Comparison of Mucosal and Systemic Humoral Immune Responses and Subsequent Protection in Mice Orally Inoculated with a Homologous or a Heterologous Rotavirus

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Rotaviruses are the single most important cause of severe diarrhea in young children worldwide, and vaccination is probably the most effective way to control the disease. Most current live virus vaccine candidates are based on the host range-restricted attenuation of heterologous animal rotaviruses in humans. The protective efficacy of these vaccine candidates has been variable. To better understand the nature of the heterologous rotavirus-induced active immune response, we compared the differences in the mucosal and systemic immune responses generated by heterologous (nonmurine) and homologous (murine) rotaviruses as well as the ability of these infections to produce subsequent protective immunity in a mouse model. Suckling mice were orally inoculated with a heterologous simian or bovine rotavirus (strain RRV or NCDV) or a homologous murine rotavirus (wild-type or tissue culture-adapted) strain EHP at various doses. Six weeks later, mice were challenged with a virulent murine rotavirus (wild-type strain EC₂₉) and the shedding of viral antigen in feces was quantitated. Levels of rotavirus-specific serum immunoglobulin G (IgG) and fecal IgA prior to challenge were measured and correlated with subsequent viral shedding or protection. Heterologous rotavirus-induced active protection was highly dependent on the strain and dose of the virus tested. Mice inoculated with a high dose (10^7 PFU per mouse) of RRV were completely protected, while the protection was diminished in animals inoculated with NCDV or lower doses of RRV. The ability of a heterologous rotavirus to stimulate a detectable intestinal IgA response correlated with the ability of the virus to generate protective immunity. Serum IgG titer did not correlate with protection. Homologous rotavirus infection, on the other hand, was much more efficient at inducing both mucosal and systemic immune responses as well as protection regardless of the virulence of the virus strain or the size of the immunizing dose.

Rotaviruses are the single most important etiologic agent causing severe infantile diarrhea worldwide. In developing countries, it has been estimated that rotavirus infection results in 870,000 deaths and millions of severe cases of diarrhea annually in children under 5 years of age (10). Rotavirus-associated mortality is rare in developed countries; however, nearly all children under 3 years of age will be infected by rotavirus, and about 70,000 cases of severe rotavirus diarrhea are reported in the United States each year (7, 8). Given the high frequency of rotavirus disease in well-developed countries, it is unlikely that the morbidity of rotavirus infection in such areas will be significantly reduced by further improving sanitation conditions. Hence, development of an effective vaccine is likely to be the most efficient way to control the disease.

Rotavirus infection occurs in many mammalian species, but in most instances infection is primarily species specific. Group A rotaviruses of animal origin have not been found to cause widespread disease or recurrent epidemics in the human population (12). The virulence of a heterologous rotavirus (virus originally isolated from a different species) and its ability to spread between susceptible individuals are reduced significantly compared with a homologous rotavirus (virus isolated from the same species). For instance, the dose of a heterologous simian rotavirus strain (RRV) required to induced diar-

rhea in suckling mice was about 10^5 to 10^6 times greater than the dose required for a homologous murine virus strain (2, 6). The attenuated phenotype produced by host range restriction of animal rotaviruses in humans has been the basis for several candidate rotavirus vaccines. This Jennerian approach to develop live rotavirus vaccines has been further modified (modified Jennerian vaccine) to incorporate genes encoding VP7 or VP4 of selected human rotavirus strains into animal rotavirus genomes by genetic reassortment. The resulting reassortant viruses have the same G or P serotype specificity as human rotaviruses but are relatively avirulent in humans (15, 16). In recent years, such vaccine candidates have been tested extensively in a variety of volunteer and field trials.

The abilities of the Jennerian and modified Jennerian rotavirus vaccine candidates to protect against subsequent symptomatic rotavirus infection in children have been variable (1, 13). Several field studies showed that oral administration of a live animal rotavirus or an animal-human rotavirus reassortant could reduce the incidence of subsequent human rotavirus induced diarrhea or severe diarrhea (1, 13). However, the degree of protection for different rotavirus vaccines or even for the same vaccine candidate in different trials varied considerably. The precise reason for the variability in vaccine efficacy has been difficult to determine. Proposed explanations have included variations in the serotype of the wild-type challenge strain, differences in the prior rotavirus exposure history of the vaccinees, differences in vaccine "take" rates depending on maternal antibody status, and differences in the timing between vaccination and wild-type rotavirus exposure.

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Despite a variety of investigations, the specific roles of the cellular and humoral immune responses in providing active protection after either homologous or heterologous immunization as well as the factors that regulate the stimulation of active immunity are incompletely understood. As listed above, the potential confounding variables and difficulties in studying active immunity following homologous and heterologous rotavirus infection in humans are multiple. To evaluate active immunity to rotavirus infection more precisely than can occur in human studies, Ward and colleagues recently developed an adult mouse model (18). In this model, adult mice were immunized and later challenged with a cell culture-adapted, moderately virulent strain of homologous murine rotavirus (EW). The extent of rotavirus infection in these mice was evaluated by measuring the viral antigen shedding in fecal samples. The mice did not develop diarrhea but shed a considerable amount of virus for several days. We have modified the adult mouse model by using a variety of highly virulent wild-type challenge murine strains that are just as infectious in adults as in sucklings and that are shed in comparable amounts and for similar periods of time in both adults and sucklings (3). In addition, we have shown that these murine rotavirus challenge strains spread efficiently from infected to uninfected adults, as they do in sucklings (3). Furthermore, we have characterized the sequences of genes encoding VP4 and VP7 of these murine strains as well as their serology (4).

