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Heterotypic Protection and Induction of a Broad Heterotypic Neutralization Response by Rotavirus-Like Particles

SUE E. CRAWFORD,[1] MARY K. ESTES,[1] MAX CIARLET,[1] CHRISTOPHER BARONE,[1] CHRISTINE M. O'NEAL,[1] JEAN COHEN,[2] AND MARGARET E. CONNER[1,3]*

Division of Molecular Virology, Baylor College of Medicine,[1] and Veterans Affairs Medical Center,[3] Houston, Texas 77030, and Laboratories de Recherché de Virologie et d'Immunologie Moléculaires, Institut National de la Recherche Agronomique, 78352 Jouy-en-Josas, France[2]

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The recognition that rotaviruses are the major cause of life-threatening diarrheal disease and significant morbidity in young children has focused efforts on disease prevention and control of these viruses. Although the correlates of protection in children remain unclear, some studies indicate that serotype-specific antibody is important. Based on this premise, current live attenuated reassortant rotavirus vaccines include the four predominant serotypes of virus. We are evaluating subunit rotavirus vaccines, 2/6/7-VLPs and 2/4/6/7-VLPs, that contain only a single VP7 of serotype G1 or G3. In mice immunized parenterally twice, G3 virus-like particles (VLPs) induced a homotypic, whereas G1 VLPs induced a homotypic and heterotypic (G3) serum neutralizing immune response. Administration of three doses of G1 or G3 VLPs induced serum antibodies that neutralized five of seven different serotype test viruses. The inclusion of VP4 in the VLPs was not essential for the induction of heterotypic neutralizing antibody in mice. To confirm these results in another species, rabbits were immunized parenterally with two doses of 2/4/6/7-VLPs containing a G3 or G1 VP7, sequentially with G3 VLPs followed by G1 (G3/G1) VLPs, or with live or psoralen-inactivated SA11. High-titer homotypic serum neutralizing antibody was induced in all rabbits, and low-level heterotypic neutralizing antibody was induced in a subset of rabbits. The rabbits immunized with the G1 or G3/G1 VLPs in QS-21 were challenged orally with live G3 ALA rotavirus. Protection levels were similar in rabbits immunized with homotypic G3 2/4/6/7-VLPs, heterotypic G1 2/4/6/7-VLPs, or G3/G1 2/4/6/7-VLPs. Therefore, G1 2/4/6/7-VLPs can induce protective immunity against a live heterotypic rotavirus challenge in an adjuvant with potential use in humans. Following challenge, broad serum heterotypic neutralizing antibody responses were detected in rabbits parenterally immunized with G1, G3/G1, or G3 VLPs but not with SA11. Immunization with VLPs may provide sufficient priming of the immune system to induce protective anamnestic heterotypic neutralizing antibody responses upon subsequent rotavirus infection. Therefore, a limited number of serotypes of VLPs may be sufficient to provide a broadly protective subunit vaccine.

Rotaviruses are the most common cause of severe gastroenteritis in young children worldwide. Rotavirus infections cause over 870,000 deaths each year among children less than 2 years of age in developing countries (25). In developed countries, dehydration in children resulting from rotavirus diarrhea is a common cause of hospitalization. Rotavirus-induced mortality and morbidity are of sufficient magnitude that the development of a rotavirus vaccine is a global priority.

Ten human rotavirus serotypes have been characterized throughout the world, but human serotypes G1 to G4 represent 95% of the viruses isolated and typed (27). Although the role of serotype-specific neutralizing antibody in immunity from rotavirus infection in children remains unclear, antibodies to VP4 and VP7 are each independently associated with protection against rotavirus challenge in various animal models (1, 32, 34, 36, 46, 47). The lack of consistent protective efficacy observed in early field trials of bovine and simian rotavirus vaccines in humans was attributed to the induction of serotype-specific immunity that did not protect against heterotypic strains of circulating rotavirus (21, 27, 38). Multivalent simian and bovine reassortant vaccines were developed to overcome this deficiency (10, 27). The need for a multivalent vaccine is supported by the finding that during a predominant G3 season of rotavirus infection, Native American children immunized with the rhesus rotavirus-tetravalent candidate vaccine showed a significant reduction in gastroenteritis and in severe disease compared to children who received a monovalent rhesus rotavirus (G1) vaccine (42). Neonatal rotavirus infection has also been reported to confer protection against clinically severe disease during the first 3 years of life unless children are reinfected with new serotypes (3). Finally, natural rotavirus infections in children induce increased protection from reinfection and diarrhea following each subsequent rotavirus infection, and secondary infection is most likely to be caused by a different G serotype (45). These findings suggest that a polyvalent vaccine that can induce protective immunity to all, or at least to the most prevalent, circulating serotypes of rotavirus may be needed.

Virus-like particles (VLPs) composed of the inner capsid layer protein VP2, the middle capsid layer VP6 and the outer shell protein VP7, with or without the VP4 spike protein, can be expressed and purified from recombinant baculovirus-infected insect cells (16). VLPs are stable and morphologically and antigenically similar to native rotavirus particles, and VLPs containing VP7 with or without VP4 can induce neutralizing antibody responses in mice, guinea pigs, rabbits, and cows (7, 14, 20, 37).

To begin to evaluate heterotypic immunity using VLPs, we cloned the G1 VP7 from a human rotavirus, Houston 8697

* Corresponding author. Mailing address: Division of Molecular Virology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030. Phone: (713) 798-3590. Fax: (713) 798-3586. E-mail: mconner@bcm.tmc.edu.

