Immunization with Baculovirus-Expressed VP4 Protein Passively Protects against Simian and Murine Rotavirus Challenge

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A baculovirus-expressed VP4 protein derived from the simian rhesus rotavirus (RRV) was used to parenterally immunize murine dams. VP4-immunized dams developed high levels of neutralizing antibodies against RRV and low levels of cross-reactive neutralizing antibodies against human strains Wa, ST3, and S2 and animal strains SA-11, NCDV, and Eb. Newborn mice suckled on VP4-immunized dams were protected against a virulent challenge dose of the simian strain RRV and against murine rotavirus Eb. The cross-reactive nature of the serum-neutralizing response generated by VP4 immunization and the protective efficacy of the immunization suggest that recombinant-expressed VP4 proteins should be considered as viable vaccine candidates.

Rotaviruses are one of the leading causes of severe gastroenteritis in infants and young children throughout the world. In developing countries, rotavirus infection is also associated with a high rate of infant mortality. Estimates suggest that 5×10^5 to 1×10^6 deaths annually might be prevented by an effective rotavirus vaccine (4).

Rotaviruses belong to the family *Reoviridae* and are composed of two capsid layers forming a 70-nm icosahedral virion (8, 13). The rotavirus genome contains 11 double-stranded RNA segments which encode structural and non-structural viral proteins (14 to 120 kilodaltons). The outer capsid is composed of two proteins, VP7 and VP4, in a 13:1 molar ratio (32). VP7 is the viral glycoprotein (37 kilodaltons) which specifies the rotavirus serotype (3, 5, 10, 12, 16, 23) and is involved in viral neutralization (10, 12, 14, 16, 20, 24). Gene segment 4 of rhesus rotavirus (RRV) encodes the nonglycosylated outer capsid protein VP4 (86.5 kilodaltons) (20). VP4 is responsible for binding the erythrocyte protein glycoporphin (19) and is the viral hemagglutinin (15, 19). VP4 is cleaved by trypsin to VP8 and VP5 polypeptide components, resulting in enhanced viral infectivity (7). VP4 has also been associated with viral virulence and penetration of the virion into the cell (9, 18, 25, 36, 37).

Antibodies directed at VP4 inhibit viral hemagglutination and neutralize the virus in vitro (3, 12, 17, 19, 20, 24, 33, 35, 39). Shaw et al. (33, 35) have mapped neutralization domains on the VP4 and VP7 proteins of RRV and demonstrated that serotype-specific and cross-reactive neutralization epitopes exist on both outer capsid proteins. We extended these studies by determining the amino acids involved in heterologous and homologous RRV neutralization by selecting a series of neutralizing monoclonal antibody (N-MAb)-resistant mutants and sequencing their VP4- or VP7-encoding gene segments (20, 21). The results of the mutational analysis of gene 4 demonstrated that a cross-reactive neutralizing domain exists in VP5, along with several largely homotypic neutralization sites in VP8 (21).

Passive-transfer studies have also been used to determine that both VP8- and VP5-specific N-MAbs are able to protect

mice from virulent rotavirus challenge (22, 27, 30). By using a passive murine challenge model (26, 28, 29), Offit and Clark (26) demonstrated that protection from rotavirus diarrhea relies on the passive transfer of protective colostral antibody from an immunized dam to suckling mice. Using this model, Offit et al. (30) and Matsui et al. (22) found that N-MAbs specific for the VP8, VP5, and VP7 proteins could protect mice from rotavirus disease. The importance of the immune response to VP4 in humans has also been demonstrated. Eighty percent of the serum neutralizing-antibody activity in sera from infected adult volunteers appears to be directed toward the VP4 protein (40), and in young children, antibodies to VP4 occur more frequently and at an earlier age than do VP7-specific antibodies (38).

To directly evaluate the role of the VP4 protein in disease prevention, a system for obtaining the isolated VP4 protein was established by expressing the protein at high levels in a recombinant baculovirus (19). Our previous findings demonstrated that both VP8- and VP5-specific neutralization sites are present on the expressed VP4 protein (19). In fact, not only is the expressed VP4 protein functionally active in hemagglutinating erythrocytes, but it also contains a complete complement of antigenically conserved neutralization domains (19). The stability of the neutralizing epitopes of the baculovirus-expressed VP4 protein suggest that immunization with expressed VP4 protein may elicit a protective immune response.

In this study we immunized dams with complete RRV VP4 protein expressed by a recombinant baculovirus. Suckling newborn mice were then challenged with the rhesus strain of simian rotavirus or the murine rotavirus strain Eb. VP4-immunized dams passively protected the newborns from either rotavirus challenge. These findings are discussed in terms of a recombinant rotavirus vaccine strategy.

