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Analysis of Homotypic and Heterotypic Serum Immune Responses to Rotavirus Proteins Following Primary Rotavirus Infection by Using the Radioimmunoprecipitation Technique

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Three sequential serum samples collected from each of 20 young children with a naturally acquired primary rotavirus infection were assayed by the radioimmunoprecipitation technique for immunoglobulin G antibodies to rotavirus structural and nonstructural proteins of the four major human rotavirus serotypes G1, P1A; G2, P1B; G3, P2; and G4, P2. Fourteen children were infected with a serotype G1 rotavirus strain and six children were infected with a serotype G4 rotavirus strain. Sera were collected from each child in the acute and convalescent periods postinfection and also approximately 4 months later. Serum immune responses to rotavirus core antigens VP2 and VP3, to the major inner capsid antigen VP6, to nonstructural proteins NS35, NS28, and NS26, and to the outer capsid neutralization antigen VP4 of all test strains were detected in the majority of patients. These responses do not appear to be influenced by the G type or P type of the rotavirus strain used in the reactions. Homologous responses to the main neutralization antigen VP7 were detected in 93% of patients with serotype G1 infections and 50% of patients with serotype G4 infections. Heterologous VP7 responses were less frequently detected and were restricted to G1, G3, and G4 serotype rotavirus strains. No responses to VP7 of the serotype G2 rotavirus strain were detected in any patients. Heterotypic immune responses to the neutralization antigens, at least following serotype G1 and G4 infections, therefore appear to consist primarily of responses to VP4 rather than to VP7.

Rotaviruses are one of the leading causes of acute infantile gastroenteritis throughout the world. The need for a safe and effective vaccine strategy is recognized following epidemiological studies in developing and developed countries. A number of live animal rotavirus strains have already been evaluated as human vaccine candidates in field trials (7, 17, 34). More recent approaches include the use of reassortant viruses. These contain a single gene which codes for the major neutralization antigen, VP7, from human virus serotypes on an animal rotavirus background (11, 15, 23). Alternatively, the use of recombinant vaccines incorporating live expression vectors or subunit vaccines of individual proteins synthesized from DNA clones is recognized as another possible approach to rotavirus vaccination (4), as is the use of naturally attenuated neonatal rotavirus strains which cause asymptomatic infection (3).

Rotavirus particles are composed of two capsid layers which surround a core containing a genome of 11 double-stranded RNA segments. These rotavirus genes code for structural proteins found in virus particles and nonstructural proteins found in infected cells but not present in mature particles. The core and inner capsid of the rotavirus particle are composed of structural proteins VP1 (125,000 Da), VP2 (94,000 Da), VP3 (88,000 Da), and VP6 (42,000 Da). The remaining structural proteins VP4 (84,000 Da) and VP7 (34,000 Da), both of which induce antibodies with neutralizing activity and thus define serotype specificity, make up the outer capsid layer of the rotavirus particle. Rotavirus strains are now beginning to be classified into specific serotypes by the use of single letters which represent these outer capsid proteins. The letter P is used to denote the protease-sensitive protein VP4, and the letter G is used to denote the glycoprotein VP7. Thus, each rotavirus strain is now assigned a specific P type and G type on the basis of these neutralizing antigens. The five nonstructural proteins of rotavirus are thought to have a diverse array of roles in the replication cycle of rotaviruses and are named according to their molecular masses, as follows: NS53 (53,000 Da), NS35 (35,000 Da), NS34 (34,000 Da), NS28 (28,000 Da), and NS26 (26,000 Da).

For a vaccine strategy to be effective, an understanding of both the serum and mucosal immune responses which develop to these structural and nonstructural rotavirus proteins following infection is of major importance. In particular, an understanding of the protein-specific immune response following naturally acquired primary infection is required. Longitudinal studies of infection in young children have found that such initial infections are likely to be the most severe and may protect against the development of clinically severe disease during reinfection (3, 27). The ultimate aim, therefore, is to develop a broader understanding of the immune response which follows these primary infections so that an attempt can be made to reproduce similar immune responses when using vaccine strains. Estimations of serum immunoglobulin G (IgG) and IgM antibodies to rotavirus are sensitive indicators of an immune response following severe primary rotavirus gastroenteritis (14). Previously, animal experiments have indicated that serum antibody levels generally cannot be correlated with protection (24, 30), although a recent report by Ward et al. (36) has shown some correlation of immunity with the magnitude of serum rotavirus IgG titers in infected mice. Similarly, studies of human subjects have sometimes indicated a correlation between preexisting titers of neutralizing homotypic antibody in serum and protection against rotavirus illness (6). We have previously

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investigated the IgG immune response to rotavirus structural proteins in three sequential serum samples obtained from each of 16 young children presenting with acute diarrhea following a primary serotype G1 rotavirus infection (28). Homotypic immune responses measured by Western blotting (immunoblotting) were associated with serum IgG antibody to four rotavirus structural proteins: VP2, VP4, VP6, and VP7. The timing and intensity of the IgG antibody responses differed for each of these proteins but showed similar trends in all patients. Antibody responses to VP2, VP4, and VP7 were maximal in convalescent-phase sera, while antibody to VP6 was usually first detected in acute-phase sera and was found to persist for more than 4 months.

