Quantification of Systemic and Local Immune Responses to Individual Rotavirus Proteins during Rotavirus Infection in Mice

SHIN-ICHI ISHIDA,^{1*} NINGGUO FENG,¹ BAOZHANG TANG,¹ JOANNA M. GILBERT,¹
AND HARRY B. GREENBERG^{1,2}

Departments of Medicine, Microbiology, and Immunology, Stanford University School of Medicine, Stanford, California 94305,¹ and Veterans Administration Palo Alto Health Care System, Palo Alto, California 94304²

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The purpose of the present study was to develop a quantitative assay that could be used to measure the local and systemic immune responses to specific rotavirus proteins following rotavirus infection of adult mice. To measure these responses, we used an immunocytochemical staining assay of *Spodoptera frugiperda* (Sf-9) cells which were infected with recombinant baculovirus expressing selected rotavirus proteins. The specificity of the assay was documented by using a series of monoclonal antibodies to individual rotavirus proteins. We observed that the assay had high levels of sensitivity and specificity for a series of VP7- and VP4-specific neutralizing monoclonal antibodies which recognized conformation-dependent epitopes on their target proteins. We also studied immunoglobulin G (IgG) immune responses in serum and IgA immune responses in the stools of mice infected with wild-type murine rotavirus strain EHPw. In both sera and stools, the most immunogenic proteins were VP6 and VP4. VP2 was less immunogenic than VP6 or VP4, and the immune responses to VP7, NSP2, and NSP4 were very low in serum and undetectable in stools.

Rotavirus is the most important cause of severe dehydrating diarrhea in children and young animals worldwide (30). In developing countries, approximately 870,000 deaths per year are caused by rotaviral diarrhea (10). In the United States, 70,000 to 100,000 children are hospitalized and 75 to 125 deaths are caused by rotavirus annually (20). Rotavirus is a member of the family *Reoviridae*. The genome contains 11 segments of double-stranded RNA, and with one or two exceptions, each segment encodes a single viral protein. Six structural proteins are found in virus particles and five nonstructural proteins are found only in infected cells (15). Gene segment 4 encodes an outer capsid spike-like protein (VP4), and gene segment 9 (or gene segment 7 or 8 in some rotavirus strains) encodes an outer capsid surface glycoprotein, VP7. Both VP4 and VP7 can induce antibodies with neutralizing activity in vitro (23, 26, 45), and antibodies to both of these proteins can passively protect experimental animals from rotavirus-induced diarrhea (41, 48). Gene segment 6 encodes VP6, which has group and subgroup antigenic specificities and constitutes 51% of the virion mass (33). Antibodies to VP6 do not have neutralizing activity in vitro but may mediate protection in vivo (7). NSP2 and NSP4 are two relatively abundant nonstructural proteins which function during viral replication and assembly (15). Antibody responses to these proteins have not been studied in detail.

Many studies have identified local immune mechanisms as key determinants of protection from rotavirus infection and disease (6, 9, 25, 38, 42, 43, 46). We recently reported that the antirotavirus intestinal immunoglobulin A (IgA) level, measured against a whole viral antigen, correlated best with protection after heterologous rotavirus immunization in the mouse model (16). However, it was not clear in those studies

which specific protein(s) was the major target of protective immunity. One reason that it has been difficult to determine which protein(s) series is the primary target of protective immunity is that a simple and reliable assay system for quantifying immune responses to individual rotavirus proteins in either serum or the intestine has not been available.

Several reports have analyzed the immune responses in serum to individual rotavirus proteins after wild-type virus infection or vaccination by either a radioimmunoprecipitation assay or a Western blot (immunoblot) assay, and some of them attempted to quantify the level of antibody response (28, 53, 54, 59, 60). However, those analyses were primarily semiquantitative and were not amenable to testing large numbers of specimens, and local immune responses were not studied. In addition, prior studies have not determined whether the individual rotavirus proteins used in Western blot or immunoprecipitation assays retained the conformation-dependent antigenic determinants present on the virion. VP7, for example, does not fold correctly under many experimental conditions (11, 12, 14, 35, 40, 62). Serum immune responses to specific epitopes on VP4 or VP7 have also been measured by monoclonal antibody (MAb) blocking tests, but these assays have not been widely applied to other proteins and are restricted to the MAb available (21, 39).

