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Immunodominance of the VP4 Neutralization Protein of Rotavirus in Protective Natural Infections of Young Children

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Natural infection by very similar strains of rotavirus during the 1988–1989 rotavirus season in Cincinnati, Ohio, provided complete protection of young children against subsequent rotavirus illnesses for a period of at least 2 years. Using this limited strain variability, we characterized the association between the titers of antibody to either the VP4 or the VP7 neutralization protein and protection against subsequent rotavirus disease. This was done by using reassortants that contained only one of the two rotavirus neutralization proteins of 89-12, a culture-adapted isolate representative of the protective rotavirus strains. The other neutralization protein in these reassortants was derived from a heterologous rotavirus (WC3 or EDIM) to which the infected subjects made little or no neutralizing antibody (titers, ≤20). The geometric mean titer (GMT) of antibody to 89-12 in convalescent-phase sera from the 21 subjects analyzed was 2,323. The GMT of antibody to a reassortant (strain WC-4) that contained the VP7 protein of 89-12 and VP4 of WC3 was 387. In contrast, the GMT of antibody to a reassortant (strain EDIM-7) that contained the VP4 protein of 89-12 and the VP7 protein of EDIM was 1,078. Thus, the major neutralization response was directed against VP4 rather than VP7, a finding that has important implications for development of appropriate rotavirus vaccines.

Rotaviruses have been established as the primary cause of severe infantile gastroenteritis, but the mechanism of immunity to rotavirus disease is poorly understood. At least eight serotypes of human rotavirus have been identified (8, 21), and neutralization by serotype-specific antibody has been suggested as one possible mechanism of protection. Accordingly, rotavirus vaccine candidates have been developed to stimulate neutralizing antibodies to circulating strains. Rotaviruses contain two outer capsid proteins, VP4 and VP7, and both are able to elicit neutralizing-antibody responses (11–13, 16). Although VP7 had been found to be immunodominant in hyperimmunized animals and was thought to be the major neutralization protein, VP4 was shown to be immunodominant in adults challenged with a nonattenuated strain of human rotavirus (24). Subsequently, it was also reported that VP4 was the immunodominant neutralization protein in infants vaccinated with human rotavirus × animal rotavirus reassortants containing only the VP7 gene from human strains (5, 18). In contrast, vaccination of infants with WC3, a bovine rotavirus that is serotypically unrelated to the major serotypes of human rotavirus, elicited neutralizing-antibody responses almost solely to VP7 (23). Likewise, immune responses to epitopes on VP7 showed significant correlations to protection against rotavirus infection or disease in adults challenged with a virulent serotype 1 human rotavirus (10). Thus, it is unclear whether VP4 or VP7 will be immunodominant following natural rotavirus infection of young children.

Natural rotavirus infections are known to provide at least partial protection against subsequent rotavirus disease. In a vaccine trial conducted with young children in Cincinnati, Ohio, placebo recipients who experienced a natural rotavirus infection were consistently protected for a period of at least 2 years (1, 2). Rotavirus isolates obtained during the first year of this study were all serotype 1 and had essentially identical electropherotypes (2). Thus, these subjects appeared to have been infected with very similar strains of rotavirus. Because it was not known whether active immunity correlates with neutralizing antibody to one or possibly both neutralization proteins, analysis of the convalescent-phase blood specimens of these subjects provided a unique opportunity to make this determination.

MATERIALS AND METHODS

Virus strains. Three strains of rotavirus were used in this study. WC3 is a serotype G6 bovine rotavirus that was obtained from F. Clark (Wistar Institute, Philadelphia, Pa.). EDIM is a murine rotavirus that was culture adapted in our laboratory (26) and found to share a weak, one-way serotypic relationship with the human rotavirus serotype G3 P strain (27). Strain 89-12 is a natural serotype G1 isolate of human rotavirus obtained during a vaccine trial (2) on the basis of its reactivity pattern with VP7-specific monoclonal antibodies (MAbs). Its VP4 or P serotype has not been specifically determined. However, the finding that infection with 89-12-like rotaviruses stimulated high titers of neutralizing antibodies to viruses belonging to P serotype 1A and much smaller amounts to those belonging to P serotype 1B (28), coupled with the results of this report showing that the major neutralization response was directed against the 89-12 VP4 protein, strongly indicated that 89-12 belonged to P serotype 1A (9). It was adapted to grow in cell culture as previously described (22). All three rotavirus strains were plaque purified three times in MA104 cells, and stock cultures were stored in aliquots at −70°C.

