobulin A (IgA) antibodies (12, 32) and the presence of serum IgA (41) have been shown to correlate with this protection. It has also been shown that serologically defined primary rotavirus infections induce heterotypic as well as homotypic neutralizing antibodies (NtAb) (5, 18, 46, 64); however, the role of these antibodies in protection is not clear. Some studies have indicated that homotypic NtAb are protective against clinical illness (7, 41), while others have found protection even in the absence of NtAb to the infecting strain (24, 57, 59, 65). Also, studies with animal models have shown that intestinal secretory IgA and serum IgA may be important to confer protection against reinfections (15, 36) and may play a role in viral clearance (37). Furthermore, the presence of a cytotoxic T-cell response was found to correlate with clearance of the virus in mice (16, 35), and an as-yet-unidentified factor, other than antibodies and CD8 cells, was also important for resolving infection (35). It is clear that designing the most effective rotavirus vaccine will require the identification of the various immunological effectors active in protection against reinfection

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TABLE 1. Origin and serotype specificity of the surface proteins from the rotavirus strains employed in the characterization of the NtAb immune response[a]

Rotavirus strain	Origin (serotype)[b] of the surface protein	
	VP7	VP4
Wa	Human (G1)	Human (P1A)
S2	Human (G2)	Human (P1B)
Price	Human (G3)	Human (P1A)
ST3	Human (G4)	Human (P2A)
D×RRV	Human (G1)	Simian (P5B)
RRV	Simian (G3)	Simian (P5B)
EDIM×RRV (3–17)	Murine (G3)[c]	Simian (P5B)
EDIM×CJN (4–10)	Murine (G3)[c]	Human (P1A)
EDIM	Murine (G3)[c]	Murine (P10)
DS1×RRV	Human (G2)	Simian (P5B)
UK×DS1	Bovine (G6)	Human (P1B)
UK	Bovine (G6)	Bovine (P7)
ST3×SA11	Human (G4)	Simian (P5B)
SA11	Simian (G3)	Simian (P5B)

[a] The reassortant and control rotavirus strain pairs used to determine the specific reactivity to each of the G and P serotypes were as follows: D×RRV and EDIM×RRV for serotype G1; DS1×RRV and EDIM×RRV for serotype G2; RRV and EDIM×RRV for serotype G3; ST3×SA11 and SA11 for serotype G4; EDIM×CJN and EDIM for serotype P1A; and UK×DS1 and UK for serotype P1B. RRV rotavirus was used to determine the NtAb response to human G3 VP7, since RRV VP7 shares neutralization specificity with human G3 VP7 proteins.

[b] Serotypes are according to the classification proposed by Estes (14). The P serotype of simian rotavirus SA11 was recently described by Hoshino et al. (25).

[c] EDIM is a G3-like virus. It has a limited and one-way cross-reactivity by neutralization with several prototype G3 strains (59).

and the optimization of the induction of the corresponding host's immune response.

In this study, we have characterized the immune response of children naturally infected with rotavirus of known G and P serotypes in an attempt to understand the specificity of the NtAb response induced by each of the two rotavirus surface proteins. Both proteins carry heterotypic as well as homotypic epitopes (26); however, their individual contributions to cross-reactive NtAbs in primary natural rotavirus infections have not been fully evaluated. By using neutralization and epitope-blocking assays, we found that both surface proteins elicited homotypic as well as cross-reactive NtAbs.

MATERIALS AND METHODS

Patients and serum specimens. We studied the immune response to rotavirus infection in paired serum samples from 71 children who were part of a larger study designed to determine the antigenic diversity of the surface proteins of rotavirus strains circulating in Mexico (44a). The patients had been admitted with acute diarrhea to hospitals or outpatient clinics in five cities of Mexico (Mexico City; Monterrey, Nuevo León; San Luis Potosí, San Luis Potosí; Tlaxcala, Tlaxcala; and Mérida, Yucatán) during the epidemic season from October 1994 to March 1995. The children had an average age of 10.3 months, with a median age of 9 months (range, 2 to 26 months). Acute-phase serum samples were collected 1 to 3 days after the onset of symptoms, and convalescent-phase sera were obtained 2 to 3 weeks later.