MATERIALS AND METHODS

Animals. Outbred CD-1 mice were obtained from Charles River Breeding Laboratories, Inc., Portage, Mich. The mice were housed in isolator cages that were maintained in a positive-pressure laminar-flow tent. They were bled on arrival and just before the pups were challenged with rotavi-

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TABLE 1. Focus reduction neutralization and hemagglutination inhibition titers of immunized dams

Immunizing antigen	Dam	HI titer against RRV ^a	FRN titer against the following viruses (serotype) ^b :										
			RRV (st3)	SA-11 (st3)	Eb (st3)	Gottfried (st4)	OSU (st5)	NCDV (st6)	Wa (st1)	DS1 (st2)	S2 (st2)	Price (st3)	ST3 (st4)
AcNPV-expressed VP4	1	102,200	102,200	400	400	<100	400	1,600	1,600	<100	400	<100	3,200
	2	25,600	25,600	400	400	<100	400	1,600	800	<100	400	<100	3,200
	3	25,600	25,600	1,600	400	400	400	1,600	1,600	<100	800	<100	6,400
	4	102,200	102,200	1,600	400	<100	400	1,600	1,600	<100	400	<100	6,400
Control AcNPV protein	1	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
	2	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
	3	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100
	4	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100	<100

^a Hemagglutination inhibition (HI) titers are expressed as the reciprocal of the serum dilution required to inhibit hemagglutination of 4 to 8 hemagglutination units of RRV.

^b Focus reduction neutralization (FRN) data represent the reciprocal of the serum dilution resulting in a 60% reduction in the number of infected MA104 cells.

rus. Prior to immunization, sera were assayed by immunoprecipitation of RRV proteins for the presence of preexisting antibodies to rotavirus (11). Only seronegative dams were used in subsequent studies.

Cells and virus. Rotaviruses were grown on a continuous line of MA104 monkey kidney cells as previously described (34). Seed stocks of rotavirus strains with known titers were used as challenge virus or in focus reduction neutralization assays (33). The animal viruses used were as follows: RRV (simian), serotype 3 (st3); SA-11 (simian), st3; Eb (murine), st3; Gottfried (porcine), st4; OSU (porcine), st5; and NCDV (bovine), st6. The following human strains were used: Wa st1; DS1, st2; S2, st2; Price, st3; and ST3, st4.

The VP4-expressing baculovirus recombinant was grown as previously described (19) on *Spodoptera frugiperda* Sf-9 cells in Excell 400 medium (JR Scientific, Woodland, Calif.).

Immunization. Seronegative female CD-1 mice were initially immunized intraperitoneally at 8 weeks of age with 5 µg of baculovirus-expressed VP4 protein as an *Autographa californica* nuclear polyhedrosis virus (AcNPV)-infected-cell sonic extract or control AcNPV-infected-cell sonic extract, as 50:50 mixtures in complete Freund adjuvant. Mice were boosted at 11 and 15 weeks with a 50:50 mixture of VP4 (5 µg, partially purified from AcNPV supernatants by DEAE-cellulose binding in 0.1 M NaCl and elution in 0.5 M NaCl) or AcNPV-infected-cell control protein in incomplete Freund adjuvant. Two females were bred with each CD-1 male at 15 weeks of age. Males were removed 2 weeks later, and dams delivered pups at 18 to 19 weeks of age. Dams were bled again 2 days after giving birth and immunized intraperitoneally with VP4 protein (5 µg, as assayed by a Coomassie blue staining band on polyacrylamide gel electrophoresis [19]) or AcNPV control protein without adjuvant.

Rotavirus challenge of suckling mice. Control and VP4-immunized litters of 4- to 5-day old suckling mice were given oral RRV (4×10^7 PFU) or Eb rotavirus (1×10^4 focus-forming units) in 0.1 ml through a gavage needle. Doses were chosen to cause diarrhea in $\geq 90\%$ of inoculated animals. Diarrheal disease was determined daily by gentle abdominal palpation (26, 30).

Focus reduction neutralization and hemagglutination inhibition assays. An immunoperoxidase staining technique for detecting infected cells was used in a neutralization assay essentially as described previously (33). Approximately 100 focus-forming units of various rotavirus strains was mixed with serial dilutions of serum or stomach contents from pups. The mixtures were incubated for 60 min before infection of MA-104 cells in 96-well microdilution plates. Infected

cells were fixed 18 h later and stained as previously described (33). The titer expressed in Table 1 represents the inverse dilution resulting in a 60% reduction in the number of infected MA-104 cells. Hemagglutination and hemagglutination inhibition assays were performed as previously described (33).