MAbs. Four MAbs, 2C9, 5E8, RV4:2 (all directed at epitopes on VP7), and HS3 (which recognized an epitope on the VP5 region of VP4), were used. The three MAbs that

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bind VP7 were derived after immunization with serotype G1 rotaviruses and specifically neutralize rotaviruses of this serotype (7, 17, 20). Nucleotide sequence analyses of escape mutants selected with 2C9 or RV4:2 revealed changes at amino acid positions 94 and 213, respectively (6, 15), indicating that they recognized different epitopes. Further analyses with additional escape mutants indicated that the three VP7-specific MAbs recognize different but overlapping epitopes (25). The VP4-specific MAb, HS3, was derived after immunization with the serotype G4 ST-3 strain but cross-neutralized serotype G1 rotaviruses (unpublished results).

Formation and selection of rotavirus reassortants. Reassortants of EDIM × 89-12 and WC3 × 89-12 were produced after coinfection of MA104 cells by using a multiplicity of infection of 20 for each coinfecting virus. Following a 24-h incubation of the coinfected cultures at 37°C, plaque purification of the desired reassortants was performed by using selective pressure. To select reassortants that contained EDIM VP7 and 89-12 VP4 genes, a 1:2,000 dilution of mouse ascites fluid containing MAb 2C9, which neutralizes serotype G1 rotaviruses through an epitope on VP7 (20), was added to the agarose overlay medium of the EDIM × 89-12 coinfected culture. This MAb was expected to neutralize parental 89-12 and all reassortants containing 89-12 VP7 but not affect viruses containing EDIM VP7. Because 89-12 forms larger plaques than EDIM, a characteristic associated with the VP4 protein, the largest plaques were selected for analysis. To select reassortants that contained 89-12 VP7 and WC3 VP4, a polyclonal antiserum to the bovine rotavirus NCDV strain (dilution, 1:10,000) was added to the overlay medium during plaque formation of the WC3 × 89-12 coinfected culture. This antiserum neutralizes WC3 through epitopes on VP7 and did not neutralize 89-12 at the dilution used. Only rotaviruses formed during coinfection that contained WC3 VP7 should, therefore, be neutralized by this antiserum. Because WC3 forms much larger plaques than 89-12, the largest plaques were selected for analysis. Approximately 20 plaque-purified isolates from both coinfections were analyzed by gel electrophoresis to detect the desired reassortants. This was done as described previously (24), except that the separation gel contained 7.5% acrylamide. Reassortants with the proper combination of segments from each coinfected culture were plaque purified an additional two times before being used in neutralization studies.

Study subjects. The 21 subjects whose serum specimens were analyzed in this study were placebo recipients enrolled in a vaccine trial conducted in Cincinnati during the 1988–1989 rotavirus season (2). Of the 103 placebo recipients included in that trial, only these 21 experienced rotavirus illness and had fourfold or greater increases in the titer of neutralizing antibody to the circulating serotype G1 rotavirus strains detectable at the end of the rotavirus season, i.e., June 1989. Their ages at the time of enrollment ranged from 2 to 12 months, and their mean age (7.0 months) was nearly equal to the mean age (6.8 months) for all of the placebo recipients. None of the 21 subjects had been infected with rotavirus prior to enrollment, as evidenced by the absence of detectable serum rotavirus immunoglobulin A at the time of placebo administration (7 September to 1 December 1988). Furthermore, only 2 of the 21 had detectable neutralizing antibodies (titers, ≥20) to representatives of the four major serotypes of human rotavirus. Because these two subjects had no rotavirus immunoglobulin A and were only 2 or 3 months of age when enrolled, it is assumed that this antibody was of maternal origin. All 21 subjects were ≥6 months of age when they experienced rotavirus illness, by which time maternal rotavirus immunoglobulin G would have decayed to negligible titers and, therefore, should have had no effect on the outcome following rotavirus infection, including immune responses.

Serum analysis. The convalescent-phase serum specimens collected from study subjects in June 1989 were heat inactivated (56°C, 30 min) and stored at −70°C. The titers of neutralizing antibodies against specified strains of rotavirus in these sera were determined as previously described (14).