In this study, we used the adult mouse model system to quantitate the efficacy and begin to identify the determinants of active immunity following live heterologous and homologous rotavirus infection. We have examined the differences in local and systemic humoral immune responses stimulated by heterologous and homologous rotaviruses. We observed that oral administration of either homologous or certain heterologous rotavirus strains protected mice from subsequent challenge with virulent homologous virus and that protection was correlated with the presence of a mucosal but not a systemic humoral immune response. Of note, the capacity of heterologous rotaviruses to induce a mucosal immune response was highly dependent on the strain and the inoculating dose of virus. On the other hand, homologous rotaviruses, regardless of their virulence or the dose of the virus given, were much more efficient in stimulating both mucosal and the systemic humoral immune responses.

MATERIALS AND METHODS

Cells. The continuous MA-104 cell line derived from African green monkey kidney was maintained in medium 199 supplemented with 2 mM L-glutamine, 100 U of penicillin per ml, 0.1 mg of streptomycin per ml, and 7% fetal calf serum.

Viruses. Wild-type murine rotavirus strains EHP_w (G3, P18) and EC_w (G3, P17) were originally obtained from H. Pereira and T. Flewett respectively (4, 6). Tissue culture-adapted murine rotavirus strain EHP_T was derived from EHP_w (3). Wild-type B150 Cody NCDV bovine rotavirus (NCDV_w; G6, P1), with a titer of 10^9 focus-forming units (FFU) per milliliter, was kindly provided by L. Saif. This strain is highly virulent for newborn calves, but the precise 50% diarrhea dose (DD₅₀) has not been determined (16a). Cell culture-adapted simian rotavirus strain RRV (G3, P3), bovine rotavirus strain NCDV (G6, P1), and EHP_T were propagated in MA-104 cells as previously described (9). Stock viruses used in this study were tissue culture homogenates that were prepared by freeze-thawing culture flasks two times and were stored at -70°C prior to use. Virus titers were determined by

plaque assay (9) or focus-forming assay and expressed as PFU per milliliter or FFU per milliliter. The titers of stock RRV or NCDV were about 2×10^8 PFU/ml.

Stock wild-type murine rotaviruses (strains EHP_w and EC_w) were prepared as intestinal homogenates derived from infected suckling mice. Seven-day-old Swiss Webster mice were orally inoculated with either EHP_w or EC_w. Forty-eight hours later, the suckling mice were sacrificed and their entire small intestines were removed and freeze-thawed. The intestines were placed in centrifuge tubes, and medium 199 supplemented with L-glutamine, penicillin, and streptomycin but without fetal calf serum (iM199) was added to make a 10% (wt/vol) preparation. The intestines were then homogenized with a tissue homogenizer (Tisseumizer, Cincinnati, Ohio) and stored at -70°C prior to use.

All viral titrations, immunizations, and challenges were carried out in BALB/c mice. To determine the infectivity of the stock wild-type viruses in suckling mice, EHP_w and EC_w were serially diluted 10 fold in iM199. One litter of 7-day-old BALB/c mice was used for each virus dilution, and each mouse was orally gavaged with 100 μl of virus. The highest dilution that caused diarrhea in 50% of suckling mice was defined as the DD₅₀. Since wild-type murine rotavirus infection spreads very efficiently among littermates, pups that developed diarrhea 24 h or later after the initial case in a litter were not considered to be infected from primary inoculation but rather to be infected from secondary spread within the litter. The DD₅₀ of EHP_w and EC_w stocks used in this study was 10^9 /ml. The titer for EHP_w stock on MA-104 cells was 7.9×10^6 FFU/ml. Hence, each DD₅₀ of EHP_w had 7.9×10^{-3} FFU (see Table 1).

The infectivity of EC_w in adult mice was determined by orally inoculating 6- to 8-week-old BALB/c mice with diluted virus (100 μl per mouse). The highest dilution that caused fecal viral antigen shedding in 50% of adult mice was considered the 50% adult mouse shedding dose (SD₅₀). The SD₅₀ of the EC_w stock used in this study was 10^9 , the same as the DD₅₀.

To isolate EHP_T, EHP_w was initially passaged four times in primary African green monkey kidney cell roller culture and then adapted to grow in the MA-104 cell line (2a). The EHP_T stock used in this study was triply plaque purified.

Animals. Pregnant BALB/c or Swiss Webster female mice were purchased from Simonsen Inc. (Gilroy, Calif.). Each animal was housed individually in a microisolator cage maintained in a laminar flow hood. After delivery, mouse pups remained with their dams for 4 weeks, at which time the litters were segregated according to sex into separate isolation cages. Each cage housed five or fewer mice. Each dam was bled prior to experimentation and screened for serum antirotavirus antibody by enzyme-linked immunosorbent assay (ELISA). All dams used in this study were seronegative for rotavirus antibody.

Primary virus inoculation. RRV, NCDV, NCDV_w, EHP_w, and EHP_T stocks were diluted in iM199 as indicated in Table 1. Five- to seven-day-old BALB/c mice were orally gavaged with 100 μl of diluted virus. Mice inoculated with murine rotaviruses and nonmurine rotaviruses were kept in separate rooms to avoid cross-contamination. Each mouse was checked for diarrhea daily for 1 week after inoculation by gentle abdominal pressure (6). The proportion of mice with diarrhea in each treatment group was recorded. Six weeks after primary inoculation, blood and fecal samples were collected from each mouse. Blood specimens were obtained by retro-orbital puncture from anesthetized mice in accordance with National Institutes of Health guidelines. Serum samples were stored at -70°C before testing. Fecal samples were made 10% (wt/vol)

by suspension in a stool diluent (10 mM Tris, 100 mM NaCl, 1 mM CaCl_2 , 0.05% Tween 20, 5 mM sodium azide, 5% fetal calf serum [pH 7.4]) and stored at 4°C. The 6-week fecal samples were tested for antirotavirus antibody within 1 week of collection.