The technique of Western blotting permits analysis of immunoglobulin class, but it has limitations in other respects. The technique incorporates the use of denatured and reduced antigenic proteins. Thus, it is possible to detect only reactions of antibodies which bind to denaturation-resistant linear epitopes of proteins which are not dependent on conformation. Antibody reactions to denaturation-sensitive epitopes are not detected. Intensity differences and detection of antibody responses may, therefore, partially reflect the degree of conservation of antigenic epitopes within each protein. The radioimmunoprecipitation (RIP) technique, however, uses milder denaturation conditions and the viral proteins are in a relatively native state when reacting with serum antibody. In addition, the use of RIP allows the detection of antibodies to nonstructural virus-associated proteins found in infected cell preparations as well as those to the viral structural proteins. Thus, a more comprehensive analysis of the immune response to a broader range of near-native rotavirus proteins can be achieved by this technique.

In the present study, the RIP technique was used to evaluate the immune responses to rotavirus structural and nonstructural proteins in the sera of young children with either a serotype G1 or a serotype G4 primary rotavirus infection. In addition, the antibody responses to rotavirus proteins were also analyzed in the sera of two children who had experienced a serotype G2 rotavirus infection. The serum antibody responses to proteins of rotavirus strains RV4 (serotype G1, P1A), RV5 (serotype G2, P1B), RV3 (serotype G3, P2), and ST3 (serotype G4, P2) were examined, allowing analysis of homotypic and heterotypic antibody responses to the majority of group-specific, serotype-specific (G and P types), and nonstructural proteins.

MATERIALS AND METHODS

Viruses. Local human rotavirus strains RV4 (G1, P1A) (subgroup II), RV5 (G2, P1B) (subgroup I), and RV3 (G3, P2) (subgroup II) were isolated in our laboratory (1). The ST3 human rotavirus (G4, P2) (subgroup II) was provided by R. G. Wyatt, Bethesda, Md. These four rotavirus strains are now considered to be standard strains representative of the four most common G serotypes of rotavirus which infect humans (9).

All viruses were routinely propagated in MA104 cells in the presence of porcine trypsin type IX (10 μg/ml to activate virus for 20 min at 37°C, 1 μg/ml in maintenance medium; Sigma Chemical Co., St. Louis, Mo.).

Patients. The majority of serum samples analyzed in the present study were a subset of those collected for a previous study in our department (14). The sera used were collected from a total of 20 patients (14 males; 6 females) aged between 2 and 39 months (median age, 20.5 months) admitted to the hospital with severe acute diarrhea caused by rotavirus. All patients were considered to be experiencing a primary rotavirus infection on the basis of an IgM-class rotavirus antibody response measured in sera by an enzyme immunoassay (14). Fourteen children were infected with a serotype G1 rotavirus strain and six children were infected with a serotype G4 rotavirus strain. Some of these children have been described in an earlier publication (28). All infections were diagnosed upon admission of the child to the hospital, and an enzyme immunoassay incorporating monoclonal antibodies (8) was used to determine the group and serotype antigens of the infecting rotavirus strain.

Acute-phase sera (collected 2 to 7 days postinfection; median, 5.5 days), convalescent-phase sera (collected 26 to 42 days postinfection; median, 34 days), and follow-up sera (collected 113 to 148 days postinfection; median, 121.5 days) were obtained. These sets of three sequential serum samples collected from each patient were assayed in the same experiment by using identical RV4, RV5, RV3, or ST3 rotavirus antigen preparations. All sera were stored at −70°C until they were used in RIP assays, and the sera were used in the assays either undiluted or at a 1:10 dilution.

In addition, a single serum sample was collected from each of two children, aged 8 months and 3 years, who were admitted to hospital with a serotype G2 rotavirus infection. Sera were collected from these children at 1 or 3 months, respectively, post-rotavirus infection and were reacted in RIP assays by using RV4-, RV5-, or ST3-labeled rotavirus proteins.

Hyperimmune sera. Seronegative rabbits were injected subcutaneously three times with a cesium chloride-purified preparation of rotavirus strain RV4, RV5, RV3, or ST3. The rabbits were exsanguinated by cardiac puncture 14 days after the final injection, and the antisera collected were divided into aliquots and stored at −70°C until they were used in RIP experiments.

Preparation of [^{35}S]methionine-labeled, rotavirus-infected cell lysates. Confluent monolayers of MA104 cells grown in 35-mm petri dishes were infected with a 0.1-ml inoculum of trypsin-activated virus at a multiplicity of 1 fluorescing cell-forming unit per cell (RV4, RV5 and ST3) or 0.2 fluorescing cell-forming unit per cell (RV3). Additional cell monolayers were mock infected. After 1 h of adsorption at 37°C, the virus inoculum was removed and 1 ml of Dulbecco's modified Eagle's medium containing 1 μg of trypsin per ml and 2 μg of actinomycin D per ml was added to each petri dish. At 6 h postinfection (RV4, RV5, and ST3) or 12 h postinfection (RV3), the medium was replaced with 0.1 ml of methionine-free Eagle's minimum essential medium (Flow Laboratories, Irvine, Scotland, United Kingdom) containing 150 mM excess NaCl, 1 μg of trypsin per ml, 2 μg of actinomycin D per ml, and 100 μCi of [^{35}S]methionine (>800 Ci/mmol; NEN Research Products) per ml for 1 h; this was followed by replacement with 1.0 ml of Dulbecco's modified Eagle's medium containing 150 mM excess NaCl, 1 μg of trypsin per ml, and 2 μg of actinomycin D per ml for 30 min at 37°C. The medium was then removed, and the cells were washed twice with ice-cold phosphate-buffered saline (PBS) and were then harvested by freezing at −70°C for a few hours or overnight.