We used a baculovirus expression system to develop a quantitative assay to detect humoral immune responses to individual rotavirus proteins in serum and feces. The assay uses fixed *Spodoptera frugiperda* (Sf-9) cells infected with recombinant baculovirus expressing individual rotavirus proteins as the antigen. This assay was found to be rapid, simple, sensitive, and specific. Of greatest importance, we found that complex, conformation-dependent antigenic epitopes on both VP4 and VP7 were preserved in this system.

MATERIALS AND METHODS

Cells and virus. The MA-104 cell line, originally derived from African green monkey kidney, was maintained in medium 199 (M199) supplemented with 2

* Corresponding author. Mailing address: School of Medicine, Stanford University, Lab Surge, P304, Stanford, CA 94305-5487. Phone: (415) 493-5000, extension 63121. Fax: (415) 852-3259. Electronic mail address: ml.hbg@forsythe.stanford.edu.

mM L-glutamine, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, and 5% fetal calf serum (FCS) as described previously (16). Sf-9 cells were maintained as described previously (37).

Wild-type murine rotavirus strain EHPw (G3, P[19]) was originally obtained from H. Pereira (24). The virus was prepared as a clarified homogenate of infected suckling mouse intestine as described previously (6, 16). Tissue culture-adapted murine rotavirus EHP_T was derived from EHPw (6). EHP_T and simian rotavirus RRV (G3, P[6]) were grown in MA104 cells, and viral titers were determined by plaque assay (27).

RRV×EHP_T reassortant viruses, R/E4 and E/R4, were isolated as described previously (4), and virus growth and titer determination were the same as those for the parental viruses. R/E4 has EHP_T gene segment 4 and the other 10 gene segments from RRV, and E/R4 has RRV gene segment 4 and the other 10 gene segments from EHP_T.

Mouse serum and stool antibodies. Pregnant rotavirus antibody-negative BALB/c female mice were purchased from Simonson Inc. (Gilroy, Calif.). Each animal was housed individually in a microisolation cage, and pups were left with their dams for 4 weeks, at which time the litters were segregated by sex in separate isolation cages. Each cage housed three mice. All mice were bled prior to experimentation and were screened for serum antirotavirus antibody by an enzyme-linked immunosorbent assay (ELISA) to verify that they were seronegative.

Six-week-old BALB/c mice were inoculated with 10⁶ diarrhea doses 50 of EHPw murine rotavirus by gavage as described previously (16). Blood and stool samples were collected prior to infection and at 1 through 6 weeks postinfection. Blood specimens were obtained by retro-orbital puncture from anesthetized mice in accordance with the guidelines of the National Institutes of Health. Serum samples were stored at -70°C before testing. Stool samples were stored at -70°C and were made 10% (wt/vol) by suspension in a stool diluent (10 mM Tris, 100 mM NaCl, 1 mM CaCl₂, 0.05% Tween 20 [Sigma, St. Louis, Mo.], 5 mM sodium azide, 5% FCS [pH 7.4]) containing a protease inhibitor cocktail which included 0.2 mM AEBSF [4-[(2-aminoethyl)benzenesulfonyl]fluoride], 1 µg of aprotinin per ml, 1 mM benzamidine, 10 µg of leupeptin per ml, and 10 µg of pepstatin A per ml. The stool suspension was centrifuged at 15,000 rpm for 6 min in an Eppendorf microfuge. The supernatant was used as the sample of stool suspension for antibody testing.

MAbs. A variety of rotavirus-specific MAbs were used to standardize and evaluate the assay. MAb 3A8/6E8 is specific for VP2 (58). MAbs 159, 4C3, 4F8, 5H3, and 3 are VP7-specific, G3 neutralizing antibodies that have been characterized previously (56). MAbs 60 and 129 are nonneutralizing, VP7-specific MAbs (56). MAb 1C10 (G2 specific), 5B8 (G5 specific), and UK7 (G6 specific) are VP7-specific neutralizing antibodies (57). MAb 2G4 (which recognizes the VP5 segment of VP4) is a heterotypic neutralizing antibody (36, 56). MAb 255/60 recognizes the subgroup I region on VP6 (22). MAb 191 recognizes NSP2 (3), while MAb B4-1/55 is an NSP4-specific antibody (51). MAbs 1A10, HS16, and HS2, which recognize type-specific or cross-reactive epitopes on human rotavirus VP4s, have been described previously (49).