Virulent murine rotavirus challenge. Six weeks after primary inoculation, mice were orally challenged with 10^4 SD_{50} of wild-type EC_{50} rotavirus diluted in iM199 following oral administration of 100 μl of 5% sodium bicarbonate solution to neutralize stomach acidity. Fecal samples were collected daily for 9 days starting 1 day prior to challenge. The fecal samples were suspended and stored as described above. The presence of viral antigen in fecal samples was determined by ELISA within 1 week of the last collection.

Detection of viral antigen shedding in feces by ELISA. The level of viral antigen in fecal samples was measured by ELISA. Ninety-six-well polyvinyl chloride microtiter plates (Dynatech, McLean, Va.) were coated with rabbit antirotavirus hyperimmune serum diluted in TNC (10 mM Tris, 100 mM NaCl, 1 mM CaCl_2 [pH 7.4]) and incubated at 37°C for 4 h. Plates were then blocked with 5% BLOTTO (5% [wt/vol] Carnation powdered milk in TNC) at 37°C for 2 h (11). The plates were washed three times with TNC plus 0.05% Tween 20 (TNC-T). Suspended stool samples were diluted 1:1 in 1% BLOTTO and added to the plates. After overnight incubation at 4°C, the plates were washed with TNC-T, and guinea pig antirotavirus hyperimmune serum (diluted 1:4,000 in 1% BLOTTO) was added to the plates for 2 h at 37°C. The plates were washed, and horseradish peroxidase (HRP)-conjugated goat anti-guinea pig immunoglobulin G (IgG) antibody (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) diluted in 1% BLOTTO was added to the plates for 1 h at 37°C. After three washes, ABTS (2,2'-azino-di[3-ethylbenzthiazoline sulfonate]) substrate (Kirkegaard & Perry Laboratories) was added to the plates, and the color reaction was developed at room temperature for 10 min. The $A_{405\text{ nm}}$ was measured with an ELISA reader (Bio-Tek Instruments, Burlington, Vt.).

The fecal viral antigen shedding data were expressed as net optical density (OD), which equals the OD from fecal samples minus the background OD reading from wells not containing a fecal suspension. A mouse was considered fecal viral antigen positive if at least two of its postchallenge samples had a net OD greater than 0.1. The net OD of prechallenge fecal samples ($n = 71$) was $0.005 (\pm 0.001)$ standard error of the mean. The area under the viral antigen shedding curve (Fig. 1) in an 8-day period after challenge was calculated for each mouse and was defined as total fecal viral antigen shedding (Table 2). This index was used for statistical analysis to estimate the total extent of intestinal viral antigen shedding after challenge.

Detection of serum rotavirus-specific IgG by ELISA. Ninety-six-well plates were coated with rabbit antirotavirus hyperimmune serum and blocked with BLOTTO as described above. To coat plates with viral antigen, stock viruses that were identical to inoculating viruses were diluted in 1% BLOTTO and added to plates for overnight incubation at 4°C. For purposes of testing the noninoculated group, RRV was used to coat plates. Serum samples were serially diluted threefold from 1:50 to 1:109,350 with 1% BLOTTO and added to plates. After incubation at 37°C for 2 h, plates were washed, HRP-conjugated goat anti-mouse IgG antibody (Kirkegaard & Perry Laboratories) was added, and the plates were incubated for 1 h at 37°C. ABTS substrate was added, and color development was stopped after 10 min at room temperature. Absorbance was measured as described above.

The titer of serum antigen-specific IgG was defined as the

highest serum dilution that had a net OD reading greater than 0.1. The net OD was calculated as the OD reading of sample serum minus the OD reading of normal control serum at the same dilution. An animal with titer below 1:50 was considered IgG seronegative. All uninoculated mice ($n = 14$) had serum titers below 1:50. The serum IgG titer of each individual mouse was $\log_3$ transformed for geometric mean titer calculation and statistical analysis. Negative samples (titer of $<1:50$) were arbitrarily assigned a titer of 1:16.7 (threefold below 1:50) for statistical calculations.

Detection of antigen-specific fecal IgA by ELISA. Ninety-six-well plates were coated with rabbit antirotavirus hyperimmune serum and blocked with BLOTTO as described above. Half of each plate was coated with stock virus diluted in 1% BLOTTO as described above (antigen-coated wells), and the other half of the plate was coated with 1% BLOTTO alone (non-antigen-coated wells). After overnight incubation at 4°C, the plates were washed with TNC-T. Suspended stool samples (10%) diluted 1:1 in 1% BLOTTO were added in duplicates to both the antigen-coated wells and the non-antigen-coated wells. After 2 h of incubation at 37°C, the plates were washed with TNC-T and HRP-conjugated goat anti-mouse IgA (Kirkegaard & Perry Laboratories) was added to plates. The plates were incubated for 1 h at 37°C and washed, and ABTS substrate was added for 10 min at room temperature. The OD measurement was the same as described above. The isotype specificity of the HRP-conjugated anti-IgG and IgA antibody (Kirkegaard & Perry Laboratories) was verified by testing these reagents in checkerboard titration versus antirotavirus IgG and IgA monoclonal antibodies (data not shown). Antigen-specific IgA levels in fecal samples were expressed as the antigen-specific OD, which was calculated as the OD from viral antigen-coated wells minus the OD from non-antigen-coated wells. The antigen-specific OD level in fecal specimens obtained from unimmunized mice ($n = 14$) was $0.014 (\pm 0.007)$ standard error of the mean.