Cell lysates were prepared by adding 100 μl of lysis buffer containing 0.8 M KCl, 10 mM Tris-hydrochloride (pH 7.8), 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1% Triton X-100 to each petri dish; this was followed by the addition of 400 μl of 10 mM Tris-hydrochloride (pH 7.8), 1 mM PMSF, and 1% Triton X-100. The cell lysates were then centrifuged

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at 166,320 × *g* (Beckman rotor TLS 55) for 1 h at 4°C, and the supernatants were collected and stored at −70°C until they were used in immunoprecipitation experiments.

RIP. Mixtures of 25 μl of the viral lysate supernatant and 25 μl of undiluted human serum (or a 1:10 dilution if insufficient serum was available) were incubated in Eppendorf tubes overnight at 4°C. The immune complexes were precipitated by the addition of 50 μl of Pansorbin cells, a 10% suspension of *Staphylococcus aureus* cells with a coat of protein A in PBS (Calbiochem). After 1 h at room temperature with frequent mixing, the precipitates were collected by centrifugation at 5,000 rpm for 5 min in an Eppendorf Microfuge and were then washed three times with RIP wash buffer (0.05 M Tris [pH 7.4], 0.15 M NaCl, 1 mM EDTA, 0.25% bovine serum albumin, 0.05% Triton X-100, and 0.02% NaN_3). The adsorbed labeled viral proteins were recovered by resuspending the pellets in 60 μl of sample buffer (62.5 mM Tris-HCl [pH 6.8], 10% glycerol, 5% β-mercaptoethanol, 2% sodium dodecyl sulfate [SDS], 0.01% bromophenol blue) and boiling for 4 min. The samples were centrifuged at 10,000 rpm for 10 min in an Eppendorf Microfuge, and the supernatants were applied to SDS-polyacrylamide gels.

SDS-PAGE. Samples were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and the discontinuous Tris-glycine buffer system described by Laemmli (18). All gels were 0.75-mm thick and consisted of a 4% acrylamide stacking gel and a 10% acrylamide resolving gel run under reducing conditions. Polypeptides were separated in the gel by using a constant current of 30 mA for approximately 3 h at room temperature. Lysates of rotavirus-infected cells labeled with [^{35}S]methionine were mixed with sample buffer, boiled for 4 min, and applied to gels as migration reference markers. Immediately after electrophoresis, the gels were processed for fluorography by immersion directly in 1 M sodium salicylate for 30 min at room temperature (5) and were then dried and exposed to Kodak X-Omat AR-5 films at −70°C for 7 days.

RESULTS

Identification of proteins. In each RIP assay, the profiles of ^{35}S-labeled polypeptides of the RV4, RV5, RV3, or ST3 rotavirus strain and the corresponding mock-infected cellular proteins were examined. The labeled viral polypeptides were identified by estimation of their molecular weights relative to markers of known molecular weights and by comparison with previously reported data. Only minor differences in migration patterns were found for the viral proteins of the four different rotavirus strains used in the reactions.

Identification of the outer capsid protein VP4 and the two trypsin cleavage products of VP4, VP5 and VP8, was confirmed following treatment of labeled viral proteins with trypsin. Labeled rotavirus proteins were treated with 1.0, 2.0, 5.0, and 10.0 μg of trypsin per ml and were then reacted with hyperimmune rabbit serum or a strongly reactive human serum in the RIP assay (Fig. 1). With increasing trypsin concentrations, the amount of VP4 precipitated by serum antibody was observed to decrease. At the same time, the amount of precipitated VP5 remained constant, clearly distinguishing it from the NS53 protein which migrates through the gel at a similar position. Reactions to a faintly labeled band migrating at a molecular mass position of approximately 27,000 Da were also detected. This latter band corresponded to the VP8 protein. Although the migration

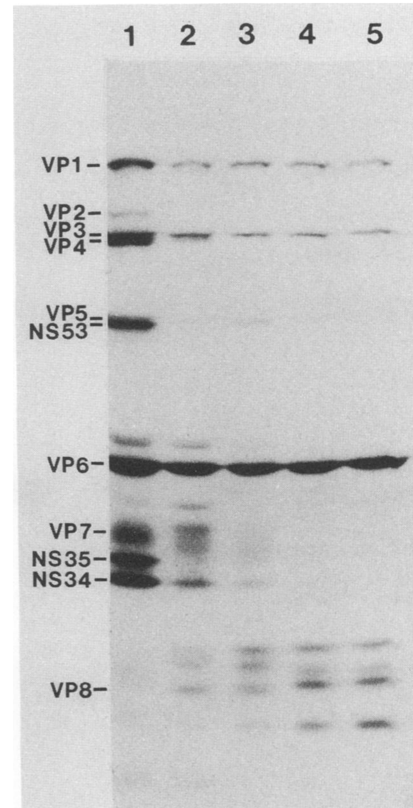

FIG. 1. SDS-PAGE analysis of [^{35}S]methionine-labeled RV4 (G1, P1A) proteins treated with increasing concentrations of trypsin and immunoprecipitated with hyperimmune rabbit serum prepared to the RV4 rotavirus strain. The hyperimmune rabbit serum was used undiluted in the assay. The lanes show untreated RV4 proteins (lane 1), and RV4 proteins treated with 1.0 μg of trypsin per ml (lane 2), 2.0 μg of trypsin per ml (lane 3), 5.0 μg of trypsin per ml (lane 4), and 10.0 μg of trypsin per ml (lane 5). The positions of the RV4 proteins, indicated in the left margin, were estimated by comparing their migrations relative to those of protein standards in a 10% acrylamide resolving gel.

positions of VP5 and VP8 could be identified following treatment with trypsin, immune responses in patient sera to these proteins could not be differentiated in routine RIP assays.