Viruses. Rotaviruses Wa, S2, Price, ST3, RRV, UK, D×RRV, and DS1×RRV were obtained from H. B. Greenberg (Stanford University, Stanford, Calif.); the isolation and characterization of reassortant viruses D×RRV and DS1×RRV have been reported previously (38); rotaviruses EDIM, EDIM×RRV (strain 3–17), and EDIM×CJN (strain 4–10) have been described previously (60); rotavirus UK×DS1 was obtained from Y. Hoshino (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.); rotavirus SA11 (clone 3) was obtained from M. K. Estes (Baylor College of Medicine, Houston, Tex.); rotavirus ST3×SA11 was obtained from I. H. Holmes (Melbourne University, Melbourne, Australia). The G and P serotypes of all these viruses are listed in Table 1. The identities of the viruses were confirmed at the beginning and end of the study by polyacrylamide gel electrophoresis of their genomic RNAs.

The G serotypes of the viruses isolated from the children included in this study have been described by Padilla-Noriega et al. (44a). Of the 71 rotavirus strains, 24 were serotype G1 and 47 were serotype G3. The VP4 proteins of the 71 rotavirus strains most probably belong to serotype P1A, based on their pattern of reactivity with VP4-specific neutralizing monoclonal antibodies (NtMAbs) and on the VP4 genotyping of a subset of these strains (44a).

IgM and IgG enzyme-linked immunosorbent assay (ELISA). For determination of IgM antibodies, 96-well microtiter plates (enzyme immunoassay [EIA]/radioimmunoassay plates; Costar) were coated with a 1:5,000 dilution of goat anti-human rotavirus strain D (kindly provided by H. B. Greenberg) in phosphate-buffered saline containing 0.05% sodium azide (PBS-Az). After overnight incubation at 4°C, the plates were washed twice with PBS-Az and blocked with 10% fetal bovine serum (FBS) in PBS-Az overnight at 4°C. The plates were then washed twice with PBS-Az and incubated for 2 h at 37°C with an undiluted MA104 cell lysate that had been infected with rotavirus RRV or mock infected. After the plates were washed four times with PBS-Az, serial dilutions (1:25 to 1:800) of the children's sera in PBS-Az containing 5% FBS (PBS-5% FBS) were added to duplicate wells and incubated for 2 h at 37°C. The plates were then washed four times and incubated with a 1:1,000 dilution in PBS-5% FBS of goat anti-human IgM conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories) for 1 h at 37°C. The plates were then washed four times, and the presence of phosphatase activity was detected by incubation for 1 h at 37°C with no. 104 substrate (Sigma Chemical Co.). The optical density was read at 405 nm.

The IgG ELISA was carried out in the same way as the IgM ELISA, with the following modifications. The undiluted RRV virus-infected and mock-infected MA104 cell lysates were bound directly to the plate by overnight incubation at 4°C. The plates were blocked with 5% nonfat dry milk in PBS-Az–Tween 0.05% (PBS-T), and the washings were done with PBS-T. The serum samples and anti-human IgG conjugated to alkaline phosphatase (1:2,000; Kirkegaard & Perry Laboratories) were diluted in PBS-T–2.5% milk. The IgM and IgG antibody titers were defined as the highest serum dilution that gave an optical density equal to or greater than 0.2 and greater than twice the negative control value obtained when mock-infected cells were used as the antigen.

Neutralization antibody assay. NtAb titers in the children's sera were measured by an immunochemical focus reduction neutralization test (1). The titer of NtAb in a serum sample was defined as the highest serum dilution at which a reduction of at least 60% in the number of infected cells was observed compared with controls in which PBS had been used instead of serum.