Statistical analysis. All statistical analyses were performed in Staviw, a statistical package for Macintosh computer (Abacus Concepts, Inc., Berkeley, Calif.). Analysis of variance was used to examine the difference in total fecal viral antigen shedding, serum IgG titer, and fecal IgA level among groups inoculated with different rotaviruses at various doses. Fisher's protected least significant difference test was used for pairwise comparison among selected groups.

RESULTS

Gastrointestinal pathogenicity in suckling mice after primary oral inoculation with heterologous and homologous rotaviruses. The proportion of 5- to 7-day-old suckling mice that exhibited diarrhea over a 7-day period following primary inoculation with infectious RRV, NCDV_w, NCDV, EHP_w, or EHP_T varied among the viruses and the doses administered (Table 1). All mice inoculated with 10^8 to 10^7 FFU of NCDV_w, 10^7 to 10^6 PFU of RRV, or 10^7 PFU of cell culture-adapted NCDV had diarrhea. The percentage of animals with diarrhea was reduced to 40% when the dose of RRV was decreased to 10^5 PFU. Mice inoculated with 10^6 FFU of NCDV_w or 10^6 PFU of NCDV had no diarrhea (data not shown).

In contrast to heterologous rotaviruses, the dose of EHP_w required to induce diarrhea in suckling mice appeared to be considerably lower (Table 1). Since EHP_w infection spreads efficiently among littermates, doses as low as 10^{-1} DD_{50} frequently caused diarrhea in 100% of suckling mice. No diarrhea was observed in animals inoculated with 10^{-2} DD_{50} of EHP_w. Suckling mice that were orally inoculated with EHP_T

TABLE 1. Percentage of suckling mice which developed diarrhea after primary oral inoculation with selected heterologous and homologous rotaviruses

Virus (species origin) used in primary infection and dose	<i>n</i>	% of suckling mice with diarrhea
RRV (simian)		
10 ⁷ PFU/mouse	17	100
10 ⁶ PFU/mouse	8	100
10 ⁵ PFU/mouse	5	40
NCDV _w (bovine)		
10 ⁸ FFU/mouse	6	100
10 ⁷ FFU/mouse	7	100
NCDV (bovine)		
10 ⁷ PFU/mouse	6	100
EHP _w (murine, wild type)		
10 ⁰ DD ₅₀ /mouse	8	100
10 ⁻¹ DD ₅₀ /mouse	5	100
10 ⁻² DD ₅₀ /mouse	8	0
EHP _T (murine, cell culture adapted)		
10 ² PFU/mouse	10	0
10 ¹ PFU/mouse	6	0
10 ⁰ PFU/mouse	6	0

at doses from 10⁴ to 10⁰ PFU per mouse did not develop diarrhea after primary infection (data for 10⁴ and 10³ PFU of EHP_T groups are not shown), indicating that this virus strain had been attenuated during tissue culture adaptation (Table 1).

Abilities of orally inoculated heterologous and homologous rotaviruses to induce protection against a subsequent virulent murine rotavirus challenge. Six weeks after primary viral inoculation, each group of mice was orally challenged with 10⁴ SD₅₀ of a virulent murine rotavirus strain (EC_w) (equivalent to 10⁴ DD₅₀ in suckling mice), and stool samples were collected daily. The presence of rotavirus antigen in fecal samples was determined by ELISA. All unimmunized controls shed rotavirus antigen in their feces (Fig. 1a and Table 2). Rotavirus antigen generally appeared in fecal samples on day 2 after EC_w challenge and remained detectable for 4 to 6 days. If primary immunization was fully successful, mice were completely protected and shed no detectable viral antigen in feces following challenge (Fig. 1b and Table 2). A mouse was considered to be partially protected if the duration and/or the total viral antigen shedding in feces was less than for unimmunized controls (Fig. 1c and Table 2).

Table 2 summarizes the rotavirus antigen shedding in feces after challenge of mice inoculated with heterologous and homologous viruses at various doses. Among the heterologous rotavirus inoculation groups, only the group that was administered 10⁷ PFU of RRV exhibited complete protection against EC_w challenge. Mice that were inoculated with 10⁶ PFU of RRV were partially protected, since the duration and the degree of viral antigen shedding were significantly less than for the noninoculated control group (Fig. 1c and Table 2).

The duration and amount of fecal viral antigen shedding in groups inoculated with 10⁵ PFU of RRV or 10⁷ PFU of NCDV were not different from results for the noninoculated group. Therefore, these groups were not protected from EC_w challenge despite the fact that they developed diarrhea during primary infection; i.e., the development of clinical disease during primary heterologous infection did not correlate with subsequent protection.

The groups of mice inoculated with 10⁰ or 10⁻¹ DD₅₀ of EHP_w were completely protected against EC_w challenge, while

FIG. 1. Fecal viral antigen shedding curve after EC_w challenge in animals inoculated with different doses of RRV. Six- to seven-day-old mice were either not inoculated (a) or orally inoculated with 10⁷ PFU (b) or 10⁶ PFU (c) of RRV per mouse. Six weeks later, mice were challenged with a virulent murine rotavirus strain (EC_w). Fecal rotavirus antigen shedding was measured by ELISA from 1 day prechallenge to 8 days postchallenge and was expressed as net OD (see Materials and Methods). Data for five mice are presented in panels a and b and data for six mice are presented in panel c.