(HRV8697), that was isolated from a child hospitalized with diarrhea in Houston in 1991. The VP7 from HRV8697 was chosen for use in the VLPs because this virus reacted with a panel of VP7 G1 monoclonal antibodies (MAbs) specific for different epitopes on G1 viruses. Additionally, preliminary results in mice hyperimmunized with G1 2/6/7-VLPs indicated that homotypic (G1) and heterotypic (G3) serum neutralizing antibodies were induced. These results indicated that the HRV8697 G1 VP7 induced cross-reactive neutralizing antibody to G3 virus. We hypothesized that if neutralizing antibodies are important for reduction in gastroenteritis or severe rotavirus disease, then the generation of heterotypic cross-reactive neutralizing antibodies to G3 VP7 by a G1 VP7 would be beneficial and important to test in animal models. To confirm that the HRV8697 G1 VP7 induced cross-reactive neutralizing antibody to G3 viruses and evaluate the role of VP4 in inducing neutralizing antibodies, the immunogenicity of 2/6/7-VLPs and 2/4/6/7-VLPs containing either the G1 or G3 VP7 were compared in mice and rabbits. Additionally, the protective efficacy of G1 2/4/6/7-VLPs, or sequentially administered G3 and G1 2/4/6/7-VLPs in rabbits against a heterotypic G3 rotavirus challenge, was determined and compared to the recently reported results that G3 2/4/6/7-VLPs induce protective immunity against a homologous (G3) rotavirus challenge (7).

MATERIALS AND METHODS

Cells and virus. The simian rotavirus SA11 clone 3 (P5B,G3) (19, 24) and the human Wa (P1A,G1), S2 (P1B,G2), ST3 (P2A,G4), VA70 (P1A,G4), 69M (P4,G8), WI61 (P1A,G9), porcine OSU (P9,G5), and bovine NCDV-Lincoln (P6,G6) and B223 (P8,G10) rotaviruses were cultivated in fetal rhesus monkey kidney (MA104) cells in the presence of trypsin as previously described (18). The lapine ALA (P[14],G3) strain, used for rabbit live rotavirus challenge inoculations and enzyme-linked immunosorbent assays (ELISAs), was passaged 10 times in MA104 cells in the presence of trypsin as described previously (12). The ALA rotavirus used in ELISAs was plaque purified three times.

Spodoptera frugiperda 9 (Sf9) cells were grown and maintained in TNM-FH medium containing 10% fetal bovine serum (FBS) (43). The baculovirus recombinants used in this study were BacRF2A (30), pVL941/SA11-4 (16), pAc461/SA11-6 (17), pVL941/SA11-9 (G3) (described below), and pVL1392/HRV8697-9 (G1) (16). The sequence of HRV8697-9 was determined by the dideoxy-chain termination method and submitted to GenBank.

The full-length SA11 clone 3 VP7 was used to produce the VLPs for this study. The SA11 clone 3 gene 9 cDNA from bp 7 to the end of the SA11 gene was inserted into pVL941 by blunt-end ligation, and recombinants were obtained as described previously (16).

Preparation of VLPs synthesized in infected Sf9 cells or inactivated rotavirus SA11 clone 3 virus. To produce each of the different VLPs, Sf9 cells were coinfected at a multiplicity of infection of 5 PFU/cell with different combinations of the baculovirus recombinants BacRF2A, pVL941/SA11-4, pAc461/SA11-6, pVL941/SA11-9, and pVL1392/HRV8697-9, which code, respectively, for the rotavirus proteins VP2, VP4, VP6, G3 VP7, and G1 VP7. The infection, purification by isopycnic CsCl gradient centrifugation, and characterization of the purified VLPs were performed as described previously (16). Briefly, particle integrity was determined by negative stain electron microscopy. The protein composition of each VLP was determined by Western blot analysis using a mouse hyperimmune serum prepared against triple-layered SA11 virus or the anti-VP4 MAb 5E4 that also reacts with the VP4 trypsin cleavage product, VP5* (5). The serotype of each VLP preparation was confirmed by immunoelectron microscopic analysis and a MAb-based serotyping ELISA. The Bio-Rad protein assay was used to determine the concentration of the VLPs. The *Limulus* amebocyte lysate test was used to quantitate the level of endotoxin in each VLP preparation, which was <0.05 endotoxin unit/dose.

SA11 clone 3 triple-layered particles (TLPs), composed of the genome and VP1 and VP3 surrounded by the inner VP2, middle VP6, and outer VP7 layer, and the VP4 spike protein, were purified by isopycnic CsCl gradient centrifugation as described elsewhere (16). Purified SA11 clone 3 TLPs were inactivated with 4′-aminomethyltrioxsalin hydrochloride (psoralen; Lee Biomolecular Research Laboratories, San Diego, Calif.) as described previously (7, 23).

Immunization of mice with G1 and G3 VLPs. Female CD-1 mice (Charles River Breeding Labs, Portage, Mich.) were confirmed to lack serum or fecal antibody reactivity to rotavirus by ELISA. Groups of five rotavirus-seronegative mice were inoculated intramuscularly with 5 μg of purified G1 or G3 2/6/7-VLPs or 2/4/6/7-VLPs in Freund's complete adjuvant. VLPs in Freund's incomplete adjuvant were administered at 14 and 61 days postinoculation (dpi). The third vaccination was performed 47 days after the second vaccination to determine if the serum total or neutralizing antibody response would be boosted or broadened and also to evaluate rotavirus-specific antibody in fecal samples. Serum was collected at 0, 28, 42, and 114 dpi from each of the mice. Fecal samples from each mouse were collected at 61 and 75 dpi. Nonimmunized mice housed in the same room remained antibody (serum and fecal) negative throughout the experiment. Mice were not challenged with rotavirus because we had not yet established the mouse challenge model in our laboratory.

Immunization of rabbits with G1 and G3 VLPs. Three- to six-month-old rotavirus antibody- and specific-pathogen-free New Zealand White rabbits of either sex were obtained from a commercial source (Charles River Laboratories, Inc., Wilmington, Mass.). For immunizations, all rabbits were housed in open cages in two isolated rooms in a BL2 containment facility at Baylor College of Medicine. For the rotavirus challenge, all rabbits were housed individually in negative-pressure isolator units.