MAbs. The VP7-specific monoclonal antibodies (MAbs) used in the epitope-blocking assay (EBA) described below were 5E8 and 2C9 (G1 specific), 2F1 (G2 specific), 4F8 (G3 specific) (48), ST-2G7 (G4 specific) (55), and 2A4 (G1 and G3 heterotypic) (44). The VP4 MAbs used were 1A10, derived from the serotype P1A strain Wa (44); RV5:2, derived from the serotype P1B strain RV5 (11); and HS6, derived from the serotype P2A strain ST3 (44). These VP4 MAbs had been preliminarily shown to be specific to human rotavirus strains having the same serotype as the immunizing virus, P1A, P1B, or P2A, respectively, when assayed by EIAs (11, 44). In addition, we used the cross-reactive VP4 MAbs 1E4, derived from the serotype P1A strain Wa (44), and YO-2C2, derived from the serotype P1A strain YO (52), both of which neutralize P2A as well as P1A rotavirus strains.

Mutations that allow viruses to escape neutralization by the above-mentioned MAbs have been mapped for several of these antibodies: MAb 2C9, amino acid 94 (21); 2F1, amino acids 94, 208, 213, and 291 (13); 4F8, amino acid 96 (31); ST-2G7, amino acid 145 (20); 1A10, amino acid 458 (42); RV5:2, amino acid 148 (30); HS6, amino acid 72 (42); 1E4, amino acid 392 (42); and YO-2C2, amino acid 305 (51).

EBA. The EBA was modified from the assay previously described by Shaw et al. (47). A 96-well plate (EIA/radioimmunoassay plates; Costar) was coated overnight at 4°C with an optimal dilution of the indicated MAb in PBS-Az. The plates were washed twice with PBS-Az and blocked overnight at 4°C with 10% FBS in PBS-Az. After the plates were washed twice with PBS-Az, a homotypic virus was added which had been previously incubated overnight at room temperature with serial dilutions (1:25 to 1:1,600) of the patient's sera. Each virus was diluted in PBS-5% FBS to give an optical density reading of 1.2 when it was tested in the absence of human sera. After 4 h of incubation at 37°C, the plates were washed four times with PBS-Az, and an equivolumetric mix of rabbit hyperimmune antisera to Wa, DS1×RRV, RRV, and ST3 rotaviruses diluted 1:2,500 in PBS-2.5% FBS was added, and the plates were incubated for 2 h at 37°C. The plates were then washed four times and incubated with a 1:1,000 dilution of goat anti-rabbit IgG coupled with alkaline phosphatase (Kirkegaard & Perry Laboratories) in PBS-2.5% FBS and incubated for 1 h at 37°C. The plates were washed four times, and the presence of phosphatase activity was detected as described above. The epitope-specific antibody titer was defined as the highest dilution of serum that gave an optical density that was less than or equal to 50% of the value of the nonblocking controls. The homotypic viruses used were Wa for MAbs 5E8, 2C9, 1A10, and 1E4; S2 for MAbs 2F1 and RV5:2; Price for MAbs 4F8, 2A4, and YO-2C2; and ST3 for MAbs ST-2G7 and HS6.

TABLE 2. ELISA IgM and IgG rotavirus antibody presence in serum samples from rotavirus-infected children

Phase of serum	No. of positive samples/total (GMT)	
	IgM	IgG[a]
Acute	62/71 (464)	16/71 (52)
Convalescent	61/71 (372)	65/71 (120)
Total[b]	70/71	69/71

[a] Sixty of 71 children seroconverted for IgG. Seroconversion was defined as a fourfold increase in titer in comparisons of the acute- and convalescent-phase sera.

[b] The numbers in this category refer to children having rotavirus-specific antibodies either in the acute- or convalescent-phase sera or in both types of sera.

RESULTS

ELISA antibody response. The aim of this study was to determine the specificity of the NtAb response directed to the individual rotavirus surface proteins VP4 and VP7. To accomplish this, it was important to determine if the children included in the study had experienced a primary or secondary rotavirus infection. To investigate the immune status of the patients, we analyzed the Ig class specificity of the serum antibody response to rotavirus. Of 71 children studied, 70 (99%) had antirotavirus IgM either in the acute- or convalescent-phase serum (Table 2). Sixty-two (87%) had detectable virus-specific IgM (titer, 1:50 to 1:>800) in the acute-phase serum, while 61 (86%) had IgM in the convalescent serum sample and 53 (75%) children had detectable IgM levels in both serum samples. As negative controls, we included two serum samples from adults and two paired serum samples from children who had experienced a proven secondary rotavirus infection (2). None of these control serum samples was positive for IgM. Since the presence of virus-specific IgM has been reported to be a reliable marker for primary rotavirus infections (18, 23), the present results suggest that all but one of the children studied were experiencing a primary infection.