Viral hemagglutinin preparation. The viral hemagglutinins used for the hemagglutinin inhibition (HI) test (see Table 2) were prepared by freezing and thawing rotavirus-infected MA104 cells when the cultures exhibited a complete or a nearly complete cytopathic effect. The culture fluid was stored at -70°C and was used as the hemagglutination antigen for HI testing. The HI assay was performed as described previously by using 8 U of hemagglutinin and human type O erythrocytes (29).

Recombinant proteins. Recombinant baculovirus expressing murine rotavirus EHP (G3, P[19]) VP4 was constructed with the PharMingen Baculogold kit, according to the manufacturer's instructions, by S. J. Dunn and T. L. Cross at Stanford University. Recombinant baculoviruses expressing bovine rotavirus RF (G6, P[7]) VP2 and VP6 were kindly provided by Jean Cohen (32, 64). The construction and characterization of recombinant baculovirus expressing simian rotavirus RRV (G3, P[3]) NSP2 and simian rotavirus SA11 (G3, P[2]) NSP4 (kindly supplied by M. K. Estes) were described previously (1, 3). Recombinant baculoviruses expressing RRV VP7 and VP4 were constructed as described previously (17, 34, 37).

Sf-9 cell immunostaining assay. An immunohistochemistry assay was used to detect immune responses to individual rotavirus proteins. Recombinant baculovirus expressing an individual rotavirus protein was adjusted to a titer of 10⁷ PFU/ml. Almost confluent Sf-9 cells in T-25 flasks were infected with recombinant baculovirus at a multiplicity of infection of 0.1. After incubation on a rocker platform at room temperature (RT) for 1 h, infected Sf-9 cells were harvested and distributed to a 96-well microtiter plate (10⁴ cells per well), and the plates were incubated at 28°C for 72 h. After incubation, the cell culture medium was discarded and the 96-well plates were dried in a vacuum oven at RT for 1 h, the cells were fixed with 10% formalin (formaldehyde, 37% solution, containing 10 to 15% methanol; Sigma) in phosphate-buffered saline at RT for 30 min, and the cells were subsequently permeabilized with 1% Triton X-100 (Sigma) in TNC (10 mM Tris, 100 mM NaCl, 1 mM CaCl₂ [pH 7.4]) at RT for 2 min. Several other fixation protocols were evaluated but were found to be less effective (data not shown).

Fixed cells were used for antibody testing within 24 h of preparation. Mouse ascites samples were serially diluted fivefold from 1:100 to 1:312,500 in M199 with 5% FCS. Mouse sera were serially diluted threefold from 1:50 to 1:2,150.

Serum was diluted starting at a 1:3 dilution for detection of antibodies to NSP4. Mouse stool suspension samples were serially diluted twofold from 1:2 to 1:512 as described above. The plates were incubated at 37°C for 2 h. The plates were then washed twice with M199. For the detection of IgG antibody in sera, horseradish peroxidase-labeled goat anti-mouse IgG(γ) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) was added as a second antibody (1:3,000 dilution in M199 with 5% FCS), and the mixture was incubated at 37°C for 1 h. For the detection of IgA in stool suspensions, biotin-labeled goat anti-mouse IgA(α) (Kirkegaard & Perry Laboratories) was used as a second antibody (1:1,000 dilution in M199 with 5% FCS), and the mixture was incubated at 37°C for 1 h; this was followed by incubation with peroxidase-streptavidin (1:1,000 dilution in M199 with 5% FCS; Kirkegaard & Perry Laboratories) at 37°C for 1 h. The antibody bound to cells was detected with 3-amino-9-ethyl-carbazole (AEC; Sigma) after washing twice with M199. The antibody titer was assigned to the highest dilution at which cell staining could easily be detected by examination of the monolayer in a microscope at ×100 magnification.

Serum focus reduction neutralization assay. The focus reduction neutralization assay has been described previously (27). Serum samples were serially diluted, and the dilutions were mixed with EHP_T, RRV, or reassortant virus (R/E4 or E/R4) for a 1-h incubation at 37°C. One hundred microliters of the serum-virus mixture was transferred to MA-104 cell monolayers in a 96-well plate, and the plate was incubated overnight at 37°C in 5% CO₂. The cells were fixed with 10% formalin for 30 min and permeabilized with 1% Triton X-100 for 2 min. After washing, rabbit antirotavirus hyperimmune serum was added to the plate for a 2-h incubation at 37°C. The plate was washed and horseradish peroxidase-conjugated goat anti-rabbit IgG (Kirkegaard & Perry Laboratories) was added. After a 1-h incubation at 37°C, a color reaction was detected with the AEC substrate. The neutralization titer of a serum sample was defined as the highest dilution at which virus-positive foci were reduced at least 50% compared with that for normal control serum samples.