Reproducibility of the RIP assay. As a test of reproducibility, the spectrum of viral polypeptides precipitated by a hyperimmune rabbit serum prepared against the particular rotavirus strain which had been labeled was examined in each RIP assay (Fig. 2). Each hyperimmune serum sample routinely precipitated bands corresponding to viral proteins VP1, VP2, VP3, VP4, VP6, VP7, NS35, and NS34. The number and intensities of the precipitated proteins were similar between assays that incorporated the same rotavirus strain.

Specificity of the RIP assay. The specificity of the RIP assay was shown by incorporating aliquots of PBS in place of serum samples in RIP assays with each rotavirus strain. These control experiments were shown to nonspecifically immunoprecipitate proteins VP1 and NS34 of each rotavirus strain. Consequently, precipitation of these two proteins by any test serum sample was excluded from the results.

Further specificity of the assay was demonstrated by incorporating serum samples collected from five rotavirus-

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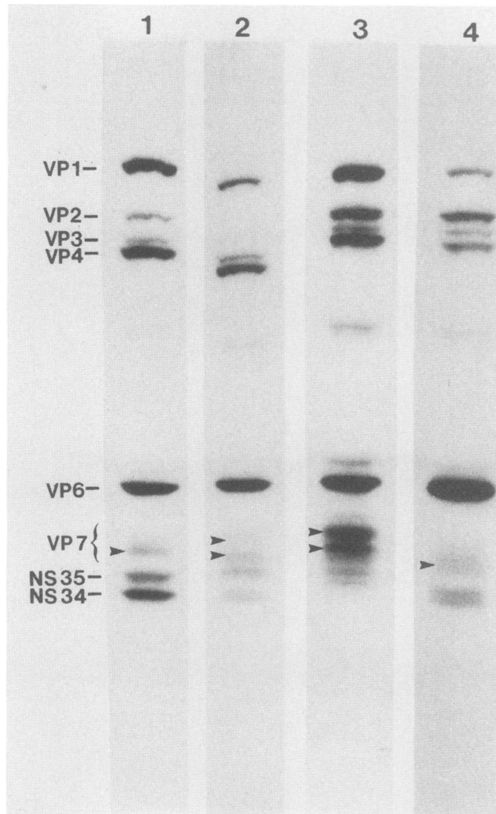

FIG. 2. SDS-PAGE analysis of [^{35}S]methionine-labeled RV4 (G1, P1A), RV5 (G2, P1B), RV3 (G3, P2), and ST3 (G4, P2) rotavirus proteins immunoprecipitated with hyperimmune rabbit serum to the homologous labeled rotavirus strain. Lanes: 1, RV4 proteins precipitated with hyperimmune serum to RV4; 2, RV5 proteins precipitated with hyperimmune serum to RV5; 3, RV3 proteins precipitated with hyperimmune serum to RV3; and 4, ST3 proteins precipitated with hyperimmune serum to ST3.

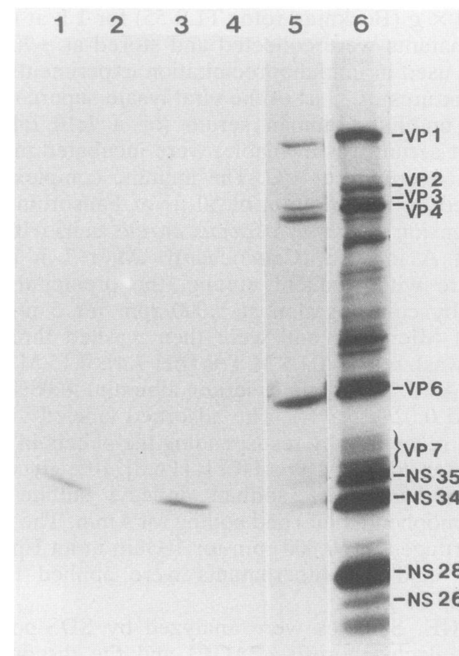

FIG. 3. SDS-PAGE analysis of [^{35}S]methionine-labeled RV5 (G2, P1B) proteins immunoprecipitated with sera from two seronegative children and with a hyperimmune rabbit serum prepared against the RV5 strain. All sera were used undiluted in the assay. Lanes: 1 and 3, RV5 proteins precipitated with sera from seronegative children; 2 and 4, nonspecifically labeled cellular proteins precipitated with the same sera from seronegative children; 5, RV5 proteins precipitated with a hyperimmune rabbit serum to RV5; 6, labeled RV5 proteins.

seronegative children who were part of an earlier rotavirus epidemiological study. The immune responses in two of these serum samples are shown in Fig. 3. These sera were reacted with labeled RV4 and RV5 proteins in the RIP assay and were observed to precipitate up to two rotavirus proteins: VP1 and NS34 when using RV4 and NS34 only when using RV5. Both proteins were previously shown to be nonspecifically precipitated in the absence of sera. No other nonstructural proteins or rotavirus structural proteins were precipitated by any of the five serum samples.

All patient sera and hyperimmune sera were also tested for antibodies to mock-infected cellular proteins. Although a number of cellular proteins were labeled with [^{35}S]methionine, even when using actinomycin D at a concentration of 2 μg/ml, no reaction of these proteins with any undiluted or 1:10-diluted patient serum or hyperimmune rabbit serum sample was evident in any RIP assay, indicating specific precipitation of viral polypeptides only.