Analysis of the IgG titers showed that 69 (97%) of the 71 children had detectable antirotavirus antibody (Table 2). However, only 16 (23%) of the children had IgG in the acute-phase serum (titer, 1:25 to 1:50 with the exception of two children who had titers of 1:100 and 1:>800), while 65 (92%) had detectable IgG in the convalescent-phase serum sample (titer, 1:25 to 1:>800). Sixty (85%) of the children seroconverted for IgG. Thus, the low frequency (23%) and quantity of rotavirus-specific IgG in the acute-phase sera and the presence of IgM antibody in 99% of the children, which appeared earlier than IgG in most patients, suggest that all but one of the rotavirus infections studied were primary infections. For the study of the specificity of the NtAb response described below, only the 70 children with a serologically defined primary infection were included.

General NtAb seroconversion to reference strains. We first analyzed the overall children's NtAb response to four reference rotavirus strains representing serotypes G1 to G4 for VP7 and serotypes P1A, P1B, and P2A for VP4 (Table 3). Twenty (28%) of the children were found to have NtAb to one or more of the reference strains in the acute sera (geometric mean titer [GMT], 146; range, 1:100 to 1:200); these antibodies are presumably either IgG of maternal origin or IgM actively induced by the virus infection. Given these findings, the conclusions in this study are based only on cases in which there were seroconversions, defined as a fourfold or greater rise in the titer of NtAb in comparisons of acute- and convalescent-phase sera.

Of the 47 children infected with a serotype G3 virus, 38 (81%) seroconverted to at least one rotavirus strain (Table 3). Thirty five (74%) seroconverted to Price, the serotype G3,P1A reference strain; 25 (53%) seroconverted to Wa (G1,P1A); and only 3 and 5 children seroconverted to rotaviruses S2 (G2,P1B) and ST3 (G4,P2A), respectively. The most frequent pattern of NtAb response was the seroconversion to both Wa and Price (16 children), followed by the single seroconversion to Price (12 children) (Table 3). Other less-frequent patterns of seroconversion were observed among the G3-infected children; these patterns, found in no more than three children each, comprised NtAb against a single reference strain other than Price or NtAb against two, three, or all four strains tested.

In the case of the 23 G1-infected children, 17 (74%) were seroconverters; 13 (57%) seroconverted to the G1 virus Wa, while 12 (52%) did so for Price. None of these children seroconverted to S2, and four seroconverted to ST3 (Table 3). Similar to the response pattern among the G3-infected children, the most frequent pattern of response among the G1-infected patients was the double seroconversion to Wa and Price (seven children), followed by the single homotypic seroconversion to Wa (four children). Other less-frequent patterns of response included seroconversion to one, two, or three of the reference viruses.

As shown in Table 3, 23 (42%) of the 55 children who seroconverted did so to both Wa and Price, suggesting a high degree of cross-reactivity between these two strains. Since both G1 and G3 strains are usually associated with a VP4 P1A protein, and the characterization of the VP4 from the infecting strains supports this assumption (44a), the observed cross-reactivity could be mediated by antibodies to this protein. It is still possible, however, that the heterotypic response is due to the presence of shared epitopes in the VP7 protein of these two serotypes. Also, three and nine of the subjects seroconverted to the heterotypic strains S2 and ST3, respectively. These seroconversions may be the result of low-level interserotypic cross-reactivity of either VP4 or VP7.