RESULTS

Assay development. Our purpose was to design a simple quantitative assay that could be used to measure humoral immune responses in serum and feces to individual rotavirus proteins. Initially, we attempted to purify selected recombinant rotavirus proteins from baculovirus-infected Sf-9 cells for use in an ELISA. However, the need to develop a separate purification scheme for each rotavirus protein and the requirement for large-scale recombinant protein production rendered this approach difficult. We therefore evaluated the utility of an immunocytochemical assay in which fixed Sf-9 cells containing recombinant rotavirus proteins expressed by baculovirus were used as the solid-phase antigen. Two technical hurdles that needed to be overcome included the development of a protocol that enhanced the binding of the nonadherent Sf-9 cells to microtiter wells and the development of a fixation strategy that preserved most or all of the conformation-dependent epitopes on surface proteins VP4 and VP7 (see below). By including an air drying step prior to fixation, we were able to obtain reproducibly immobilized monolayers of Sf-9 cells for staining in the microtiter plates. This format permitted us to use the fixed cells efficiently in quantitative assays (Fig. 1). Use of a multiplicity of infection of 0.1 or less provided a solid-phase target in which infected cells could easily be identified against a background of uninfected cells (Fig. 1A). The variations seen in the staining intensities of individual cells (Fig. 1A) are probably due to the presence of cells undergoing secondary infection following spread from cells infected primarily at the start of the experiment.

Sensitivity and specificity. Once we determined that we could efficiently immobilize and immunostain baculovirus-infected Sf-9 cells, we sought to determine the specificity of our assay for detecting antibodies to the four major structural proteins of the virus and for antibodies to two of the five nonstructural proteins (Table 1). We specifically sought to determine if conformation-dependent epitopes on VP7 and VP4 were preserved in this assay system (Tables 2 and 3, respectively). We examined by the immunostaining assay the titers of a variety of previously described MAbs against individual recombinant rotavirus proteins. In all cases, the titer of

FIG. 1. Immunocytochemical staining of Sf-9 cells. Cells were fixed and analyzed as described in Materials and Methods. (A) Sf-9 cells infected with baculovirus expressing RRV VP4 were stained with VP4-specific MAb 2G4 diluted 1/62,500. Bar, 50 μ m. (B) Sf-9 cells infected with baculovirus expressing rotavirus RF VP6 were stained with MAb 2G4. Magnifications, $\times 50$.

the antibody against its homologous protein target was 100-fold or more the titer against the heterologous proteins (Table 1). In fact, with the exception of VP2, which appeared to cross-react nonspecifically at a low level with several of the MAbs, the assay was extremely specific (Table 1).

In the past, several assays to specifically detect antibodies to individual rotavirus proteins underestimated responses to VP4 and VP7 because the conformational epitopes on these proteins, including neutralization epitopes, were poorly reproduced on the test antigen (12). Hence, we sought to determine whether fixed Sf-9 cells expressing recombinant rotavirus VP4 and VP7 displayed a variety of conformation-dependent epitopes that had previously been shown to be involved in viral neutralization. Neutralizing MAbs directed at the A (159, 4C3, 4F8) and C (5H3) regions of RRV VP7 reacted at a high titer in an immunohistochemistry assay with Sf-9 cells infected with baculovirus expressing RRV VP7, while MAbs to serotype 2, 5, or 6 rotaviruses were nonreactive (Table 2). The titer of the

reactive antibodies by immunostaining was substantially higher than the titer of the same antibodies tested by the HI assay (Table 2). Antibodies to various regions of RRV VP4, including regions localized to both VP8 and VP5, reacted specifically with Sf-9 cells expressing recombinant RRV VP4, while antibodies to human VP4 serotypes were nonreactive (Table 3). Again, the titer of the VP4 MAbs by immunostaining was substantially higher than the titer by the HI assay.