Polyacrylamide gel electrophoresis and immunoprecipitation. Immunoprecipitations of RRV proteins and polyacrylamide gel electrophoresis of proteins were performed as previously described (11, 19). Briefly, MA104 cells were infected with RRV at a multiplicity of infection of 10 and 1 h later were starved for methionine and treated with 5 µg of dactinomycin per ml. At 2 h postinfection 50 µCi of [³⁵S]methionine per ml was added, and RIPA cell lysates were collected after an additional 6-h incubation at 37°C (11). Radiolabeled proteins were immunoprecipitated as previously described (11), with hyperimmune anti-RRV guinea pig serum, monoclonal antibodies to VP2 and VP6, or sera from VP4-immunized or control immunized mice. Proteins were resolved on 10% polyacrylamide gels, and bands were identified by fluorography of the dried gel.

RESULTS

As a first step in evaluating the efficacy of recombinant VP4 as a vaccine candidate, we sought to characterize the extent and specificity of the humoral immune response to this protein in mice. Dams were immunized parenterally with either the RRV VP4 expressed from a baculovirus recombinant or control wild-type AcNPV-infected-cell proteins. To be sure that the neutralizing activity detected in the immune sera was due exclusively to antibody to VP4, we tested these sera by immunoprecipitation, using a metabolically labeled rotavirus-infected cell lysate. Mice immunized with the recombinant VP4 protein developed serum antibody responses to VP4 that were capable of immunoprecipitating only the VP4 protein from RRV-infected cell lysates (Fig. 1). The VP4 postimmunization sera did not contain antibodies which recognize VP6 and VP2 proteins, indicating that the mice had not previously been exposed to rotaviruses.

Dams immunized with RRV VP4 developed high levels of serum neutralizing and hemagglutination-inhibiting antibodies to the homologous RRV strain (Table 1). To determine whether immunization with VP4 stimulated cross-reactive neutralizing antibodies, we tested sera from immunized dams for neutralizing antibodies against a number of animal and human rotavirus strains. In focus reduction neutralization assays, dams immunized with VP4 developed only low

FIG. 1. Immunoprecipitation of RRV proteins from [35 S]methionine-labeled MA104 infected-cell lysates (11). MA104 cells were infected with RRV at a multiplicity of infection of 10. At 1 h later, cells were starved for methionine and 5 μ g of dactinomycin per ml was added for 1 h prior to the addition of 50 μ Ci of [35 S]methionine per ml. RIPA cell lysates were collected 8 h postinfection, and 100,000 $\times$ g supernatants were used for immunoprecipitation with hyperimmune anti-RRV guinea pig serum (lane HI RRV), MAbs to VP2 or VP6 (lanes mAb vp2 and mAb vp6), or an example of serum from mice immunized with the VP4 protein expressed in baculovirus (lane HI vp4). A mixture of both the VP6 MAb and the anti-VP4 mouse serum demonstrates that immunoprecipitations were performed from equal amounts of labeled rotavirus protein.

to moderate levels of neutralizing antibodies against several viruses other than RRV (SA-11, Eb, OSU, NCDV, Wa, S2, and ST3). Unlike RRV, neutralization titers directed at other st3 viruses were low (SA-11, Eb) or undetectable (Price). Neutralizing antibodies against the Gottfried strain (st4) were detected in only one dam and at low serum dilutions (Table 1). In addition, neutralizing antibodies against human strains DS1 and Price were absent from the hyperimmune sera of the dams. Control immunized dams did not have detectable neutralizing antibody titers to any virus strain tested.

Since VP4 immunization elicited a strain-specific and

cross-reactive neutralizing antibody response, we next investigated the ability of VP4 immunization to confer passive immunity to suckling mice in the murine challenge model. To determine whether VP4 immunization could induce protective immunity against simian and murine rotavirus challenge, we bred immunized dams with seronegative CD-1 males and challenged the pups with the RRV or Eb strain. Neonates from unimmunized dams developed diarrhea when challenged with RRV (18 of 19) or Eb (12 of 14) (Table 2). RRV-induced diarrhea occurred 1 to 2 days postchallenge only, with the peak incidence of disease (18 of 19) on day 1. However, none of the neonates from VP4-immunized dams developed diarrhea when challenged with RRV (0 of 17). In this case, the high level of serum neutralizing antibody of the dams correlated with rotavirus protection.