TABLE 3. Neutralizing antibody seroconversion patterns in children with serologically defined primary rotavirus infections

Serotypes of infecting virus (no. of samples)	No. of children showing the indicated NtAb seroconversion patterns (GMT)[a]								
	Wa	P	ST3	Wa, P	P, ST3	Wa, S2, P	Wa, P, ST3	Wa, S2, P, ST3	Total (%)
G3, P1A (47)	3 (800)	12 (356)	0	16 (418, 497)	1 (400, 200)	2 (1,131, 283, 566)	3 (317, 1,008, 200)	1 (1,600, 800, 1,600, 400)	38 (81)
G1, P1A (23)	4 (476)	2 (283)	1 (400)	7 (345, 328)	1 (200, 400)	0	2 (566, 400, 400)	0	17 (74)
Total (70)	7 (594)	14 (345)	1 (400)	23 (400, 438)	2 (283, 283)	2 (1,131, 283, 566)	5 (400, 696, 264)	1 (1,600, 800, 1,600, 400)	55 (79)

[a] Seroconversion was defined as a fourfold or greater rise in the titer of NtAbs in comparisons of the acute- and convalescent-phase sera. The GMTs for the strains indicated in the boxheads are shown sequentially in the body of the table. The G and P serotypes of the reference strains are as follows: for Wa (G1, P1A), for S2 (G2, P1B), for P (G3, P1A), and for ST3 (G4, P2A).

TABLE 4. Neutralizing antibody seroconversion patterns for rotavirus VP4 and VP7 proteins in children with serologically defined primary rotavirus infections

Serotypes of infecting virus (no. of samples)	No. of children showing the indicated NtAb seroconversion patterns to rotavirus surface protein indicated (GMT)[a]									
	VP7						VP4			
	G1	G3	G1, G3	G2, G3	G1, G3, G4	Total (%)	P1A	P1B	P1A, P1B	Total (%)
G3, P1A (47)	0	21 (575)	4 (200, 1,131)	1 (800, 800)	0	26 (55)	34 (289)	1 (200)	3 (317, 200)	38 (81)
G1, P1A (23)	2 (400)	4 (200)	6 (252, 504)	0	2 (283, 200, 400)	14 (61)	16 (238)	0	1 (200, 200)	17 (74)
Total (70)	2 (400)	25 (486)	10 (230, 696)	1 (800, 800)	2 (283, 200, 400)	40 (57)	50 (272)	1 (200)	4 (282, 200)	55 (79)

[a] Seroconversion was defined as a fourfold or greater rise in the titer of NtAb in comparisons of the acute- and convalescent-phase sera. The GMTs for the serotypes indicated in the boxheads are shown sequentially in the body of the table. All 70 paired sera were analyzed for the presence of NtAb to G1 and G3 VP7s and NtAb to P1A VP4. For G2 and G4 VP7s and for P1B VP4, we analyzed only the sera in which antibodies to S2 (19 children) or ST3 (17 children) had been previously detected, including sera for which no seroconversion occurred.

NtAb seroconversion to individual surface proteins. We studied the balance of the NtAb response to the rotavirus surface proteins VP4 and VP7 and defined the relative contribution of each of these proteins to the observed NtAb cross-reactivity. To accomplish this, we determined the NtAb titers of the children's sera versus those of a panel of rotavirus reassortants having either VP4 or VP7 from the epidemiologically relevant human rotavirus serotypes G1 to G4 for VP7 and P1A and P1B for VP4 and the second outer capsid protein from a heterologous animal rotavirus strain to which little or no NtAb is made (see Table 1). For the analysis of the response to G3 VP7, the simian rotavirus RRV strain was used, since RRV VP7 shares neutralization specificity with human rotavirus VP7 serotype G3. Seroconversion to a specific VP7 or VP4 protein serotype was given a positive score when the subject seroconverted to the reassortant virus having the relevant surface protein but did not seroconvert to the control rotavirus strain (Table 1).

As indicated in Table 4, more infants seroconverted to VP4 (79%) than to VP7 (57%), although the NtAb GMTs (calculated from the convalescent-phase serum titers of the subjects who seroconverted) were higher to VP7 (504) than to VP4 (266). The analysis of the individual response to VP7 showed that 26 (55%) of the G3-infected children seroconverted to G3 VP7, while only 4 (9%) and 1 (2%) of these children had detectable antibodies to G1 and G2 VP7s, respectively. On the other hand, similar to what was observed in the analysis of the general NtAb response, the G1-infected children induced a more heterotypic response to VP7. Ten (44%) of these children responded to G1 VP7, while 12 (52%) seroconverted to G3 VP7.