We also evaluated the antigenicities of several other rotavirus proteins. Both group- and subgroup-specific regions on VP6 were preserved (Table 1 and data not shown). We were unable to thoroughly test the preservation of antigenic domains on VP2, NSP2, and NSP4 because of the limited number of MAbs to these proteins. However, the expressed proteins clearly reacted with the available antibodies (Table 1).

Protein-specific immune responses in vivo. Since our assay appeared to be specific and sensitive, we evaluated its utility in measuring the immune responses to homologous infection in a

TABLE 1. Specificity of immunocytochemical staining of Sf-9 cells infected with recombinant baculovirus expressing individual rotavirus proteins

Baculovirus-expressed rotavirus protein	Reciprocal titer of the following MAbs to indicated rotavirus proteins ^a					
	3A8/6E8 (VP2)	2G4 (VP4)	255/60 (VP6)	60 (VP7)	191 (NSP2)	B4-1/55 (NSP4)
RF VP2	$\geq 312,500$	2,500	2,500	<100	500	2,500
EHP VP4	<100	312,500	<100	<100	<100	<100
RF VP6	<100	<100	$\geq 312,500$	<100	<100	<100
RRV VP7	<100	<100	<100	$\geq 312,500$	<100	<100
RRV NSP2	<100	<100	<100	<100	$\geq 312,500$	<100
SA11 NSP4	<100	<100	<100	<100	<100	$\geq 312,500$

^a Rotavirus proteins are indicated in parentheses.

TABLE 2. Titers of neutralizing and nonneutralizing VP7-specific MABs by Sf-9 cell immunostaining

VP7-specific MAB (G specificity)	Titer vs RRV VP7 by Sf-9 cell staining	HI titer (target virus)
159 (3)	$\geq 312,500$	12,800 (RRV)
4C3 (3)	$\geq 312,500$	1,600 (RRV)
4F8 (3)	$\geq 312,500$	12,800 (RRV)
5H3 (3)	$\geq 312,500$	800 (RRV)
3 (3)	$\geq 312,500$	6,400 (RRV)
60 CR ^a	$\geq 312,500$	<50 (RRV)
129 CR	$\geq 312,500$	<50 (RRV)
1C10 (2)	<100	1,600 (DSI $\times$ RRV)
5B8 (5)	<100	3,200 (OSU)
UK7 (6)	<100	1,600 (NCDV)

^a CR, cross-reactive.

mouse model of rotavirus infection. Adult mice (6 weeks old) were infected with the wild-type murine rotavirus EHPw at a dose of 10^6 diarrhea doses 50 per mouse. Serum and stool specimens were assayed for antibody responses on a weekly basis (Fig. 2). As test antigen, we used the homologous recombinant VP4 cloned from EHP rotavirus. Since EHP VP7 has the greatest sequence and antigenic similarity to G3 strains, we used RRV VP7 (G3) as a test antigen (13). VP2 and VP6 have previously been demonstrated to be antigenically cross-reactive. Therefore, we used heterologous genes to express antigen derived from the bovine RF strain. Although the antigenic diversities of the NSP2 and NSP4 proteins have not been well studied, by sequence analysis these proteins are highly conserved among most rotavirus strains (50, 65). Therefore, to measure immune responses, we used baculovirus expressing RRV NSP2 and SA11 NSP4.

In the serum specimens, immune responses to VP4 and VP6 were first detected 7 days after infection and reached maximal levels at 2 to 3 weeks after infection (Fig. 2). The responses to VP2 were somewhat delayed at the onset and the overall titer was lower, while the responses to VP7 were at a low level and were only detectable 5 weeks after the primary infection (Fig. 2A). The IgA immune responses in the feces generally mirrored the IgG immune responses in the serum except that they were at a lower level and we could not detect IgA immune responses to VP7, NSP2, or NSP4 (Fig. 2B). Fecal IgG responses were detectable only against VP6 and were very low in titer ($\leq 1:4$). Of note, VP7-specific immune responses could not be detected in feces.