Occurrence of immune responses to rotavirus nonneutralizing group antigens (VP2, VP3, VP6). Immune responses to two of the three proteins which make up rotavirus core particles, VP2 and VP3, and to the major group-specific polypeptide located on the inner capsid of virus particles, VP6, were frequently detected in sera after infection with either serotype G1 or serotype G4 rotavirus (Table 1). These results represent the summation of the number of children with a detectable response to each protein, regardless of the timing of collection of the serum specimen in which the responses were detected.

TABLE 1. Number of serotype G1- and serotype G4-infected patients with detectable immune responses to group antigens VP2, VP3, and VP6 of rotavirus strains RV4, RV5, RV3, and ST3

Rotavirus strain (serotype)	Serotype G1-infected patients				Serotype G4-infected patients			
	No. of patients	% with detectable response to:			No. of patients	% with detectable response to:		
		VP2	VP3	VP6		VP2	VP3	VP6
RV4 (G1, P1A)	14	100	64	100	6	100	33	100
RV5 (G2, P1B)	9	100	67	100	6	67	17	100
RV3 (G3, P2)	14	100	79	100	6	100	50	100
ST3 (G4, P2)	13	100	92	100	6	100	100	100

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TABLE 2. Number of serotype G1- and serotype G4-infected patients with detectable immune responses to neutralization antigens VP4 and VP7 of rotavirus strains RV4, RV5, RV3, and ST3

Rotavirus strain (serotype)	Serotype G1-infected patients			Serotype G4-infected patients		
	No. of patients	% with detectable response to:		No. of patients	% with detectable response to:	
		VP4	VP7		VP4	VP7
RV4 (G1, P1A)	14	100	93	6	100	33
RV5 (G2, P1B)	9	100	0	6	100	0
RV3 (G3, P2)	14	100	50	6	100	17
ST3 (G4, P2)	13	100	62	6	100	50

An antibody response to VP2 of all test strains was evident in at least one serum sample collected from all patients with a serotype G1 rotavirus infection. Patients with a serotype G4 rotavirus infection also showed serum antibody responses to VP2 of all test strains, with the exception of two patients in whom there was no response to VP2 of the serotype G2 test strain.

In comparison with VP2, VP3 was precipitated less frequently by serum antibody. After a serotype G1 rotavirus infection, VP3 was immunoprecipitated in 64 to 92% of patients, depending on the rotavirus strain used. Sera obtained after a serotype G4 rotavirus infection immunoprecipitated VP3 in 17 to 100% of patients.

A serum antibody response to VP6 of all rotavirus strains was detected in all patients examined, irrespective of the infecting serotype.

Occurrence of immune responses to rotavirus neutralizing antigens (VP4, VP7). The number of serum antibody responses to the two outer capsid proteins which induce neutralizing antibody and which therefore define serotype specificity, VP4 and VP7, is shown in Table 2.

A serum antibody response to VP4 of each test strain was detected in at least one serum sample from all patients with either a serotype G1 or serotype G4 rotavirus infection.

Antibody responses to the major neutralizing antigen VP7 were detected less frequently. In the group of patients with a serotype G1 rotavirus infection, a serum immune response was evident in 93% of patients to VP7 of RV4. In the serotype 4-infected patients, 50% showed responses to VP7 of ST3. Responses to VP7 of noninfecting serotypes were recorded in 17 to 62% of patients. An antibody response to VP7 of the RV5 strain (serotype G2, P1B) was not detected in any serum sample collected from any patient with either a serotype G1 or a serotype G4 infection.

Occurrence of immune responses to nonstructural proteins of rotavirus (NS35, NS28, NS26). Antibody responses to the nonstructural proteins NS35, NS28, and NS26 of the four rotavirus strains were detected in sera collected from a proportion of patients with a serotype G1 rotavirus infection or a serotype G4 rotavirus infection (Table 3).

An immune response to NS35 of each rotavirus strain was detected in at least one serum sample collected postinfection from all patients, irrespective of the serotype of the infecting rotavirus strain.

Serum immune responses to the nonstructural protein NS28 were also detected in the majority of patients tested. Patients with a serotype G1 rotavirus infection showed serum antibody to NS28 in 67 to 100% of cases, depending on the rotavirus strain used. Patients with a serotype G4 rotavirus infection had antibody to NS28 of RV4, RV3, and ST3 strains in 67 to 100% of cases. The NS28 protein of the RV5 strain was not precipitated by any serum sample collected from serotype G4-infected patients.

Responses to the third nonstructural protein, NS26, were detected in 79, 22, 64, and 85% of serotype G1-infected patients when we used the RV4, RV5, RV3, or ST3 strain, respectively. Immune responses to NS26 were generally less common in sera collected from patients with a serotype G4 rotavirus infection. As found for protein NS28, antibody to NS26 of the RV5 strain was not detected in any serum sample collected from serotype G4-infected patients.

An example of the spectrum of detected antibody responses to the structural and nonstructural proteins of the four labeled human rotavirus preparations RV4, RV5, RV3, and ST3 in one follow-up serum sample collected from a serotype G1-infected patient is shown in Fig. 4.

Timing of serum immune responses to VP4 and VP7 following serotype G1 or serotype G4 infection. The number of immune responses to VP4 and VP7 detected in acute-phase sera, convalescent-phase sera, and sera collected 4 months postinfection in patients with either a serotype G1 or a serotype G4 rotavirus infection is shown in Tables 4 and 5, respectively. A similar timing in the appearance of antibody to VP4 and VP7 was seen for both the serotype G1-infected patients and the serotype G4-infected patients.