With regard to the NtAb seroconversion to VP4, a heterotypic response to P1B VP4 (considering that the VP4 serotype of the infecting strains is P1A) was observed in only 4 children and 1 child infected with serotype G3 and G1 viruses, respectively. However, the homotypic NtAb response to VP4 P1A, which was found in 79 and 74% of the G3 and G1 virus-infected children, respectively, can be considered heterotypic with regard to the VP7 protein (G serotype), since in principle this antibody response could neutralize G1,P1A and G3,P1A strains as well as G4,P1A viruses.

The majority (81%; 21 of 26) of the G3-infected children who responded to VP7 responded exclusively to G3 VP7 (Table 4). On the other hand, of 14 G1-infected children who responded to VP7, only 2 (14%) recognized G1 VP7 in an exclusive manner; 4 recognized only G3 VP7, and 6 recognized both G1 and G3 VP7 proteins. These observations confirm that G1 VP7 induced a more heterotypic response than G3 VP7. On the other hand, as mentioned above, G1 and G3 viruses elicited very similar patterns of NtAb response to VP4.

Epitope-specific seroconversion to the surface proteins. To further dissect the immune response to the rotavirus surface proteins, we analyzed the response to six and five individual neutralizing epitopes on VP4 and VP7, respectively, using an EBA (47).

A subset of 37 paired sera selected at random (25 from G3 and 12 from G1 virus-infected children) was analyzed (Table 5). In 22 (59%) of the children studied, we detected seroconversion (a fourfold increase in the antibody titer) to at least one of the VP7 epitopes tested, while 26 (70%) of the children showed seroconversion to at least one VP4 epitope. Similar to what was observed for the NtAb response, the response to VP7 epitopes was less frequent than that to VP4 epitopes, although the antibody GMT was higher for VP7 (1:152) than for VP4 (1:96). The children infected with G3 rotaviruses seroconverted more frequently and with a higher GMT to the G3-

TABLE 5. Antibody seroconversion to rotavirus VP4 and VP7 proteins by an EBA

Serotypes of infecting virus (no. of samples)	No. of children showing seroconversion to the indicated protein and epitope (GMT)[a]								
	VP7 epitope					VP4 epitope			
	5E8 (G1)	2F1 (G2)	4F8 (G3)	2A4 (G1, G3)	Total [%]	1A10 (P1A)	1E4 (P1A, P2A)	YO-2C2 (P1A, P2A)	Total [%]
G3, P1A (25)	6 (56)	3 (126)	9 (400)	5 (114)	14 [56]	8 (109)	15 (87)	2 (141)	17 [68]
G1, P1A (12)	1 (50)	2 (71)	6 (252)	1 (50)	8 [67]	7 (149)	4 (84)	2 (50)	9 [75]
Total (37)	7 (55)	5 (100)	15 (333)	6 (99)	22 [59] (152)	15 (126)	19 (80)	4 (84)	26 [70] (96)

[a] The GMT was calculated from the convalescent-phase serum titers of the subjects whose sera seroconverted. The highest titer was used when there were seroconversions to more than one epitope. None of the serum samples competed with MAbs 2C9 (G1 specific), ST-2G7 (G4 specific), RV5:2 (P1B specific), or HS6 (P2A specific). The viruses used as antigens were as follows: Wa for MAbs 5E8, 2C9, 1A10, and 1E4; S2 for MAbs 2F1 and RV5:2; Price for MAbs 4F8, 2A4, and YO-2C2; and ST3 for MAbs ST-2G7 and HS6.