Immune responses to two nonstructural proteins were also measured. Responses to NSP2 were present but were low in

TABLE 3. Titers of neutralizing and nonneutralizing VP4-specific MABs by Sf-9 cell immunostaining assay and rotavirus HI test

VP4-specific MAB (P specificity)	Titer vs RRV VP4 by Sf-9 cell staining	HI titer vs RRV
M11 (3)	62,500	50
A15 (3)	$\geq 312,500$	200
1A9 (3)	$\geq 312,500$	12,800
5D9 (3)	$\geq 312,500$	3,200
7A12 (3)	$\geq 312,500$	1,600
2G4 (3)	$\geq 312,500$	3,200
1A10 (8)	<100	<50
HS16 (6)	<100	<50
HS2 (CR ^a)	$\geq 312,500$	<50

^a CR, cross-reactive.

FIG. 2. Systemic and fecal humoral immune responses to individual rotavirus proteins in adult mice infected with murine rotavirus strain EHPw. Six-week-old adult mice ($n = 3$) were orally inoculated with EHPw at 10^6 diarrhea doses 50 per mouse. Blood and stool samples were collected at the indicated times postinfection (PI). IgG immune responses to rotavirus RF VP2, EHP VP4, RF VP6, RRV VP7, and RRV VP7 in serum specimens (A) and IgA immune responses to rotavirus RF VP2, EHP VP4, and RF VP6 in stool specimens (B) were measured by the Sf-9 cell staining assay. The titer is the mean, and the standard deviations of the mean are indicated by vertical bars. $\square$, VP6; $\diamond$, VP4; $\circ$, VP2; $\triangle$, VP7; $\boxplus$, NSP2.

titer in serum and were not detectable in feces (Fig. 2). Only serum specimens from week 6 were available to measure responses to NSP4. Very low level responses (titers, between 1:9 and 1:27) were observed.

Serum neutralization titer. We carried out neutralization assays with serum specimens obtained from mice infected with the EHPw murine rotavirus in order to determine how viral protein-specific antibody responses to VP7 and VP4 correlated with this biologic activity. The serum neutralizing titers were 450 to the EHP virus and 50 or less to RRV (Table 4). The titers to reassortant virus R/E4, which has EHP_T VP4 with RRV VP7, were higher than the titers to reassortant virus E/R4, which has RRV VP4 and EHP_T VP7, indicating that

TABLE 4. Comparison of serum rotavirus VP4 and VP7 antibody responses and neutralizing activity in mice 6 weeks after a murine rotavirus EHPw infection

Mouse	Serum IgG titers to recombinant rotavirus proteins ^a		Serum neutralizing titers to selected rotaviruses ^b			
	EHP VP4	RRV VP7	EHP	R/E4 ^c	E/R4 ^d	RRV
A	1,350	150	450	150	50	50
B	1,350	50	450	150	50	50
C	1,350	50	450	50	50	<50

^a Serum IgG responses to recombinant baculovirus-expressed EHP VP4 and RRV VP7 were determined by an immunocytochemical staining assay (see Materials and Methods).

^b Serum neutralizing activities to indicated rotaviruses were determined by focus reduction neutralization assay (see Materials and Methods).

^c Reassortant which has EHP_T gene segment 4 and the other 10 gene segments from RRV.

^d Reassortant which has RRV gene segment 4 and the other 10 gene segments from EHP_T.

most of the neutralizing activity in these serum specimens was directed at VP4 (Table 4).

DISCUSSION

The viral targets of protective immunity following either natural rotavirus infection or vaccination have not been fully identified. To aid in this effort, we have developed a simple, sensitive, and specific assay that can be used to quantitatively measure humoral immune responses to individual rotavirus proteins by using recombinant baculovirus-expressed proteins.

Prior studies of recombinant rotavirus structural proteins have indicated that they do not always assume the conformations present on the virion. This has been especially true for VP7 (11, 12). In the present study we show that many, if not all, conformation-dependent epitopes present on VP4 and VP7 are well preserved in the fixed Sf-9 cells infected with recombinant baculovirus (Tables 2 and 3). Although it is unclear why this expression system works better than prior techniques, it is likely that the very high concentration of recombinant rotavirus protein present in individual Sf-9 cells, the presence of calcium in the buffers, and the specific fixatives that were used all play a role. Regardless of the explanation, it seems clear that neutralization epitopes in both the A and C regions of VP7 and on both VP8 and VP5 are antigenically preserved in this system.