Antibody to VP4 was detected in a proportion of acute-

TABLE 3. Number of serotype G1- and serotype G4-infected patients with detectable immune responses to nonstructural proteins NS35, NS28, and NS26 of rotavirus strains RV4, RV5, RV3, and ST3

Rotavirus strain (serotype)	Serotype G1-infected patients				Serotype G4-infected patients			
	No. of patients	% with detectable response to:			No. of patients	% with detectable response to:		
		NS35	NS28	NS26		NS35	NS28	NS26
RV4 (G1, P1A)	14	100	93	79	6	100	67	67
RV5 (G2, P1B)	9	100	67	22	6	100	0	0
RV3 (G3, P2)	14	100	86	64	6	100	100	50
ST3 (G4, P2)	13	100	100	85	6	100	100	67

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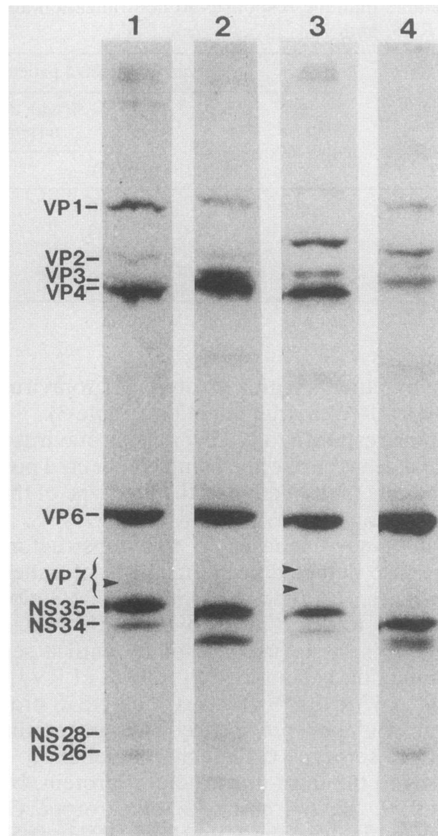

FIG. 4. SDS-PAGE analysis of [^{35}S]methionine-labeled RV4 (G1, P1A), RV5 (G2, P1B), RV3 (G3, P2), and ST3 (G4, P2) rotavirus proteins immunoprecipitated with a follow-up serum sample collected 133 days postinfection from a serotype G1-infected patient. The serum was used undiluted in all assays. Lanes: 1, RV4 proteins; 2, RV5 proteins; 3, RV3 proteins; and 4, ST3 proteins.

phase sera (collected 2 to 7 days postinfection), although only to a minimal intensity: 15 to 89% for serotype G1-infected patients and 33 to 67% for serotype G4-infected patients, depending on the rotavirus strain used. However, all convalescent-phase sera (collected 26 to 42 days postinfection) and follow-up sera (collected 113 to 148 days postinfection) contained antibody to VP4 of each rotavirus serotype.

TABLE 4. Number of immune responses to neutralizing antigen VP4 of four rotavirus strains in serum samples collected from patients with a serotype G1 or G4 rotavirus infection

Infection with:	Serum sample	No. of detectable immune responses to VP4/total no. tested for the indicated rotavirus strain (serotype):			
		RV4 (G1, P1A)	RV5 (G2, P1B)	RV3 (G3, P2)	ST3 (G4, P2)
Serotype G1	Acute[a]	10/14	8/9	5/14	2/13
	Conv[b]	14/14	9/9	14/14	13/13
	Follow-up[c]	14/14	9/9	14/14	13/13
Serotype G4	Acute	4/6	2/6	3/6	2/6
	Conv	3/3	3/3	3/3	3/3
	Follow-up	6/6	6/6	6/6	6/6

[a] Acute-phase sera were collected 2 to 7 days postinfection.
[b] Convalescent (Conv)-phase sera were collected 26 to 42 days postinfection.
[c] Follow-up sera were collected 113 to 148 days postinfection.

TABLE 5. Number of immune responses to neutralizing antigen VP7 of four rotavirus strains in serum samples collected from patients with a serotype G1 or G4 rotavirus infection

Infection with:	Serum sample	No. of detectable immune responses to VP7/total no. tested for the indicated rotavirus strain (serotype):			
		RV4 (G1, P1A)	RV5 (G2, P1B)	RV3 (G3, P2)	ST3 (G4, P2)
Serotype G1	Acute[a]	0/14	0/9	0/14	0/13
	Conv[b]	8/14	0/9	5/14	5/13
	Follow-up[c]	12/14	0/9	6/14	7/13
Serotype G4	Acute	2/6	0/6	0/6	1/6
	Conv	2/3	0/3	1/3	2/3
	Follow-up	1/6	0/6	1/6	3/6

[a] Acute-phase sera were collected 2 to 7 days postinfection.
[b] Convalescent (Conv)-phase sera were collected 26 to 42 days postinfection.
[c] Follow-up sera were collected 113 to 148 days postinfection.

In patients with a serotype G1 rotavirus infection, antibody responses to VP7 of RV4 (serotype G1), RV3 (serotype G3), and ST3 (serotype G4) were not evident in acute-phase sera but appeared in 36 to 57% of convalescent-phase sera and were detectable in 43 to 86% of follow-up sera. Responses to VP7 in patients with a serotype G4 rotavirus infection were detectable in some acute-phase sera (33% to VP7 of RV4 and 17% to VP7 of ST3). The number of responses increased to 33 to 67% in convalescent-phase sera, but then decreased to 17 to 50% in follow-up sera. No antibody responses to VP7 of RV5 (serotype G2) were evident in any serum sample collected from any child with either a serotype G1 rotavirus infection or a serotype G4 rotavirus infection.