TABLE 2. Rotavirus antigen shedding in feces of mice challenged with 10^4 SD₅₀ of EC_w rotavirus 6 weeks after primary oral inoculation with selected heterologous and homologous rotaviruses

Virus (species origin) used in primary infection and dose	n	% of mice shedding antigen	Average shedding duration in days	Mean total antigen shedding (SEM)
None	14	100	4.6	3.41 (2.28)
RRV (simian)				
10^7 PFU/mouse	5	0	0	0.07 (0.01) ^a
10^6 PFU/mouse	7	85.7	3.4	1.80 (0.32) ^a
10^5 PFU/mouse	4	100	4.5	3.42 (0.11) ^b
NCDV (bovine)				
10^7 PFU/mouse	6	100	4.8	4.27 (0.43) ^b
EHP _w (murine, wild type)				
10^0 DD ₅₀ /mouse	8	0	0	0.02 (0.01) ^a
10^{-1} DD ₅₀ /mouse	5	0	0	0.02 (0.01) ^a
10^{-2} DD ₅₀ /mouse	6	100	4.5	3.53 (0.10) ^b
EHP _T (murine, cell culture adapted)				
10^2 PFU/mouse	7	0	0	0.02 (1.01) ^a
10^1 PFU/mouse	5	0	0	0.01 (0.01) ^a
10^0 PFU/mouse	4	75.0	4	2.54 (0.97) ^c

^a Levels of total fecal viral antigen shedding were significantly different from those in the noninoculated group ($P < 0.0001$).

^b Levels of total fecal viral antigen shedding were not significantly different from those in the noninoculated group ($P > 0.05$).

^c One mouse was fecal viral antigen negative (0.075), and the other three mice were positive (mean = 3.356).

the group given 10^{-2} DD₅₀ of EHP_w was not protected, as the total amount of fecal viral antigen shedding was not significantly different from that in the control group (Table 2).

Mice inoculated with 10^2 or 10^1 PFU of EHP_T also displayed complete protection against EC_w challenge even though these mice did not develop diarrhea during primary infection. In the group inoculated with 10^0 PFU of EHP_T, one of four mice was completely protected from challenge, while the other three mice were not protected.

Rotavirus-specific serum IgG response in mice inoculated with heterologous and homologous viruses. The rotavirus-specific serum IgG responses in groups inoculated with different rotaviruses at various doses are shown in Table 3. In mice inoculated with heterologous rotaviruses, the level of serum IgG after primary infection was only modestly influenced by the strain and dose of virus inoculated. Every animal inoculated with 10^7 PFU of RRV developed a serum IgG response to rotavirus, while the responses were reduced to 83 and 66.7% for mice inoculated with 10^6 and 10^5 PFU, respectively, of RRV. Fifty percent of the mice inoculated with 10^7 PFU of NCDV developed antirotavirus serum IgG (Table 3), but no antirotavirus serum IgG was detectable in animals inoculated with 10^6 PFU of NCDV (data not shown). Therefore, induction of clinical diarrhea by heterologous rotavirus after oral inoculation was not a sufficient condition for stimulating a detectable serum IgG response.

At any given dose, the titer of rotavirus-specific serum IgG varied appreciably in mice inoculated with a heterologous rotavirus (Table 3). For example, the serum IgG titer in the group inoculated with 10^7 PFU of RRV varied from 1:150 to 1:36,450. The serum IgG titers in groups inoculated with lower doses of RRV or 10^7 PFU of NCDV varied from undetectable (<1:50) to 1:12,150. However, the mean IgG titers among groups of mice given different doses of heterologous rotaviruses were not significantly different. Moreover, some animals

TABLE 3. ELISA serum IgG response to rotavirus in mice 6 weeks after oral inoculation with selected heterologous and homologous rotaviruses

Virus (species origin) used in primary infection and dose	n	% of mice with serum IgG response	Geometric mean titer (range)
None	14	0.0	<50
RRV (simian)			
10^7 PFU/mouse	14	100.0	912 (150–36,450) ^a
10^6 PFU/mouse	12	83.3	237 (<50–4,050) ^a
10^5 PFU/mouse	9	66.7	450 (<50–4,050) ^a
NCDV (bovine)			
10^7 PFU/mouse	6	50.0	312 (<50–12,150) ^a
EHP _w (murine, wild type)			
10^0 DD ₅₀ /mouse	8	100.0	3,078 (1,350–4,050) ^b
10^{-1} DD ₅₀ /mouse	5	100.0	3,252 (1,350–4,050) ^b
10^{-2} DD ₅₀ /mouse	6	0.0	<50
EHP _T (murine, cell culture adapted)			
10^2 PFU/mouse	7	100.0	329 (150–1350) ^a
10^1 PFU/mouse	5	100.0	450 (450–450) ^a
10^0 PFU/mouse	4	25.0	<50 (<50–150) ^c

^a Serum IgG titers in these groups were not significantly different from each other ($P > 0.05$).

^b Serum IgG titers in these groups were not significantly different from each other ($P > 0.05$) but were significantly different from groups defined in footnote a ($P < 0.05$).