It is difficult to measure precisely the sensitivity of the present assay. It certainly exceeds by several orders of magnitude the sensitivities of HI assays. We did not repeat neutralization assays with the identical aliquots of the MAbs tested in the immunostaining assay. However, in general, it appears that the present assay is at least 10- to 100-fold more sensitive than the neutralization assay for the VP4 and VP7 MAbs tested (56). It is impossible to measure accurately the sensitivity for detecting anti-VP4 or anti-VP7 responses in infected serum because we do not have a "gold standard." However, the sensitivity for anti-VP6 detection is roughly comparable to that by ELISA with whole virus used as antigen.

The specificity of the assay for individual protein responses is high, at least when measured with MAbs (Table 1). We are unsure why the VP2 antigen appears to bind to several MAbs nonspecifically. However, this should not be a major problem with the assay, given the relatively low level of this reactivity compared with that in sera with homologous titers and the

absence of reactivity to VP2 in serum or stool specimens from naive animals (Fig. 2).

In this initial study, we chose to study the humoral immune responses in the sera and feces of adult mice infected with murine rotavirus. Several findings deserve comment. Clearly, the immunostaining assay is capable of detecting protein-specific responses to both structural and nonstructural rotavirus proteins in sera and structural proteins except VP7 in feces after primary infection. The magnitude and specificity of the responses which would occur after secondary exposure, after exposure to heterologous rotavirus strains, or after exposure at a younger age remain to be determined. In the present investigation, we studied only the response to homotypic VP4 (EHP) and VP7 (RRV) proteins. It seems reasonable to assume that the responses to heterotypic VP4 or VP7 proteins would be less. In fact, according to a preliminary analysis of children infected with either the rhesus rotavirus vaccine or wild-type human rotaviruses, it appears that the responses to homotypic VP4 proteins are predominant (27a).

Because of the limited availability of recombinant baculovirus expressing several murine rotavirus genes, we used recombinant baculoviruses that expressed heterologous rotavirus proteins to measure antibody responses in mice inoculated with murine EHPw rotavirus. Therefore, the levels of antibody reported here might have been somewhat higher if viral proteins from the same (homologous) murine strain (EHP) were used. However, previously reported data indicate that the VP2, VP6, NSP2, and NSP4 gene segments have highly conserved amino acid sequences among mammalian group A rotavirus strains (8, 44, 50, 61, 65). In addition, the RRV VP7 gene segment has 88% amino acid sequence homology with EHP VP7 (13).

After wild-type infection in the mouse, VP4 is far more immunogenic than VP7 at both the systemic and the local levels. Early studies have emphasized that VP7 is the "major" neutralization antigen, and *in vitro* studies of neutralizing MAbs indicate that antibodies to VP7 are more efficient at neutralization than antibodies to VP4 (55). Some studies with humans indicate that VP7 is the major immunogen after infection (21, 63). However, the present immunostaining data and data from vaccine and epidemiologic studies with humans seem to indicate that VP4 is the dominant immunogen following primary infection (5, 18, 19, 47, 52, 66, 67). The dominance of VP4 as an immunogen is supported by neutralization studies of EHP_T, RRV, and RRV×EHP_T monoreassortants that also identify VP4 as the primary antibody target (Table 4). Whether alternative immunization strategies or protocols would enhance the immunogenicity of VP7 remains to be seen.

Very few data concerning the roles of antibodies to other viral proteins, whether they be structural or nonstructural, in immunity or pathogenesis are available. Recent and preliminary studies have called attention to the possible roles of antibody to VP6 in protection (7, 31) and the effect of NSP4 on diarrhea (2). The availability of a quantitative assay that can be used to measure immune responses to these and other proteins should help to identify correlates of protection that will improve our ability to evaluate vaccine candidates. In the present study, VP6 was the most immunogenic rotavirus protein studied. This observation has been made before (53, 54, 59, 60). It is unclear whether this is due entirely to the large antigenic mass of VP6. Antigenic mass may not be the only consideration, however, since VP4 is present in smaller amounts than VP7, yet VP4 appears to be more immunogenic. The ability of VP6 to self-assemble and create a particulate, multivalent structure might also play a role in its immunogenicity.

We observed very weak but detectable responses to two

nonstructural proteins, proteins NSP2 and NSP4. Presumably, these responses are due to proteins produced during viral replication in enterocytes. It will be interesting to see if infection with heterologous rotaviruses, in which replication is abortive, is also associated with immunity to the nonstructural proteins. Given the recent data indicating a potential role for NSP4 in the etiology of diarrhea, it will also be interesting to determine if antibody to NSP4 plays a role in protection.

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