An example of the timing of appearance of immune responses to the structural and nonstructural proteins of rotavirus in the acute-phase, convalescent-phase, and follow-up serum samples collected from one patient is illustrated in Fig. 5. For this example, we used a labeled RV3 antigen preparation and three serum samples collected from a serotype G1-infected patient.

Immune response of two patients with a serotype G2 infection. Serum samples collected from two children with a serotype G2 rotavirus infection were incorporated in RIP assays with labeled RV4, RV5, or ST3 rotavirus proteins. There were insufficient sera for incorporation into the RIP assay with labeled RV3 rotavirus proteins. Both patients showed antibody responses to the major neutralizing antigen VP7 of RV5 (serotype G2), in addition to other RV5 proteins, VP2, VP3, VP4, VP6, NS35, NS28, and NS26. The serum sample collected from one child (age, 8 months) at 1 month after a serotype G2 rotavirus infection also precipitated the VP4 and VP7 neutralization antigens of the RV4 and ST3 strains, in addition to group antigens VP2, VP3, and VP6 and the nonstructural protein NS35. The serum sample collected from the second child (age, 3 years) at 3 months after a serotype G2 infection precipitated only the major group antigen VP6 of the RV4 and ST3 rotavirus strains.

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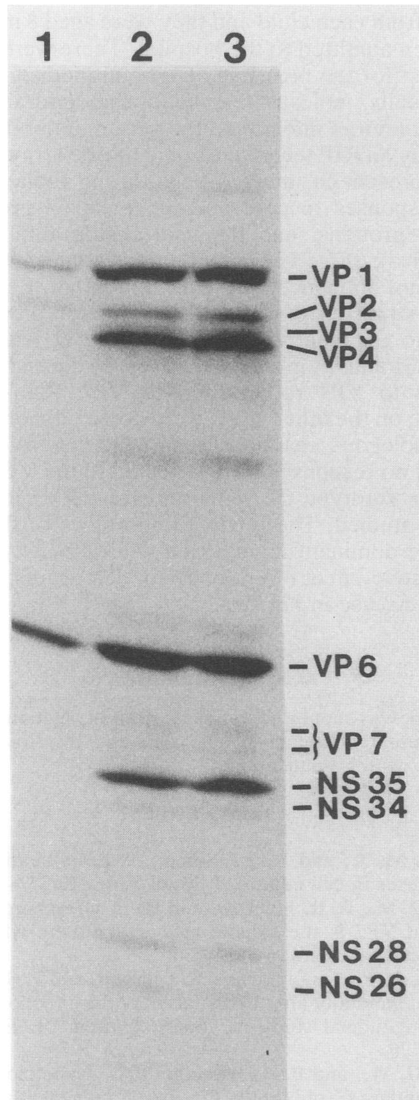

FIG. 5. SDS-PAGE analysis of [35S]methionine-labeled RV3 (G3, P2) proteins immunoprecipitated with acute-phase, convalescent-phase, and follow-up serum samples collected from a serotype G1-infected patient. Sera were used undiluted in the assay. Positions of RV3 proteins were estimated by comparing their migrations relative to those of standard proteins of known molecular weight. Lanes: 1, acute-phase serum (7 days postinfection); 2, convalescent-phase serum (39 days postinfection); and 3, follow-up serum (113 days postinfection).

DISCUSSION

RIP assays were developed to investigate the immune response to the polypeptides of four human rotavirus strains (representing four G types and three P types) following naturally acquired rotavirus infection with serotypes G1, G2, or G4. The technique allows the analysis of the serum immune response to a broad spectrum of individual rotavirus proteins, including those carrying group-specific determinants (VP2, VP3, VP6), those which induce antibodies with neutralizing activity (VP4, VP7), and the nonstructural proteins found in infected cells but not present in mature particles (NS35, NS28, NS26). It was not possible to detect specific antibody responses to the third core antigen of rotavirus, VP1, or to the nonstructural protein NS34, since these were obscured by nonspecific reactions.

Serum immune responses were detected to rotavirus core antigens VP2 and VP3 and to the major inner capsid antigen VP6 of all test strains, irrespective of the G type or the P type, in the majority of patients. All three proteins carry group determinants of rotavirus (10). Antibody to VP3 was detected less frequently than was that to VP2 or VP6. This result may reflect in part the relative amounts of each protein found in rotavirus particles (19). VP6 is the major structural protein in virus particles located on the outer surface of single-shelled particles and makes up 51% of the total virion protein. VP2 is the most abundant structural protein found in core particles and the third most abundant protein in rotavirus particles (~15% of the total virion protein). Both VP6 and VP2 have been shown to be highly immunogenic and carry epitopes associated with subgroup specificity (16, 33). In comparison with VP6 and VP2, VP3 is a minor structural protein and makes up only approximately 0.5% of the total virion protein.