Four to six weeks prior to all rotavirus or VLP inoculations, all rabbits were vaccinated twice with a *Clostridium spiroforme* toxoid (developed and kindly supplied by R. Carman, TechLabs, Inc., Blacksburg, Va.), to prevent disease due to endemic *C. spiroforme* and its associated toxin (4, 7, 11).

Rabbits were vaccinated twice intramuscularly at 0 and 28 dpi with 50 μg of either live or psoralen-inactivated purified SA11 clone 3 rotavirus, G3 2/6/7-VLPs, or 2/4/6/7-VLPs, buffer (7), G1 2/4/6/7-VLPs, or G3 2/4/6/7-VLPs for the first dose and G1 2/4/6/7-VLPs for the second dose. The vaccines were mixed with either Freund's complete adjuvant for the first dose and Freund's incomplete adjuvant for the second dose or QS-21 adjuvant (kindly supplied by Wyeth-Lederle Vaccine and Pediatrics, Pearl River, N.Y.), a purified triterpene glycoside (saponin) of quillaic acid derived from the bark of the South American Molina tree *Quillaja saponaria* (28, 29). All rabbits were challenged at 56 or 69 dpi with 3.5×10^5 PFU (800 50% infective doses) of lapine ALA rotavirus. Serum and fecal samples were collected from all rabbits at 0, 28, 56, or 69 and at 84 or 97 dpi. To assess protective efficacy, virus antigen shedding was determined by ELISA in fecal samples from individual rabbits collected 0 to 14 days postchallenge (dpc) (7). The heterotypic neutralizing antibody responses for each group and the protective efficacy and serum and fecal neutralizing antibody homotypic responses for rabbits inoculated with the G1 or the G1 and G3 VLPs in QS-21 are reported in this study for the first time. The protective efficacy and the serum and fecal antibody responses for rabbits receiving the G3 VLPs administered in QS-21 or Freund's adjuvant were reported previously (7).

Neutralization assays. Plaque reduction neutralization (PRN) or fluorescent focus neutralization (FFN) assays were performed as described elsewhere (9, 19). Since the G1 HRV8697 virus is not adapted to cell culture, neutralizing antibody responses to another G1 virus, Wa, were determined.

Serum and fecal antirotavirus total antibody (immunoglobulin A [IgA], IgM, and IgG) ELISAs. ELISAs were performed to determine the amount of antibody in the mouse or rabbit serum or fecal samples as described previously (7, 13, 37).

Statistical analysis. Statistical analyses were performed with SPSS version 7.5 for Windows (SPSS, Inc., Chicago, Ill.). Antibody titers within a given group at different time points were compared by the Wilcoxon signed ranks test. Antibody titers between groups were compared by the Kruskal-Wallis test followed by the Mann-Whitney *U* test. Percent reductions in rotavirus antigen shedding were compared by the Mann-Whitney *U* test. Correlation coefficients were calculated by Pearson's correlation coefficients.

Nucleotide sequence accession number. The nucleotide sequence data reported here have been submitted to the GenBank nucleotide sequence database and have been assigned accession no. U88717.

RESULTS

Preparation and characterization of VLPs. 2/6/7-VLPs and 2/4/6/7-VLPs containing either the G1 or G3 VP7 were produced by coinfecting insect cells with different combinations of the baculovirus recombinants encoding the bovine RF VP2, simian SA11 clone 3 VP4, VP6, G3 VP7, or the HRV8697 G1 VP7. The serotype of the VLPs containing VP7 was confirmed by immunoelectron microscopy and serotyping ELISA (data not shown). Western blot analysis after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was used to verify the presence of each of the proteins in the VLPs (Fig. 1). Individual proteins were detected with a polyclonal hyperimmune mouse antiserum prepared against SA11 TLPs (Fig. 1A) or a purified anti-VP4 MAb, 5E4 (Fig. 1B). Each of the different particles contained proteins expressed to form the VLPs. Migration of the G3 VP7 in SA11 (Fig. 1A, lane 1) or the VLPs (Fig. 1A, lanes 2 and 3) was slower than that of the G1 VP7 of the VLPs (Fig. 1A, lanes 4 and 5). The 2/4/6/7-VLPs containing either the G3 or G1 VP7 contained VP4 (Fig. 1B, lane 3) or both VP4 and VP5* (Fig. 1B, lane 5).

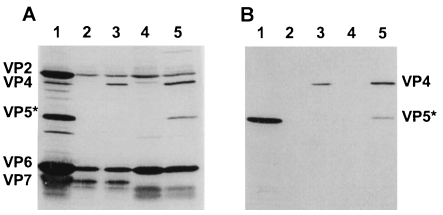

FIG. 1. SDS-PAGE and Western blot analysis of VLP preparations. The proteins in purified SA11 (lane 1) or VLPs (G3 2/6/7-VLPs [lane 2], G3 2/4/6/7-VLPs [lane 3]), G1 2/6/7-VLPs [lane 4], and G1 2/4/6/7-VLPs [lane 5]), made in infected insect cells and purified, were separated by SDS-PAGE, transferred to nitrocellulose, and detected with a hyperimmune anti-SA11 mouse serum (A) or an anti-VP4 monoclonal antibody, 5E4 (B). Locations of the individual proteins are shown at the left and right.