^c One mouse had a serum IgG titer of 1:150, and titers for the other three were <1:50.

in the groups inoculated with 10^6 or 10^5 PFU of RRV or 10^7 PFU of NCDV generated serum IgG titers comparable to those of the group inoculated with 10^7 PFU of RRV. If we compared only those animals with detectable antirotavirus serum IgG, we found no difference in the mean titers among the different groups inoculated with heterologous rotaviruses. Therefore, the serum antirotavirus IgG titer in those animals that seroconverted was not highly dependent on the virus strain or the dose of heterologous rotavirus administered.

To examine the correlation between the systemic immune response generated after live heterologous rotavirus infection and the subsequent protection against EC_w challenge, we calculated a correlation coefficient between serum IgG titer and total fecal viral antigen shedding in all animals that were inoculated with RRV or NCDV. The correlation coefficient was 0.167 ($P > 0.05$), indicating that the systemic immune response was not directly associated with the protective response induced after primary infection with heterologous rotaviruses.

All mice that were inoculated with 10^0 or 10^{-1} DD₅₀ of EHP_w developed positive serum IgG responses to rotavirus (Table 3). The serum IgG titers in these animals appeared higher and more uniform than in the groups inoculated with heterologous rotaviruses. The mean serum IgG titers between the two EHP_w-inoculated groups were not significantly different. Moreover, administration of a higher dose of EHP_w to suckling mice did not increase the antigen-specific serum IgG titer (data not shown). Mice inoculated with 10^{-2} PFU of EHP_w were apparently not infected, since these mice developed neither diarrhea nor IgG responses after primary immunization and were not subsequently immune to EC_w challenge.

Mice inoculated with 10^2 or 10^1 PFU of EHP_T also showed a 100% serum conversion rate. The serum antirotavirus IgG titers in these mice were lower than in the groups inoculated with 10^0 or 10^{-1} DD₅₀ of EHP_w but were also relatively homogeneous (Table 3). The mean serum IgG titers in the two

TABLE 4. Rotavirus-specific fecal IgA response in mice 6 weeks after oral inoculation with selected heterologous and homologous rotaviruses

Virus (species origin) used in primary infection and dose	<i>n</i>	ELISA-determined fecal IgA response mean OD (SEM)
None	14	0.014 (0.007)
RRV (simian)		
10 ⁷ PFU/mouse	14	0.543 (0.085) ^a
10 ⁶ PFU/mouse	12	0.042 (0.013) ^b
10 ⁵ PFU/mouse	9	0.011 (0.006) ^b
NCDV _w (bovine)		
10 ⁸ FFU/mouse	6	0.027 (0.014) ^b
10 ⁷ FFU/mouse	4	0.021 (0.004) ^b
NCDV (bovine)		
10 ⁷ PFU/mouse	6	0.019 (0.009) ^b
EHP _w (murine, wild type)		
10 ⁰ DD ₅₀ /mouse	8	1.482 (0.078) ^c
10 ⁻¹ DD ₅₀ /mouse	5	1.476 (0.066) ^c
10 ⁻² DD ₅₀ /mouse	6	0.051 (0.017) ^b
EHP _T (murine, cell culture adapted)		
10 ² PFU/mouse	7	1.045 (0.102) ^a
10 ¹ PFU/mouse	5	1.182 (0.119) ^a
10 ⁰ PFU/mouse	4	0.272 (0.254) ^d

^a Fecal IgA levels in these groups were significantly different from those in the noninoculated group ($P < 0.0001$).

^b Fecal IgA levels in these groups were not significantly different from those in the noninoculated group ($P > 0.05$).

^c Fecal IgA levels in these group were significantly different from those in both the noninoculated group and the groups defined in footnote *a* ($P < 0.0001$).

^d The fecal IgA level in one mouse was 1.033 (OD), and the levels in other three mice were 0.018 (mean OD).

EHP_T groups did not differ from each other or from those in the heterologous rotavirus-inoculated groups. In the group inoculated with 10⁰ PFU of EHP_T, the mouse that was protected from EC_w challenge had a positive IgG response, with a titer of 1:150. The other three mice were apparently not infected during primary immunization, since they were serum IgG negative and were not protected following subsequent challenge.

Rotavirus-specific IgA response in feces of mice inoculated with selected heterologous and homologous rotaviruses. The ELISA-determined fecal IgA response to rotavirus in different groups of mice 6 weeks after primary inoculation varied greatly (Table 4). Among groups inoculated with heterologous rotaviruses, only in the group immunized with 10⁷ PFU of RRV did detectable levels of fecal rotavirus-specific IgA develop in all of the animals. The mean fecal IgA level in this group was about 40 times higher than that in the control group. The mean rotavirus-specific fecal IgA level in the group inoculated with 10⁶ PFU of RRV was slightly but not statistically higher than the level in uninoculated controls. Animals that were inoculated with 10⁵ PFU of RRV, 10⁸ or 10⁷ FFU of NCDV_w, or 10⁷ PFU of NCDV showed no detectable IgA responses.

The correlation coefficient between the antirotavirus fecal IgA level (antigen-specific OD) and total viral antigen shedding in animals inoculated with RRV or NCDV was 0.76 and was highly significant ($P < 0.0001$). Therefore, as opposed to the serum IgG response, the rotavirus-specific intestinal IgA response was highly correlated with protection from virulent murine virus challenge in mice immunized with heterologous viruses. Generation of an intestinal IgA response was dependent on both the strain and the dose of heterologous rotavirus inoculated. Mice inoculated with 10⁰ or 10⁻¹ DD₅₀ of EHP_w developed levels of fecal IgA that were significantly higher

than those in either the noninoculated group or the group inoculated with 10⁷ PFU of RRV. The levels of fecal IgA in the two EHP_w-inoculated groups were similar, suggesting that the intestinal IgA responses in EHP_w-inoculated animals were not influenced by the virus dose administered. The level of antirotavirus fecal IgA in the group inoculated with 10⁻² DD₅₀ of EHP_w was not different from that of the uninoculated group, confirming that the group was not infected during primary inoculation.