RESULTS

Because natural rotavirus isolates obtained from symptomatically infected subjects during a WC3 vaccine trial conducted in Cincinnati during the 1988–1989 rotavirus season were all serotype G1 and had essentially identical electropherotypes (2), they all appeared to be the same strain or very similar strains. In addition, the relative neutralizing-antibody responses of subjects infected with these viruses to representatives of the four major serotypes of human rotavirus were very similar (28). For these reasons, one of these isolates, 89-12, was chosen to represent the group. To determine whether subjects symptomatically infected with 89-12-like rotaviruses had neutralizing-antibody responses primarily to VP4 or primarily to VP7, convalescent-phase sera collected at the end of the rotavirus season were examined to determine their titers of antibodies against the two 89-12 neutralization proteins. This was done with reassortants that each contained the gene segment for only one 89-12 neutralization protein. The gene for the other neutralization protein in each reassortant was derived from a rotavirus strain that was heterotypic with respect to 89-12. These heterotypic neutralization proteins were not expected to be recognized by antibodies made in subjects following infection with 89-12-like rotaviruses. It was necessary to use two different heterotypic rotaviruses (WC3 and EDIM) to make reassortants with 89-12 because all three strains had dissimilar growth properties and VP4 of the strain that grew better was consistently selected in reassortants.

Formation and characterization of reassortants. Two rotavirus reassortants were generated for this study. EDIM-7 was a reassortant between EDIM and 89-12, which contained all but segment 9 of 89-12 and only segment 7 or 8 of EDIM, as determined by electrophoretic analysis of viral RNAs (Fig. 1). Although the origin of segment 1 in EDIM-7 could not be determined in the gel profile shown, further analyses with additional gels showed that it was derived from 89-12. Because EDIM-7 was not neutralized by MAb 2C9, which specifically neutralizes serotype G1 rotaviruses through an epitope on the VP7 protein (20), this reassortant contained the EDIM gene encoding VP7. The gene segment encoding VP4 (segment 4) of EDIM-7 was derived from 89-12.

WC-4, the other reassortant developed for this study, contained only segment 9 of the 89-12 strain and other gene segments from WC3 (Fig. 1). The origins of segments 8 and 10 of WC-4 were not determinable in the gel pattern shown, but additional gel analyses revealed that they were derived from WC3. Because segment 9 of 89-12 encodes VP7, as determined above, and segment 4 encodes VP4, WC-4 contained the VP7 gene of 89-12 and the VP4 gene of WC3.

Neutralization of reassortants by antisera from rotavirus-infected subjects. Postinfection serum specimens from 21 subjects symptomatically infected with rotavirus during the 1988–1989 rotavirus season were examined (2). These in-

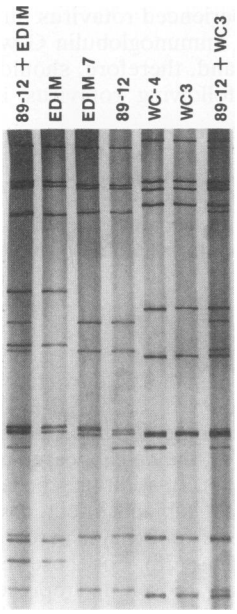

FIG. 1. Electrophoretic profiles of genomic RNA segments of parental viruses (89-12, EDIM, and WC3) and reassortants.

cluded all placebo recipients who had fourfold or greater increases in serum neutralizing antibody to the Wa strain of human rotavirus following infection. All subjects had high titers of neutralizing antibody to 89-12 (range, 661 to 6,060), with a geometric mean titer of 2,323 (Table 1). As anticipated, all subjects had low or undetectable titers (≤20) of antibodies to the EDIM and WC3 strains of animal rotaviruses. Thus, serum neutralizing antibodies to reassortants made between 89-12 and either EDIM or WC3 should be directed almost solely to epitopes on the 89-12 neutralization proteins.

The titers of neutralizing antibody to EDIM-7 in the serum specimens were consistently higher than the titers of neutralizing antibody to WC-4 (Table 1). The EDIM-7/WC-4 antibody titer ratios in all subjects were between 1.4 and 7.3. On the basis of the geometric mean titers of these antisera to the two reassortants, approximately three-fourths of the neutralizing antibody made against 89-12 was directed at VP4. Thus, this protein appeared to be immunodominant in young children naturally infected with 89-12-like rotaviruses.