Immunogenicity and induction of neutralizing antibodies in mice immunized twice with VLPs in Freund's adjuvant. To determine if the inclusion of VP4 affected the immunogenicity of the VLPs, 2/4/6/7-VLPs or 2/6/7-VLPs containing VP7 from either SA11 (G3) or HRV8697 (G1) that were greater than 90% intact TLPs were used to immunize mice. Sera collected 14 days following the second inoculation were assayed for the ability to neutralize Wa (P1A,G1), SA11 (P5B,G3), S2 (P1B,G2), or ST3 (P2A,G4) by PRN. None of the VLPs induced serum neutralizing antibodies to G2 (S2) or G4 (ST3) viruses (data not shown). Immunization with G3 VLPs induced serum neutralizing antibody to (G3) SA11 but not to (G1) Wa virus (Fig. 2). In contrast, the VLPs containing the G1 VP7 induced serum neutralizing antibodies to both SA11 and Wa. The level of G3 serum neutralizing antibody induced by the G1 VLPs was only fourfold lower than that of the homotypic G1 serum neutralizing antibody. The titer of neutralizing antibody to SA11 induced by the G1 VLPs was significantly lower than the titers of neutralizing antibody induced by the G3 VLPs [G3 2/6/7-VLPs ($P = 0.023$, G1 2/6/7-VLPs) or G3 2/4/6/7-VLPs ($P = 0.014$, G1 2/6/7-VLPs; $P = 0.049$, G1 2/4/6/7-VLPs)]. However, these titers differed <20-fold. Heterotypic neutralizing antibodies were induced in the mice by the G1 VLPs whether or not VP4 was present in the VLPs. Therefore, the HRV8697 VP7 alone displayed epitopes able to induce heterotypic antibodies to the G3 SA11 virus.

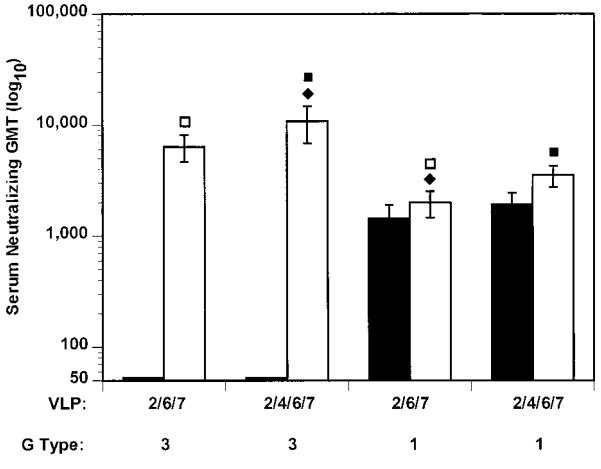

FIG. 2. G1 VLPs induce heterotypic neutralizing antibody in mice. Groups of five CD-1 mice were parenterally immunized with the indicated VLPs in Freund's adjuvant. The serum (28 dpi) neutralizing antibody titers against SA11(P5B,G3) (white bars) and Wa (P1A,G1) (solid bars) were assayed by PRN; the geometric mean of the titers are compared. Statistical differences ($P < 0.05$) in GMTs between groups are indicated by the same symbol (■, ◆, or □). The GMT against Wa for the G3 VLPs are statistically different from all other groups and are not denoted by a symbol. The error bars represent 1 standard error of the mean.

Induction of serum heterotypic neutralizing antibody in mice following a third immunization of VLPs in Freund's adjuvant. To determine whether neutralizing antibody titers could be boosted or broadened, a third vaccine dose was administered at 61 dpi to each of the mice. Serum collected 8 weeks later (114 dpi) was tested for neutralizing antibody by FFN against Wa (P1A,G1), S2 (P1B,G2), SA11 (P5B,G3), VA70 (P1A,G4), OSU (P9,G5), NCDV (P6,G6), 69M (P4,G8), and WI61 (P1A,G9) (Table 1). Following the third parenteral immunization, G3 VLPs induced an increase in both homotypic and heterotypic (G1 Wa) neutralizing antibody; G1 VLPs induced an increase in homotypic but not heterotypic (G3 SA11) neutralizing antibody. The reason for the lack of boost of heterotypic neutralizing antibody by G1 VLPs is unclear but was observed with both 2/6/7- and 2/4/6/7-VLPs. Each of the different VLP combinations also induced low levels of serum heterotypic neutralizing antibody to Wa, S2, SA11, NCDV, and WI61 (Table 1). Low titers of neutralizing antibody to OSU were induced in a few animals in each group, except in the G1 2/4/6/7-VLP immunized mice. The G1 VLPs, but not the G3 VLPs, induced serum antibodies that neutralized 69M.

Induction of heterotypic neutralizing antibodies by VLPs did not require VP4, but the presence of VP4 did increase the geometric mean neutralizing titers to homotypic and a subset of heterotypic viruses. Inclusion of the SA11 VP4 (P5B) in the G1 2/4/6/7-VLPs increased the neutralizing geometric mean titer (GMT) to Wa (P1A,G1), SA11 (P5B,G3), and 69M (P4,G8) up to 13-fold over that obtained with G1 2/6/7-VLPs. When we compared the heterotypic neutralizing antibody response induced by G1 2/6/7- and 2/4/6/7-VLPs against three P1A viruses [Wa (G1), VA70 (G4) and WI61 (G9)], the presence of VP4 resulted in changes in the GMT ranging from an eightfold increase to Wa to a twofold decrease to WI61; no heterotypic neutralizing antibody to VA70 was generated by either G serotype of the 2/6/7- or 2/4/6/7-VLPs.

Because a rise in homotypic antibody titers and induction of heterotypic serum neutralizing antibody titers was seen following the third immunization of the G1 or G3 VLPs, we also determined if significant increases in serum ELISA antibody titers occurred following the third immunization. ELISA antibody titers significantly increased only in the group that received the G3 2/6/7-VLPs (Fig. 3, $P = 0.043$). Therefore, increases in neutralizing antibody occurred in the absence of increases in antibody titers measured by ELISA.