All animals inoculated with 10² or 10¹ PFU of EHP_T developed intestinal IgA responses to rotavirus with similar intensities. The mean fecal antigen-specific IgA levels in these groups were significantly higher than in the group inoculated with 10⁷ PFU of RRV but somewhat lower than the group inoculated with 10⁰ or 10⁻¹ DD₅₀ of EHP_w. In the group inoculated with 10⁰ PFU of EHP_T, the single mouse that developed a positive serum IgG response and was protected also showed a positive fecal antirotavirus IgA response (OD = 1.033). The other three mice, which were negative for serum IgG, did not generate detectable fecal IgA responses either. Of note, the nonvirulent cell culture-adapted EHP_T strain was more efficient at generating intestinal IgA responses than the RRV strain, which caused clear-cut diarrhea at the doses given.

DISCUSSION

Several live rotavirus vaccine candidates have been extensively evaluated in phase II and III trials in young children (1, 13). The most successful candidates have all relied on host range restriction (Jennerian or modified Jennerian approach) as the basis for attenuation in humans. The results of these clinical trials clearly demonstrate that host range barriers reliably offer vaccine candidates a substantial degree of attenuation and safety. In addition, immunization with animal rotaviruses or animal-human rotavirus reassortants frequently, but not necessarily reliably, induces moderate to substantial protective immunity to subsequent disease. Interestingly, this immunity can be both homotypic and heterotypic (1, 13, 17). The immunologic and molecular basis for the success or failure of the various Jennerian-type vaccines in humans has been very difficult to determine, primarily because of inherent variabilities involved in clinical trials.

We have approached this problem by beginning to characterize and quantitate the immune response to homologous and heterologous (Jennerian) rotavirus immunizations in an adult mouse model (3). In other studies, we have demonstrated that adult mice are just as susceptible to our challenge murine virus as suckling mice and that they shed rotavirus for comparable times and in similar amounts as sucklings. In addition, adult mice can efficiently transmit infection to uninoculated cage mates just as sucklings do. Hence, we reasoned that although adult mice do not develop overt diarrhea, the determinants of protection from infection are likely to be qualitatively and perhaps quantitatively similar or identical to the determinants of protection in younger mice.

In this study, we have attempted to quantitatively compare live rotavirus immunization with homologous and heterologous strains in a mouse model in order to begin to identify and define correlates of protective immunity. As we have described previously, homologous infection can be far more virulent in terms of DD₅₀ than heterologous infection (Table 1) (2). The differences in virulence are probably not a result of the fact that most of the heterologous strains under study have been adapted to cell culture, since a wild-type bovine isolate (NCDV_w) was only marginally more virulent than the cell culture-adapted NCDV strain and far less virulent than the wild-type

murine strains in suckling mice (Table 1 and data not shown). We also noted that in the homologous mouse model, simple adaptation to cell culture of a murine rotavirus strain exerts a moderate to substantial attenuating effect on the DD_{50} (Table 1, EHP_T). The genetic changes involved in this attenuation are currently the subject of ongoing investigation in our laboratory. However, several attempts to isolate virulent revertants from the cell culture-adapted EHP_T stock have been unsuccessful, indicating that these attenuating mutations are relatively stable (1a).

To characterize and quantitate the ability of oral live virus immunization to induce protective immunity, mice infected with various doses of homologous or heterologous rotavirus strains were challenged 6 weeks after primary infection with a relatively large dose (10^4 SD_{50} , equivalent to 10^4 DD_{50}) of wild-type murine strain EC_w. We waited 6 weeks for challenge to ensure that nonimmune, acute-phase reactants such as interferon or various cytokines would be unlikely to effect the challenge outcome. We used a large challenge dose in the hope that we might identify major, as opposed to minor, correlates of protection. The G serotype of our challenge virus (G3) was identical to that of RRV, highly related but not identical to that of EHP VP7, and distinct from that of NCDV (4). The VP4 P specificity (by sequence analysis) of our challenge virus (tentatively P17) was distinct from those of all three immunizing strains (4).

Just as has been observed in humans, we found that both homologous and heterologous viruses were capable of inducing active immunity in mice. Interestingly, the ability to induce active immunity was not directly correlated with the pathogenicity of the live immunizing virus. For example, infection of suckling mice with 10^6 or 10^5 PFU of RRV or 10^7 PFU of NCDV induced considerable diarrhea in the mouse pups but did not provide substantial protective immunity to rechallenge. On the other hand, infection of suckling mice with as little as 10^1 PFU of cell culture-adapted, avirulent murine rotavirus completely prevented infection with the challenge strain (Table 2, EHP_T). The apparent dissociation, in this model, of pathogenicity and immunogenicity was unexpected and remains to be explained. One must assume, however, that in the mouse at least, the rotaviral determinants of virulence are not necessarily identical to those of immunogenicity. One would hope that this dissociation of pathogenicity and immunogenicity might be further exploited in developing live virus vaccine candidates.