TABLE 6. Correlation between EBA and neutralization assay for the analysis of the immune response of 37 rotavirus-infected children

Assay	G serotype of infecting virus (no. of samples)	Mean age (mo)	No. of children showing seroconversion to the indicated virus strain			
			Wa	S2	Price	ST3
EBA[a]	G1 (12)	9	9	2	10	0
	G3 (25)	10	21	3	18	0
	Total (37)	9.6	30	5	28	0
Neutralization	G1 (12)		7	0	6	0
	G3 (25)		14	2	16	0
	Total (37)		21	2	22	0

[a] The children's sera examined by the EBA seroconverted to either VP4 or VP7.

specific 4F8 epitope than to the G1-specific 5E8 epitope or to any of the other three VP7 epitopes analyzed. Likewise, the G1-infected children seroconverted more frequently to the G3-specific epitope 4F8 than to the G1-specific epitope 5E8, in agreement with the overall NtAb response to VP7 (the G1-infected children responded more frequently to G3 VP7 than to G1 VP7). None of the children seroconverted to the G1-specific epitope 2C9 or the G4-specific epitope ST-2G7. With regard to VP4, the G3-infected children seroconverted more frequently to the P1A/P2-specific epitope 1E4 (15 of 25; 60%), while the G1-infected subjects recognized the homotypic epitope 1A10 more efficiently (7 of 12; 58%).

A good correlation was found between EBA and neutralization assays for the analysis of the immune response. The most frequent response detected by either method was against viruses Wa and Price (Table 6), and the number of children seroconverting to these two viruses was similar for a given assay: 30 (81%) and 28 (76%) children seroconverted by EBA, while 21 (57%) and 22 (59%) seroconverted by the neutralization assay. Only 3 and 1 of the children who seroconverted for neutralizing antibodies to Wa and Price, respectively, did not seroconvert by EBA. In the case of the G2,P1B S2 rotavirus strain, 7 subjects seroconverted, 5 (14%) by EBA and 2 (5%) by neutralization assay. Neither of the two methods detected a response to virus ST3.

DISCUSSION

In this study, we have analyzed the antibody immune response of children with serologically defined primary rotavirus infections, with emphasis on understanding the homotypic and heterotypic NtAb response elicited by the individual surface proteins VP4 and VP7. The data presented in this study support the hypothesis that a primary rotavirus infection is able to induce heterotypic NtAb responses and that the magnitude of these responses is intrinsic to the particular infecting rotavirus strain (2, 9, 18, 33, 40, 46, 54, 60). The rotavirus infections characterized in this study were caused by strains having a VP7 protein with either G1 or G3 specificity and a VP4 protein most probably having a serotype P1A specificity. In addition to the homotypic response, both G1 and G3 viruses were able to induce heterotypic responses to one or more strains. The most frequent pattern of response was the double seroconversion to the serotype G1,P1A strain Wa and the serotype G3,P1A strain Price. The NtAb GMTs to both strains were similar, regardless of the G serotype of the infecting strain. In some cases there was a heterotypic seroconversion in the absence of a homotypic response. This result might be due to antigenic differences between the surface proteins of the reference strains and the field infecting strains, as has been noted by others (40).

Previous studies have provided conflicting results on the relative immunodominance of VP4 and VP7 in humans. VP4 has been reported to be the immunodominant protein that induces NtAbs in adults experimentally inoculated with attenuated human rotavirus (60) as well as in children orally vaccinated with a rotavirus reassortant strain that had only VP7 of human origin (8, 45). VP4 was also the immunodominant protein in children naturally infected with human rotavirus strains of serotype G1 (62). In contrast, children vaccinated with virus WC3, a rotavirus bovine strain, responded with NtAb almost exclusively to VP7 (59a). Likewise, the immune response to VP7 epitopes showed a significant correlation with protection against infection and symptom development in adults challenged with a serotype G1 human rotavirus strain (20). More recently, the immunodominance of VP4 and VP7 was shown to vary in three children studied (19). In the present study, more children were found to seroconvert to VP4 (79%) than to VP7 (57%), indicating that in natural infections with rotavirus strains belonging to two different G serotypes (G1 and G3), both surface proteins elicit NtAb, although VP4 seems to be more frequently detected by the immune systems of the infected children.