Fecal antibody responses in mice immunized with VLPs. We also examined fecal antibody responses in the mice immunized parenterally. Fecal samples were collected from the mice at 61 and 75 dpi (the day of the third inoculation and 14 days later). The total fecal antibody titers at 61 and 75 dpi and the neutralizing fecal antibody against SA11 (G3) virus at 75 dpi were determined (Table 2). At 61 dpi, 8 of 9 mice that received the G1 VLPs but only 5 of 10 mice that received the G3 VLPs had detectable total fecal antibodies. The third immunization boosted the total fecal GMT in all the groups except the G1 2/6/7-VLP immunized mice, but not significantly ($P > 0.5$). Due to the small amount of fecal sample collected from individual mice, fecal samples were pooled for each group and tested by PRN for neutralizing antibody against SA11 only; testing against other serotypes was not possible. Homotypic and heterotypic neutralizing antibody was detected for the G3 and G1 VLP-inoculated groups, respectively. Neutralizing an-

TABLE 1. Induction of broadly heterotypic serum neutralizing antibody in mice immunized parenterally with VLPs

Inoculum	P serotype	G serotype	Neutralizing GMT[a] (range) of sera against indicated test virus (P[b] G type)							
			Wa (P1A,G1)	S2 (P1B,G2)	SA11 (P5B,G3)	VA70 (P1A,G4)	OSU (P9,G5)	NCDV (P6,G6)	69M (P4,G8)	WI61 (P1A,G9)
2/6/7-VLPs[c]	NA[d]	1	12,800 (6,400–25,600)	800 (800)	606 (<100–3,200)	50 (<100)	57 (<100–100)	800 (400–1,600)	459 (<100–800)	1,600 (800–3,200)
2/4/6/7-VLPs[c]	5B	1	102,400 (102,400)	174 (100–400)	1,838 (1,600–3,200)	50 (<100)	50 (<100)	400 (400)	6,400 (6,400)	696 (400–800)
2/6/7-VLPs[c]	NA	3	919 (800–1,600)	459 (400–800)	102,400 (102,400)	50 (<100)	87 (<100–200)	1,056 (800–1,600)	50 (<100)	528 (400–800)
2/4/6/7-VLPs[c]	5B	3	919 (800–1,600)	919 (400–1,600)	58,813 (51,200–102,400)	50 (<100)	87 (<100–200)	919 (400–1,600)	50 (<100)	919 (800–1,600)
2/4/6/7-VLPs[e]	5B	1/3	3,200 (800–6,400)	459 (200–800)	7,352 (1,600–12,800)	87 (<100–200)	76 (<100–400)	1,838 (1,600–3,200)	50 (<100)	1,213 (800–1,600)

[a] For titers of <100, 50 was used to calculate the GMT. A GMT of 50 is considered negative.
[b] P serotype is indicated (18).
[c] Serum neutralizing antibody titers measured by FFN in sera collected 114 dpi from five mice/group immunized three times with VLPs with Freund's adjuvant.
[d] NA, not applicable.
[e] Serum neutralizing antibody titers measured by FFN in sera collected 42 dpi in five mice/group immunized twice with chimeric G1/G3 2/4/6/7-VLPs with Freund's adjuvant.

TABLE 2. Induction of mucosal heterotypic neutralizing antibody by G1 VLPs

VLP	P serotype	G serotype	Fecal antibody titer[a] (range)		
			ELISA GMT[b] (range)		NAb at 75 dpi[c]
			61 dpi	75 dpi	
2/6/7	NA[d]	1	30 (<10–80)	30 (<10–160)	200
2/4/6/7	5B	1	24[e] (10–160)	40 (20–80)	100
2/6/7	NA	3	8 (<10–20)	34[e] (10–160)	100
2/4/6/7	5B	3	17 (<10–40)	28[e] (<10–80)	100

[a] Five mice/group were immunized parenterally 0, 14, and 61 dpi with the indicated VLPs with Freund's adjuvant. Fecal samples were collected 61 and 75 dpi and assayed for total antibody (IgA, IgM, and IgG) by ELISA. Nonimmunized mice housed in the same room remained serum and fecal antibody negative throughout the experiment.
[b] For titers of <10, 5 was used to calculate the GMT. A GMT of 5 was considered negative.
[c] The 75-dpi fecal samples for each group were pooled and tested by PRN for neutralizing antibody (NAb) against G3 SA11.
[d] NA, not applicable.
[e] The antibody titer of one mouse was not tested due to insufficient fecal sample.

tibody titers were similar in magnitude to ELISA titers. Similar responses in fecal neutralizing antibody and ELISA titers have been observed previously in rabbits (11, 13).

Induction of heterotypic serum neutralizing antibody in mice immunized with chimeric VLPs. FFN also were performed on sera collected from mice immunized twice with 5 μg of chimeric 2/4/6/7-VLPs, VLPs containing both G1 and G3 VLPs on each particle (16). We previously reported the serum neutralization titers at 28 dpi; we now report the results on sera collected 2 weeks later (42 dpi). Sera from mice vaccinated with chimeric G1/G3 VLPs neutralized Wa and SA11 but not S2 and VA70 viruses at 28 dpi (16); in contrast, by 42 dpi, mice developed low titers of neutralizing antibody to S2, NCDV, and WI61 viruses (Table 1). Low titers of neutralizing antibody to VA70 also were induced in a few animals (2/5) immunized with chimeric VLPs but not with any of the other VLPs. The heterotypic neutralizing responses to the various test viruses induced by two doses of the chimeric VLPs generally were intermediate between the titers generated by the G1 and G3 2/4/6/7-VLPs (Table 1). A significant increase in ELISA antibody titers was seen following two doses of chimeric G1/3 VLPs between serum samples collected at 28 and 42 dpi (Fig. 3, $P = 0.034$).