Complete protective immunity could be induced following infection with either a homologous or heterologous rotavirus. However, the ability to induce a protective response was far more dependent on immunizing dose following heterologous versus homologous infection (Table 2). Relatively small changes in immunizing dose (≤ 10 -fold reduction) of a heterologous rotavirus had profound effects on protective efficacy. Such a tight linkage of dose and efficacy was much less apparent during homologous infection with either a virulent or an attenuated murine strain. In fact, it appeared that once a mouse became infected with a murine strain, protection was induced irrespective of the immunizing dose. Presumably the homologous virus replicates efficiently in mice, and hence the immunizing dose becomes somewhat irrelevant as long as enough virus is given to initiate infection. It is worthwhile noting that the small window of protective efficacy afforded by heterologous immunization is one potential explanation for the variability in efficacy observed in different human trials using Jennerian vaccines. Relatively small differences in the actual dose delivered to infants in different trials, compounded perhaps by variations in maternal antibody level in these

infants which would further modify the actual infectious dose delivered, might have substantial effects on efficacy.

In this initial study, we attempted to correlate protection with two common measures of immune response to vaccination. The serum IgG response to whole virus is a generally used assay to detect and quantitate take rates after vaccination. As shown, infection with heterologous strains was relatively efficient at inducing serum IgG responses, and the responses were relatively similar over the immunizing dosage range administered. Similar observations have been made in humans (1, 5, 13). At all dosages of heterologous virus delivered, a relatively broad range of serum responses was detected (Table 3). The reason why heterologous infection routinely induced a more heterogeneous immune response remains to be determined. Nevertheless, the serum IgG response correlated poorly with protection in the mice immunized with RRV or NCDV. The lack of a strong association of serum IgG response and protection has also been observed following human virus vaccination. Infection with wild-type murine rotavirus produced a higher and more homogeneous serum immune response than the heterologous infections. However, the serum IgG geometric mean response following infection with the cell culture-adapted murine strain was indistinguishable from responses following heterologous infection. Taken as a whole, the overlap in serum IgG responses following homologous and heterologous infection was substantial, and in any given instance it would be hard to differentiate between these two groups on the basis of serum response alone. Whether other serum markers of response such as total IgA, protein specific responses, or neutralization responses correlate better with protection remains to be determined.

On the other hand, we observed a highly significant correlation between fecal IgA antibody levels to rotavirus and protection (Table 4). This association was significant following either homologous or heterologous infection. Others have also described this significant association following homologous infection in humans (14). Of note, although the serum IgG response was not highly dependent on the dose of heterologous virus given, the fecal IgA response appeared to be highly related to this variable. In fact, the only animals that developed a detectable local response after heterologous infection were those given the highest dose (the protective dose) of RRV. The explanation for the dissociation of serum and fecal responses in the mice immunized with heterologous viruses is not clear. Presumably a greater dose of virus is required to activate helper T-cell populations in the gastrointestinal tract than in the systemic circulation. Whether heterologous viruses localize and/or replicate more efficiently in regional lymph nodes or the spleen than they do in the gastrointestinal tract remains to be seen. It is clear, however, that heterologous infection is a rather inefficient, although potentially effective means of inducing local immunity in mice.

Murine rotaviruses, either virulent or cell culture adapted and attenuated, were highly efficient at inducing local responses irrespective of immunizing dose (Table 4). Hence, the ability to induce protective immunity was not only associated with wild-type virus. As demonstrated for protective immunity, there was an apparent disassociation between virulence and the ability to induce a local IgA response after homologous rotavirus infection. The reason(s) why homologous infection is so much more efficient at generating a local response irrespective of the virulence of the infecting strain needs to be further explored. Probably a variety of factors are involved, including the level of viral replication, the location of viral replication, and the length of viral replication. It is not clear from this study whether the total IgA response (primarily VP6 specific in this

study) is the actual mediator of protection or simply a surrogate marker for some other aspect of the local response such as the neutralizing response or a cytotoxic cellular response. Studies to address this question are under way. Regardless of the basic mechanism involved, however, in the mouse, homologous infection is a much more consistent and efficient means of inducing local immunity than heterologous infection.

It is interesting to speculate on the role that serotype-specific neutralizing responses might have played in inducing protection after heterologous infection in this model. If one looks just at the groups of mice given 10^7 PFU of NCDV or RRV and the subsequent serum IgG responses, one might assume that G-type specific responses were likely to be involved in protection. However, if one looks at the local responses, one realizes that the doses of NCDV given were probably inadequate to simulate local immunity. Hence, the failure of NCDV to induce protection in this model may be due not to its serotype specificity but rather to its lack of local immunogenicity. We are now attempting to carry out active protection studies in which we use serotypically distinct viruses that are equivalent in the ability to induce local responses. Accurate evaluation of the role of the serotype specificity of different immunizing strains can be ensured only if the local immune responses to different strains are comparable. It is conceivable that results of a variety of studies in the past that simply compared the type specific immune responses in serum following immunization with serotypically distinct strains were misinterpreted because individual test strains varied substantially in the level of local immunogenicity.

The direct relevance of these studies to human rotavirus vaccine efforts remains to be determined. The great advantage of the Jennerian approach, which is reflected in this study, is the substantial and reliable attenuation afforded by the use of animal rotaviruses as heterologous immunogens. Unfortunately, this study indicates that this attenuation may come at a considerable price vis-à-vis the effectiveness and efficacy of immunization. Certainly a homologous mouse rotavirus infection appears to provide more reliable and efficient protective immunity than heterologous vaccination. In addition, this immunity does not appear to be directly correlated with the virulence of the infecting murine strain. It will be interesting to determine if attenuated human rotavirus strains or reassortant rotavirus strains with a human host range are also more effective and efficient inducers of protective immunity than heterologous Jennerian vaccine candidates.

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