A possible explanation for the reduced titers of antibody to WC-4 relative to the titers of antibody to EDIM-7 was that the WC3 background proteins may have decreased the antibody accessibility to the 89-12 VP7 protein on WC-4 or the EDIM VP7 protein may have increased the antibody accessibility to the 89-12 VP4 protein of the EDIM-7 strain. If this occurred, neutralizing MAbs to epitopes on the 89-12 VP4 or VP7 protein should have altered titers when these proteins were incorporated into EDIM-7 or WC-4. To test this possibility, neutralization titers of three MAbs directed at epitopes on VP7 and one MAb that recognized an epitope on VP4 of serotype 1 rotavirus were determined for the reassortants and their parents. 89-12 and reassortants containing the appropriate 89-12 neutralization protein were neutralized at comparable titers by all four MAbs (Table 2). This supports the conclusion that VP4 of 89-12-like rotaviruses was the immunodominant neutralization protein following natural infection in these subjects.

TABLE 1. Neutralization titers of convalescent-phase antisera from 21 young children following symptomatic infection with 89-12-like rotaviruses

Virus strain	Gene origin		Titer	
	VP7	VP4	Geometric mean[a]	Range
89-12	89-12	89-12	2,323	661–6,060
EDIM	EDIM	EDIM	12	<20–20
WC3	WC3	WC3	11	<20–20
EDIM-7	EDIM	89-12	1,078[b]	287–2,900
WC-4	89-12	WC3	387	151–1,220

[a] Titers of <20 were considered 10 for determination of geometric mean titers.
[b] $P < 0.001$ compared with WC-4, as determined by Student's t test.

TABLE 2. Neutralization titers of VP4- and VP7-specific MAbs against reassortants and parental strains

Virus strain	Titer of MAb[a]:			
	2C9	5E8	RV4:2	HS3
89-12	36,100	28,700	28,000	5,770
EDIM	500	<100	<100	<100
WC3	<100	<100	<100	<100
EDIM-7	100	<100	<100	3,840
WC-4	60,800	25,200	25,600	<100

[a] MAbs 2C9, 5E8, and RV4:2 are specific for epitopes on VP7; MAb HS3 is directed to an epitope on VP4.

DISCUSSION

The importance of serotype-specific neutralizing antibodies to circulating strains of rotavirus has not been established, but their production has been routinely used as an indicator of successful vaccination. If neutralization by antibody proves to be an important mechanism of protection against rotavirus, the most effective methods to stimulate this antibody need to be utilized. Because rotaviruses contain two proteins known to elicit production of neutralizing antibody, neutralizing antibodies to either or both proteins may be important mediators of protection against rotavirus disease. Reassortant rotavirus vaccine candidates that contain only the VP7 protein gene from human rotavirus, while the VP4 gene is from a heterologous strain (5, 18), may not stimulate sufficient immune responses to human rotavirus strains to provide lasting protection.

We recently demonstrated that natural serotype 1 rotavirus infection in young children in Cincinnati during the 1988–1989 rotavirus season resulted in excellent protection against subsequent rotavirus disease due predominantly to serotype G1 strains (1, 2). Furthermore, the circulating strains responsible for this protection were very similar if not identical. In the present study, we have used a representative rotavirus isolate, 89-12, obtained from one of the symptomatically infected subjects to determine whether neutralizing antibody elicited after natural infection was directed primarily to VP4 or primarily to VP7. Using reassortants that contained only one of the two neutralization protein genes of 89-12, we found that the significantly greater share of neutralizing antibody stimulated in these subjects was directed to VP4. Because these natural infections were protective, this finding suggests that stimulation of neutralizing antibody

to the VP4 protein of circulating rotaviruses may be important for successful vaccination. It is also possible, however, that if neutralizing antibody is involved in immunity, only that made against VP7 is protective.