Induction of homotypic neutralizing antibody responses to G1 and G3 viruses in rabbits immunized twice with VLPs and orally challenged. To determine if heterotypic neutralizing serum and fecal antibodies could be induced by VLPs in another animal model and to compare the immune response generated with Freund's adjuvant and QS-21, rabbits were immunized twice with VLPs, SA11, or buffer in each adjuvant. For the G3 VLPs, protective efficacy and the total serum and fecal antibody responses are reported elsewhere (7). First, we examined homotypic neutralizing antibody responses against G1 and G3 viruses. We have noted in previous reports that higher serum neutralizing antibody responses are detected when the immunizing and virus test strain are the same (7, 11, 13). Therefore, the magnitude of G1 neutralizing antibody titers are likely underestimated in this report because the titers were determined against Wa, not HRV8697, which has not been adapted to growth in vitro. Serum collected 0 dpi, 56 dpi (0 dpc), and 28 dpc were tested for serum neutralizing antibody against SA11, ALA, and Wa (Table 3; data for 0 and 28 dpc shown). All VLPs and virus induced homotypic neutralizing antibody re-

FIG. 3. Serum antibody responses in mice immunized parenterally with the indicated VLPs in Freund's adjuvant. The serum antirotavirus antibody titers were assayed by ELISA (five mice per group); the GMTs determined for mice twice immunized 28 dpi (solid bar) and 42 dpi (hatched bar) or three times immunized 114 dpi (white bar) are compared. Statistical increases ($P < 0.05$) in GMTs in each of the groups are indicated by the same symbol (■ or ◆). The error bars represent 1 standard error of the mean.

sponses before challenge. The homotypic serum neutralizing antibody titers were up to 21-fold higher in rabbits immunized with G3 VLPs in Freund's than with G3 VLPs or SA11 in QS-21. G1 2/4/6/7-VLPs in QS-21 generated neutralizing antibody titers to ALA and SA11 which were approximately 10- to 20-fold lower, respectively, than those induced by G3 VLPs in QS-21. In contrast, the rabbits sequentially inoculated with G3 and G1 2/4/6/7-VLPs in QS-21 generated high titers of neutralizing antibodies to SA11, ALA, and Wa.

Following challenge, slight increases in neutralizing antibody GMTs were seen but did not exceed fourfold in rabbits immunized with 2/6/7- or 2/4/6/7-VLPs given in Freund's adjuvant (Table 3); fourfold increases were observed in four of nine rabbits immunized with 2/6/7-VLPs given in Freund's adjuvant. Although anamnestic mean serum neutralizing immune responses to SA11 and ALA were not observed in the rabbits immunized with 2/6/7-VLPs in Freund's adjuvant, all other mean serum and fecal antibody responses showed an anamnestic response after challenge (7). The lack of antibody boost was likely due to a near-maximal induction of high levels of serum antibody to rotavirus (7). In rabbits inoculated with SA11 in QS-21, a fourfold increase against ALA but not SA11 and low titers against Wa of neutralizing antibody were induced. In contrast, in rabbits vaccinated with VLPs in QS-21, ≥4-fold increases were seen in the neutralizing antibody titers against Wa, SA11, and ALA.

Induction of heterotypic neutralizing antibody responses in rabbits immunized twice with VLPs and challenged orally. The heterotypic serum neutralizing antibody responses to seven non-G1 and non-G3 viruses with six different P serotypes were also determined before and after challenge (Table 4). As was seen in the mice, immunization of rabbits with G3 VLPs in Freund's adjuvant induced variable but generally low levels of neutralizing antibodies to all the heterotypic viruses tested. Low levels of antibodies that neutralized three to five heterotypic viruses also were induced by VLPs administered in QS-21 (Table 4). Sequential immunization with G3 and G1 2/4/6/7-VLPs with QS-21 induced antibodies that neutralized all of the test viruses except B223. However, SA11 in QS-21 adjuvant induced heterotypic antibodies that neutralized WI61 and B223 only. Based on neutralizing antibody responses to

TABLE 3. Serum neutralizing antibody responses to G3 and G1 viruses in rabbits immunized parenterally with VLPs before and after oral challenge with G3 ALA rotavirus

Inoculum	Adjuvant	P serotype	G serotype	Neutralizing GMT[a] (range) of sera against indicated test virus (P,[b] G type)						No. of rabbits	% Protection (range)
				SA11 (P5B,G3)		ALA (P[14],G3)		Wa (P1A,G1)			
				0 dpc	28 dpc	0 dpc	28 dpc	0 dpc	28 dpc		
2/4/6/7-VLPs	Freund's	5B	3	77,605[d] (25,600–204,800)	77,605 (25,600–204,800)	16,890[d] (12,800–51,200)	19,401 (12,800–51,200)	1,056 (800–1,600)	2,111 (1,600–3,200)	5	89[d] (68–100)
2/6/7-VLPs	Freund's	NA[c]	3	75,250[d] (25,600–204,800)	87,782 (51,200–102,400)	10,973[d] (3,200–51,200)	21,945 (1,600–51,200)	126 (<100–400)	467 (<100–3,200)	9	58[d] (42–100)
2/4/6/7-VLPs	QS-21	5B	3	4,222[d] (1,600–12,800)	**72,408** (12,800–204,800)	1,213[d] (400–6,400)	**63,035** (12,800–102,400)	50 (<100)	**1,393** (200–6,400)	10	56[d] (31–98)
2/4/6/7-VLPs	QS-21	5B	1	200 (<100–400)	**9,701** (3,200–12,800)	100 (<100–400)	**44,572** (12,800–102,400)	1,600 (800–3,200)	**51,200** (25,600–102,400)	5	32 (11–52)
2/4/6/7-VLPs	QS-21	5B	3 + 1[e]	9,051 (6,400–12,800)	**86,108** (51,200–102,400)	566 (400–800)	**51,200** (51,200–102,400)	1,345 (800–1,600)	**60,887** (51,200–102,400)	4	46 (36–73)
2/6/7-VLPs	QS-21	NA	3	4,222[d] (1,600–12,800)	**63,035** (12,800–102,400)	1,493[d] (400–3,200)	**72,408** (51,200–102,400)	50 (<100)	**800** (<100–3,200)	10	26[d] (0–44)
SA11	QS-21	5B	3	3,553[d] (3,200–6,400)	11,593 (6,400–51,200)	1,767[d] (800–3,200)	**8,604** (3,200–25,600)	50 (<100)	181 (<100–400)	7	83[d] (65–100)
Buffer	QS-21	NA	NA	50[d] (<100)	**883** (800–1,600)	50[d] (<100)	**10,159** (6,400–51,200)	50 (<100)	50 (<100)	7	0[d]