When neonates from VP4-immunized mothers were challenged with the murine rotavirus strain Eb, only 1 of 12 pups developed diarrhea. Diarrhea was detected in this single pup only on day 4 after infection. In pups from dams immunized with control AcNPV-infected cells, Eb-induced diarrhea was detected from days 3 to 6 after challenge, with 12 of 14 diarrhea positives on day 4 representing the peak of the disease. Hence, RRV VP4-immunized dams passively protected pups from rotavirus diarrhea caused by a virulent simian rotavirus, RRV, and by a highly infectious murine rotavirus, Eb. The protection against clinical disease with Eb occurred even though RRV VP4 immunization induced only low levels of cross-reactive neutralizing antibodies to this strain.

DISCUSSION

Recent studies have established that neutralizing antibodies directed at the VP4 protein are important for protecting animals from rotavirus disease (14, 22, 26, 30) and constitute the dominant neutralizing immune response to normal rotavirus infections in adults (40). For this reason we were interested in studying the role of a single rotavirus protein, VP4, in stimulating neutralizing antibody and in inducing protective immunity to rotavirus challenge. Our expression of the VP4 protein in a recombinant baculovirus has allowed us to directly address this issue. The baculovirus-expressed VP4 protein retained antigenic reactivity with a library of N-MAbs and also functioned as a hemagglutinin in the absence of other rotavirus proteins. The stability of antigenic

TABLE 2. Protection of suckling mice against RRV and Eb diarrhea by inoculation of dams with baculovirus-expressed VP4 protein

Immunizing antigen	Dam	HI titer against RRV ^a	HI titer of pup's stomach contents versus RRV	FRN titer against ^b :		No. of mice with diarrhea/no. of mice challenged with ^c :	
				RRV	Eb	RRV	Eb
AcNPV-expressed VP4	1	102,200	400	102,200	400	0/8	
	2	25,600	400	25,600	400	0/9	
	3	25,600	ND ^d	25,600	400		1/6
	4	102,200	ND	102,200	400		0/6
Control AcNPV protein	1	<100	<100	<100	<100	10/10	
	2	<100	<100	<100	<100	8/9	
	3	<100	ND	<100	<100		5/5
	4	<100	ND	<100	<100		7/9

^a Hemagglutination inhibition (HI) titers are expressed as the reciprocal of the serum dilution required to inhibit hemagglutination of 4 to 8 hemagglutination units of RRV.

^b Focus reduction neutralization (FRN) data represent the reciprocal of the serum dilution resulting in a 60% reduction in the number of infected MA104 cells.

^c Pups were challenged with 10⁶ PFU of RRV or 10⁴ infectious doses of Eb 5 days after birth.

^d ND, Not determined.

and functional characteristics of baculovirus-expressed VP4 prompted us to ask the following three questions. (i) Is the expressed VP4 protein immunogenic in animals? (ii) Does immunization with VP4 generate cross-reactive neutralizing antibodies? and (iii) Does immunization with the VP4 protein protect animals from rotavirus disease?

The serum from VP4-immunized animals was first analyzed for rotavirus-specific neutralizing antibodies. Our results demonstrate that VP4-immunized animals generate high-level serum neutralizing antibodies directed at the immunizing VP4 protein and low-level cross-reactive neutralizing antibodies toward a variety of rotaviruses with different VP4 proteins (Table 1). VP4 is apparently highly immunogenic and induces both strain-specific and cross-reactive neutralizing antibodies in immunized animals. This suggests that epitopes on both the largely serotype-specific VP8 portion of VP4 and the cross-reactive VP5 portion of VP4 (21) are immunogenic as well as antigenic. Apparently RRV VP4 immunization does not induce a neutralizing-antibody response to all strains of rotavirus. In fact, cross-reactivity with the human strains studied was limited. To produce a response directed at human rotaviruses, the use of alternative VP4 proteins, the inclusion of several VP4 protein types, or the expression of truncated VP4 polypeptides involved in cross-reactive neutralization could be considered in future immunization schemes.

Neutralizing titers against viruses other than RRV appear to reflect the level of cross-reactive antibodies generated by VP4 immunization and also to define a lineage of related VP4 neutralizing epitopes on these diverse rotavirus strains. It appears that serotype 4 virus ST3 contains the most similarity with the RRV VP4 neutralization epitopes (Table 1). Neutralizing-antibody responses to widely diverse rotavirus strains SA-11 (st3), NCDV (st6), and Wa (st1) are similar in titer and represent conserved VP4 epitopes with RRV. Immunization with the RRV VP4 also generated a low level of neutralizing antibody to Eb (st3), OSU (st5), and S2 (st2), whereas virtually no neutralizing-antibody response was observed to DS1 (st2), Price (st3), and Gottfried (st4) strains (Table 1). On the basis of cross-reactive VP4 neutralization responses elicited by RRV VP4 immunization, the relationship of viral VP4s is RRV $\gg$ ST3 > Wa, SA-11, NCDV > Eb, OSU, S2 > Gottfried, DS1, Price.