The high incidence of viruses belonging to two G serotypes (G1 and G3) during the season studied and the frequent NtAb heterotypic response found allowed us to explore the contribution of VP4 and VP7 to inducing heterotypic antibodies. The response to VP4 was found to be mostly homotypic, since only 7% of the subjects seroconverted to heterotypic P1B VP4, while 77% children seroconverted to the homotypic P1A VP4. However, this frequent response of homotypic NtAb to VP4 can be considered heterotypic with regard to VP7, since G1, G3, and G4 viruses usually have a P1A VP4 protein (10, 17, 49, 50, 56). Thus, if NtAbs are confirmed to be at least one of the immunological effectors that protect against symptomatic reinfections (57), the homotypic VP4 NtAb response observed might be of great relevance for the induction of protection against three of the four epidemiologically relevant human rotavirus strains.

With regard to VP7, G3 viruses induced mostly a homotypic response, since only 9% of the subjects seroconverted to G1 VP7. On the other hand, G1 viruses induced a highly heterotypic response, with 52% of the patients responding to G3 VP7. Altogether, these data provide the first evidence that VP7 as well as VP4 can induce a heterotypic NtAb response in children with primary natural rotavirus infection and thus may contribute to the induction of heterotypic protection.

To understand more precisely the nature of the homotypic and heterotypic immune responses observed, we studied the immune response to specific neutralization epitopes on both VP4 and VP7 by an EBA (47). In agreement with previous studies, we observed a good correlation between the EBA and neutralization assays (21, 22, 33, 47, 54). The VP7 epitope most frequently recognized by the sera from both G1 and G3 virus-infected children was defined by the G3-specific MAb 4F8. In the case of the G1 virus-infected subjects, the competition of their sera with MAb 4F8 might be explained by the induction of heterotypic VP7 antibodies by the G1 viruses that react with G3 VP7 within or near the 4F8 epitope. In this regard, it is important to mention that EBA cannot be considered truly epitope specific in all cases due to the overlapping nature of

some rotavirus neutralization epitopes (26, 31). The heterotypic competition of serum antibodies from G3 virus-infected children with MAb 5E8 (G1 specific) and of both G1 and G3 virus-infected subjects with MAb 2F1 (G2 specific) might be explained by the same mechanism. The heterotypic seroresponse to VP7, whether measured by neutralization or EBA, was not a function of the age of the children or the presence of rotavirus antibodies in the acute-phase sera, as has been observed by others (22, 33, 54), suggesting that this is truly a heterotypic primary response to VP7.

The absence of antibodies in the children's sera that block the binding of MAb 2C9 is in agreement with the low amount of this epitope (3%) among G1 rotavirus strains characterized previously in Mexico compared to that of the 5E8 epitope (84%) (43). Similarly, only a low percentage of G1 isolates found among rotavirus-infected Bangladeshi children was recognized by 2C9 compared to other serotype G1-specific VP7 MAbs, including 5E8 (61). It is of interest that although only 1 of the 12 children (whose serum was tested by EBA) infected with a serotype G1 virus had antibodies that competed with MAb 5E8, all 12 G1 viruses reacted with MAb 5E8 in the serotyping ELISA (data not shown). This result could be explained if the 5E8 epitope, although present in the viruses, were not very immunogenic in a natural infection or if the 5E8 epitope were immunogenic but the antibodies elicited did not efficiently recognize rotavirus Wa, the virus used as the antigen in the EBA.

With regard to VP4, the two most frequently recognized epitopes were 1A10 (P1A specific) and 1E4 (P1A/P2A heterotypic). Despite the fact that 19 of 37 (51%) children responded against epitope 1E4, none of them neutralized ST3 virus, which has a VP4 protein that belongs to serotype P2A. The blocking of MAb 1E4 by the patient's sera might be due to competition by homotypic antibodies to VP4 P1A, since MAb 1E4 was tested with Wa as the target virus, or to truly heterotypic antibodies that recognize both P1A and P2A viruses but fail to neutralize the reference strain ST3.

The presence of heterotypic MAbs directed to both VP4 and VP7 in the children's sera, as evidenced by the EBA and neutralization assays, suggests this as a mechanism for the cross-reactive NtAb response observed in primary natural rotavirus infections. This information should be useful in the design of rational vaccines to increase their potential to protect against more than one rotavirus serotype. Further studies are needed, however, to establish the roles of NtAbs and other immunological (humoral and cellular) effectors in protection against natural rotavirus infections before an efficient immunogen can be designed.

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