[a] Serum neutralizing antibody measured by FFN in sera collected 0 dpc (56 dpi) and 28 dpc from rabbits immunized twice with VLPs with Freund's adjuvant or 20 μg of QS-21, as indicated. For titers of <100, 50 was used to calculate the GMT. A GMT of 50 is considered negative. Boldface indicates fourfold GMT increase in serum neutralizing antibody titers following challenge.

[b] P serotype is indicated where known (18). If P serotype has not been determined, P genotype (in brackets) is indicated.

[c] NA, not applicable.

[d] Protection and serum neutralizing antibody titers reported by Ciarlet et al. (7).

[e] Rabbits were immunized sequentially with G3 2/4/6/7-VLPs and G1 2/4/6/7-VLPs as the first and second doses of immunogen, respectively.

TABLE 4. Serum neutralizing antibody responses to non-G1 and non-G3 rotaviruses in rabbits immunized parenterally with G1 and G3 VLPs and challenged orally with G3 ALA rotavirus

Inoculum	Adjuvant	P serotype	G serotype	Neutralizing GMT[a] (range) of sera against indicated test virus (P,G type) collected 0 and 28 dpc					
				S2 (P1B,G2)		VA70 (P1A,G4)		OSU (P9,G5)	
				0 dpc	28 dpc	0 dpc	28 dpc	0 dpc	28 dpc
2/4/6/7-VLPs	Freund's	5B	3	132 (<100–400)	264 (<100–800)	66 (<100–200)	192 (<100–800)	100 (100–800)	348 (100–800)
2/6/7-VLPs	Freund's	NA[c]	3	73 (<100–400)	159 (100–400)	54 (<100–100)	**294** (<100–1,600)	63 (<100–400)	**343** (<100–1,600)
2/4/6/7-VLPs	QS-21	5B	3	93 (<100–200)	**459** (100–800)	50 (<100)	**1,600** (200–6,400)	57 (<100–200)	**696** (100–3,200)
2/4/6/7-VLPs	QS-21	5B	1	100 (100)	**606** (200–1,600)	50 (<100)	**800** (200–1,600)	50 (<100)	174 (<100–800)
2/4/6/7-VLPs	QS-21	5B	3 + 1[d]	59 (<100–100)	**476** (400–800)	84 (<100–200)	**2,263** (1,600–3,200)	100 (<100–200)	**476** (200–800)
2/6/7-VLPs	QS-21	NA	3	50 (<100)	141 (<100–400)	57 (<100–200)	**857** (100–3,200)	50 (<100)	**373** (100–1,600)
SA11	QS-21	5B	3	50 (<100)	**200** (<100–400)	50 (<100)	74 (<100–100)	50 (<100)	50 (<100)
Buffer	QS-21	NA	NA	ND	50 (<100)	ND	50 (<100)	ND	50 (<100)

[a] Serum neutralizing antibody measured by FFN in sera collected 0 dpc (56 dpi) and 28 dpc from rabbits immunized twice with VLPs with Freund's adjuvant or 20 μg of QS-21, as indicated. For titers of <100, 50 was used to calculate the GMT. A GMT of 50 is considered negative. Boldface indicates fourfold GMT increase in serum neutralizing antibody titers following challenge.
[b] Reported previously by Ciarlet et al. (7).
[c] NA, not applicable.
[d] Rabbits were immunized sequentially with G3 2/4/6/7-VLPs and G1 2/4/6/7-VLPs as the first and second doses of immunogen, respectively.

non-G1 and non-G3 viruses, immunization of rabbits with G1 and G3 VLPs primed for neutralizing antibody responses to a broader range of heterotypic viruses than SA11 virus.

Following a G3 ALA rotavirus challenge, we determined whether the heterotypic neutralizing antibody responses were significantly enhanced (≥4-fold increase). Significant increases in neutralizing antibody GMTs were observed in only a few of the rabbits immunized with either G3 2/4/6/7-VLPs (69M) or G3 2/6/7-VLPs (VA70 or OSU) with Freund's adjuvant (Table 4). However, significant increases in neutralizing antibody titers were observed in most rabbits immunized with VLPs in QS-21. These increases were against seven, six, or four of seven test viruses in sera from rabbits immunized with G3 2/4/6/7-VLPs or immunized sequentially with G3 and G1 2/4/6/87-VLPs, G1 2/4/6/7-VLPs, or G3 2/6/7-VLPs, respectively. In contrast, virus challenge of rabbits immunized with SA11 with QS-21 failed to induce broadly reactive anamnestic neutralizing antibody responses; significant increases in neutralizing antibody GMTs were observed against only two test viruses (S2 and 69M). Taken together, these data indicate that VLPs prime the immune system against a broad range of heterotypic viruses and that subsequent homotypic or heterotypic virus challenge induces anamnestic heterotypic neutralizing antibody responses.