The model chosen to study the utility of VP4 in disease prevention is the murine passive protection model established by Offit et al. (26, 28, 29). In this system, protection from rotavirus diarrhea relies on the passive transfer of protective colostral antibody from an immunized dam to suckling mice (26). Colostral antibody from the mother rather than transplacentally transferred antibody protects pups from rotavirus diarrhea in this model, since mice born to rotavirus-immunized mothers are not protected when foster nursed on seronegative dams (26).

In this study, dams were immunized with the expressed VP4 protein or control protein. Pups were delivered and challenged with either RRV or Eb 5 days after birth. VP4-immunized mothers completely or nearly completely protected pups from rotavirus disease due to RRV and Eb, respectively (Table 2). Hence, VP4 immunization of dams is an effective method for providing protection from rotavirus strains with the same (RRV) or conserved (Eb) VP4 neutralization epitopes.

Protection from the Eb strain of rotavirus was surprising, since the serum neutralizing titers to Eb were low in the dams (Table 1). However, a cross-reactive neutralizing epitope defined by MAb 2G4 (21, 33) recognizes both RRV

and the Eb rotavirus strain, and serum from VP4-immunized dams contains antibody directed at the 2G4 epitope as assayed in a solid-phase competition assay. It is possible that conservation of this cross-reactive VP5 epitope (21) and perhaps others contributed to the protection of pups from the murine Eb rotavirus strain.

The Eb strain of murine rotavirus is highly infectious for suckling mice, undergoes multiple rounds of replication in the mouse intestine, and spreads efficiently among littermates. In this study, more than 1,000 50% diarrhea doses of the Eb virus were administered as a challenge, and suckling pups from dams immunized with VP4 were protected. In trying to further characterize the nature of protection induced by the passive transfer of antibody from the mothers, we examined the sera of the pups following Eb challenge. Although the pups were protected from Eb challenge, the 30-day-old mice developed antibodies to rotavirus VP6 protein. This probably indicates that the mice were protected against illness but not against local intestinal infection. If this were the case, the asymptomatic Eb infection could be expected to produce a localized intestinal priming event, resulting in an enhanced immune response upon subsequent viral exposure.

The immunogenicity and ability to induce a neutralizing immune response has been studied with other recombinant rotavirus proteins (1, 2, 6). Consistent with prior genetic and MAb studies, immunization with baculovirus-expressed VP6 did not elicit a neutralizing-antibody response (6). Interestingly, studies with a recombinant-expressed VP7 (1) and *Escherichia coli* expression of half of VP4 (2) have produced only low levels of neutralizing antibody. The reasons for the low levels of antibody obtained by others are not entirely clear, but may include the conformational context of the expressed protein (VP7) or an associated problem stemming from the fusion of the viral polypeptide to an *E. coli* protein (VP4).

Our results demonstrate that a recombinant-expressed VP4 protein can elicit a neutralizing, protective immune response in animals in the absence of other viral proteins or an active virus infection. The immunogenicity of VP4 does not seem to be dependent on the presence of other viral proteins, and VP4 immunization elicits neutralizing antibodies that react with a diverse group of rotaviruses when introduced into animals. The passive transfer of protective immunity from mother to infant, using a recombinant vaccine, is a potentially viable vaccine alternative for livestock populations. However, in humans, breast feeding does not occur widely in all population groups and frequently does not extend into the peak periods of rotavirus disease. As a result, it seems likely that active immunization will be necessary to prevent rotavirus disease in human populations.

Further studies are needed to determine whether systemic immunization of humans with recombinant VP4 will induce localized protective immunity or prime for a protective immune response in an active immunization format. Conceivably, systemic and local immunization with a recombinant VP4 protein, similar to the approach advocated for cholera toxin (31), could prove to be a viable vaccine strategy. As an immunogen, the RRV VP4 is able to protect mice from challenge with a heterotypic murine strain. However, our data suggest that immunization with the RRV VP4 is not likely to induce appreciable levels of neutralizing antibody to most human strains. A more direct approach, using one or more expressed VP4 proteins or the expression

of cross-reactive polypeptides from human viruses, is indicated for future human vaccine studies.

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