Antibody responses to the outer-coat rotavirus proteins VP4 and VP7, which are known to induce neutralizing antibody, are of major interest. There is evidence that one or both of these antibodies are protective against rotavirus infection and/or disease in experimental animals (21, 22, 25, 26). Responses to VP4 of all test strains (including three different P types) were detected in all patients regardless of the infecting rotavirus serotype. Responses were frequently rapid and were detectable 2 to 7 days postinfection in 15 to 89% of cases, although only to a minimal intensity. In addition, responses to VP4 appeared to be persistent and were evident in all patients up to 4 months postinfection. In a previous work in which the investigators used the immunoblotting technique (28), anti-VP4 responses were detected less frequently in children with a serotype 1 infection to a homologous rotavirus strain. This result may be attributed to the limitations of the immunoblotting technique, in which only reactions of antibody to denaturation-resistant, linear epitopes can be detected. Other previous studies have shown antibody responses to VP4 to be a major component of the immune response following either natural rotavirus infection in children or following vaccination with an animal rotavirus strain. In a study of sera collected from eight children infected with a human subgroup I or II rotavirus, Svensson et al. (31) detected antibody responses to the VP4 protein of a subgroup II rotavirus strain in all convalescent-phase serum samples. Similarly, studies following vaccination of infants or adults with rotavirus strains RRV, RIT-4237, or CJN (13, 29, 35) have found the VP4 protein to be a strong immunogen that stimulates homologous and heterologous neutralizing antibody during infection of humans.

It was not possible to distinguish the serum immune responses to the two trypsin cleavage products of VP4, VP5 and VP8. Responses to a protein band migrating at a position analogous to that of both VP5 and NS53 were evident in at least one serum sample from each patient studied but could not be reliably differentiated from each other. From previously published work investigating the sequences of escape mutants of VP4 selected with neutralizing monoclonal antibodies (20, 32), it is known that VP5 contains predominantly cross-reactive sites involved in heterologous neutralization. These sites on VP5 have been found to be clustered in a relatively limited area between amino acids 306 and 440. Antibodies to these cross-reactive sites may in fact form the basis of the homologous and heterologous serum immune responses detected in all serotype G1-infected and serotype

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G4-infected patients to VP4 of each of the four human rotavirus strains used in the present study.

In the present study, homologous responses to VP7 appeared after both serotype G1 and serotype G4 infections in 93 and 50% of patients, respectively. Heterologous VP7 responses were also detected but with less frequency and were restricted to serotypes G1, G3, and G4. The timing of collection of serum specimens may have influenced the detection of anti-VP7 antibody, particularly following serotype G4 infections. Only three convalescent-phase serum samples were available from patients with a serotype G4 infection in the present study, and thus, anti-VP7 responses may have been missed because of a lack of appropriate serum specimens. Anti-VP7 responses were delayed when compared with those to VP4 and were observed to be maximal in intensity in convalescent-phase sera, as found in a previous study with the same sera by an immunoblotting technique (28). Thus, although the number of homologous and heterologous anti-VP7 responses following serotype G1 infection appeared to remain high beyond the convalescent phase, the reaction intensities were noted to decrease in the follow-up serum sample compared with that in the convalescent-phase serum sample. No responses to VP7 of RV5 (serotype G2, P1B) were detected in any patients. This finding may relate to the fact that the VP7 protein of serotype G2 rotavirus strains has been shown to have a lower degree of sequence homology when compared with strains of serotypes G1, G3, and G4 than when these other serotypes are compared with each other (12). This lack of heterotypic responses to VP7 of serotype G2 implies that cross-protection between serotypes G1, G3, and G4 and serotype G2 (if it exists) could be mediated only via the VP4 protein.

One advantage of the RIP assay, in addition to the detection of antibody to conformation-dependent epitopes, is the detection of antibodies to the nonstructural rotavirus proteins found in infected cells. Immune responses to three of the five nonstructural proteins (NS35, NS28 and NS26) were detected in the present study. Responses to NS35 of all test strains were detected in at least one serum sample collected from each patient examined. This nonstructural protein has also been found to be relatively immunogenic following natural infection in another study that used the RIP technique (31). This finding is interesting in view of a report suggesting that this protein (and not VP7) is the cellular attachment protein (2), raising the question of whether this is really a nonstructural protein. Responses to the other two nonstructural proteins (NS28 and NS26) were relatively common in children with a serotype G1 infection and slightly less common in children with a serotype G4 infection. This lack of detection of responses in the serotype G4-infected children may again be due to insufficient appropriately timed serum specimen collection.

In order to ascertain that the lack of detection of serum responses to VP7 of the serotype G2 strain was not due to errors inherent in the technique used, two children with a serotype G2 rotavirus infection were also examined. A strong homotypic immune response to both the VP4 and VP7 proteins of the RV5 (serotype G2) rotavirus strain was detected in the sera collected from these two children. In addition, one of these serotype G2-infected children showed heterotypic responses to the VP4 and VP7 proteins of strains RV4 (G1, P1A) and ST3 (G4, P2). This heterotypic response could possibly have been caused by a preexisting infection with one (or both) of these serotypes. It was not known whether the serotype G2 infections in these children were primary infections, since only a single serum sample was obtained from each child and they were aged 8 months and 3 years when admitted to the hospital. There were insufficient sera to test for the presence of IgM antibodies.

Our results indicate that following naturally acquired primary rotavirus infections, the serum antibody responses detected by an RIP technique are to a wide array of rotavirus proteins present in infected cells during replication of the virus. Responses to core and inner capsid proteins, nonstructural proteins, and the neutralizing antigen VP4 are similar for the three G-type infections studied (G1, G2, G4) and are not influenced by the G type or P type of the rotavirus strain used in reactions. Heterotypic immune responses to the neutralization antigens, at least following serotype G1 and G4 infections, appear to consist primarily of responses to VP4 rather than to VP7. Anti-VP7 serum responses, on the other hand, were detected more frequently with homologous reactions than with heterologous reactions, and no responses were detected to the VP7 antigen of the human serotype G2 rotavirus strain RV5 in any serum specimen studied. The relative importance of the VP4 protein as the dominant immunogen will ultimately depend on the protective effect of antibody to VP4 against subsequent rotavirus disease in humans.

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