Although rotavirus illnesses in the 21 subjects whose antisera were examined in this study occurred between December and March, the convalescent-phase blood specimens were not collected until June. This provided the possibility that, in addition to experiencing a symptomatic rotavirus infection, these subjects may have experienced asymptomatic infections between the time of enrollment and the time of collection of their convalescent-phase blood specimens. This is unlikely, because a symptomatic or asymptomatic natural rotavirus infection in these subjects appeared to confer almost complete protection against reinfection for a 2-year period (1). If this did occur, however, these asymptomatic infections were probably caused by rotavirus strains that were the same or very similar to those responsible for the symptomatic infections and, therefore, would not have been expected to alter the relative titers of neutralizing antibodies to VP4 and VP7. This is based on the observation that only serotype G1 rotaviruses were found in Cincinnati during the 1988–1989 rotavirus season and almost all, including 22 isolates collected from children not in the study who were admitted to Cincinnati Children's Hospital, belonged to the same electropherotype (2). Therefore, the conclusion that VP4 is the immunodominant neutralization protein in subjects in this study should not have been affected even if they did experience two or more rotavirus infections between the time of enrollment and the time of collection of convalescent-phase blood specimens.

The relatively low amounts of neutralizing antibody to the VP7 protein of 89-12 detected in this study could also have been affected by the use of reassortants. Other researchers have reported that the expression of neutralization epitopes on rotaviruses can be altered by differences in background structural proteins (3, 4). Thus, it was possible that incorporation of the 89-12 VP7 protein into a background of WC3 proteins in reassortant WC-4 may have reduced its antigenicity. This possibility is unlikely, because three MAbs that bind to serotype G1 rotaviruses, each of which apparently recognizes a different epitope on VP7 (25), neutralized WC-4 as effectively as they neutralized 89-12. The antigenicity of 89-12 VP4 in the EDIM-7 strain may also have been increased because of incorporation of the EDIM VP7 protein. This, however, was also unlikely, because MAb HS3, which binds to an epitope on the VP4 protein, did not neutralize EDIM-7 to a titer higher than that for 89-12. Therefore, we found no evidence to suggest that changes in background proteins accounted for the observed immunodominance of VP4.

Previous studies have yielded conflicting results regarding the relative immunogenicities of VP4 and VP7. VP7 appeared to be immunodominant in hyperimmunized animals, while the VP4 protein was found to be immunodominant in humans who had been orally inoculated with infectious rotaviruses (5, 18, 24). This suggested that the difference may be due to the route of immunization. Oral administration of the WC3 vaccine to infants, however, resulted in production of neutralizing antibody almost solely to VP7 (23). Thus, the route of immunization could not fully explain differences in the relative immunogenicities of VP4 and VP7.

Interestingly, the results showing that VP7 was immunodominant following oral inoculation with WC3 (23) while VP4 was immunodominant following natural infection with 89-12-like rotaviruses (this study) were determined with subjects included in the same vaccine trial (2). The first set of results was obtained with vaccinees following infection with a heterologous bovine rotavirus, while the other set of results was obtained with placebo recipients who were naturally infected with a wild-type human rotavirus. The main variables that determined whether VP4 or VP7 would be immunodominant appeared, therefore, to be the viruses themselves. The differences between WC3 and 89-12-like rotaviruses that were responsible for this effect could have been intrinsic properties of the structural proteins of these viruses that altered the relative antigenicities or immunogenicities of their VP4 and VP7 molecules. However, WC3 replicated poorly in vaccinated infants, produced no disease, and stimulated only low titers of neutralizing antibody (2). In contrast, the 89-12-like strains replicated to high titers, produced diarrheal disease, and stimulated strong immune responses in infected infants (2). Therefore, it was more probable that the relative amounts of neutralizing antibodies to VP4 and VP7 stimulated were determined by differences in the replication properties of these viruses. Because the rotavirus particle contains many fewer molecules of VP4 than of VP7 (19), immunization with this particle may stimulate a greater share of neutralizing antibody to VP7. If the virus replicates efficiently, however, the quantities of VP4 would be considerably increased. Furthermore, newly synthesized VP4 may more effectively stimulate the immune system than virion-associated VP4. Together, these factors may have caused stronger antibody responses to VP4 than to VP7.

Although the results reported here did not fully explain why different rotaviruses stimulated relatively different titers of neutralizing antibodies to the VP4 and VP7 proteins, they did show that natural rotavirus infections in infants which resulted in excellent protection against subsequent rotavirus disease were associated with large neutralizing-antibody responses to VP4. It will now be important to determine whether these immune responses are causally associated with protection.

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