Immune response and protection from challenge of rabbits parenterally immunized twice with G1 or G3/G1 2/4/6/7-VLPs in QS-21. To determine if VLPs containing a heterotypic G1 VP7 could induce protective immunity against a G3 challenge, we also examined the total serum and intestinal immune responses and protective efficacy of G1 or sequentially administered G3 and G1 2/4/6/7-VLPs in rabbits immunized parenterally. These data were compared with the immune response of rabbits immunized parenterally with SA11 or buffer (Fig. 4) and with previously determined protective efficacy (7) of rabbits immunized parenterally with SA11, buffer, G3 2/6/7-VLPs, or 2/4/6/7-VLPs (Tables 3 and 4).

Before challenge, the levels of total serum and fecal IgG antibodies of rabbits immunized with G1 or sequentially with G3 and G1 2/4/6/7-VLPs were similar ($P > 0.05$), but were significantly lower than the titers induced by SA11 ($P < 0.02$) (Fig. 4A and B). As expected based on previous results (7, 11), little or no fecal IgA antibodies were detected for any of the groups before challenge (Fig. 4C). Following oral ALA (G3) challenge, no significant antibody rises ($P = 0.066$) were seen in rabbits vaccinated sequentially with G3 and G1 2/4/6/7-VLPs (Fig. 4), but all antibodies increased significantly in rabbits vaccinated with G1 2/4/6/7-VLPs or SA11 ($P < 0.05$).

Virus reassortants with different VP4-VP7 combinations were used to measure the VP4-specific immune response to SA11 (7). Sequential immunization with the G3 and G1 VLPs or immunization with G1 VLPs induced VP4-specific neutralizing antibody against SA11 (GMT = 168 [range, 50 to 400] or 152 [range, 50 to 800], respectively [data not shown]). VP4-specific neutralizing antibody responses to ALA virus were not measured because appropriate virus reassortants are not available.

Following challenge, similar levels of protection were achieved by two immunizations with G1 2/4/6/7-VLPs (32%) or by sequential immunization with G3 and G1 2/4/6/7-VLPs (46%) in QS-21 ($P = 0.325$). The mean protective efficacy afforded by the G1 or G3/G1 VLPs were compared to the protection efficacy afforded by the G3 2/4/6/7-VLPs or by G3 2/6/7-VLPs obtained previously (7). The protection afforded by the G1 2/4/6/7-VLPs (32%) was similar to protection afforded by either G3 2/4/6/7-VLPs (56%) or G3 2/6/7-VLPs (26%) (7) ($P = 0.115$ or <0.623, respectively). Sequential immunization with G3 and G1 2/4/6/7-VLPs afforded similar levels of protection (46%) as two immunizations with G3 2/4/6/7-VLPs (56%, $P = 0.202$) and significantly greater protection than immunization with G3 2/6/7-VLPs (26%, $P = 0.046$).

Correlates of protection from infection were calculated with results from a series of experiments reported here and previously (7). Previously we only examined a correlation with serum neutralizing antibody to SA11 VP4 (7). The analyses were

TABLE 4—*Continued*

Neutralizing GMT[a] (range) of sera against indicated test virus (P,G type) collected 0 and 28 dpc								% Protection (range)
NCDV (P6,G6)		69M (P4,G8)		WI61 (P1A,G9)		B223 (P8,G10)		
0 dpc	28 dpc	0 dpc	28 dpc	0 dpc	28 dpc	0 dpc	28 dpc	
115 (100–200)	264 (200–400)	348 (200–800)	**1,600** (800–6,400)	2,425 (1,600–6,400)	3,676 (1,600–6,400)	528[b] (400–800)	696 (400–1,600)	89[b] (68–100)
50 (<100)	86 (<100–400)	50 (<100)	171 (100–800)	2,016 (400–6,400)	5,486 (1,600–25,600)	147[b] (<100–800)	317 (<100–1,600)	58[b] (42–100)
50 (<100)	**606** (<100–3,200)	100 (<100–200)	**2,986** (400–12,800)	303 (100–800)	**5,198** (1,600–12,800)	54[b] (<100–100)	**373** (100–800)	56[b] (31–98)
50 (<100)	**606** (100–3,200)	76 (<100–200)	**1,393** (200–6,400)	66 (<100–100)	**800** (400–3,200)	50 (<100)	**230** (100–800)	32 (11–52)
119 (<100–200)	**951** (200–3,200)	200 (100–400)	**2,691** (1,600–3,200)	673 (400–1,600)	**6,400** (3,200–12,800)	50 (<100)	**400** (200–800)	46 (36–73)
50 (<100)	132 (<100–400)	54 (<100–100)	**230** (100–400)	115 (<100–400)	**1,493** (400–6,400)	57[b] (<100–100)	174 (<100–800)	26[b] (0–44)
50 (<100)	100 (<100–200)	50 (<100)	**362** (<100–1,600)	55 (<100–100)	149 (<100–800)	61[b] (<100–100)	100 (<100–200)	83[b] (65–100)
ND	50 (<100)	ND	50 (<100)	ND	159 (100–400)	ND	50 (<100)	0[b]

performed on rabbits immunized with SA11, VLPs (G3, G3/G1, or G1 2/4/6/7-VLPs; G3 2/6/7-VLPs; 2/6-VLPs), or buffer. When results with both QS-21 and Freund's adjuvant were analyzed together, the degree of protection from ALA challenge correlated significantly with the level of serum neutralizing antibody against SA11 ($r = 0.415$, $P = 0.001$), VP4-specific serum neutralizing antibody to SA11 ($r = 0.55$, $P < 0.001$), total serum antibody ($r = 0.532$, $P < 0.001$), or fecal IgG antibody ($r = 0.48$, $P < 0.001$) (Pearson's correlation coefficient). When results with QS-21 and Freund's adjuvant were analyzed separately, significant correlations for each of these immune responses were also observed (data not shown).

DISCUSSION

This is the first report of the induction of heterotypic protection in rabbits by G1 VLPs against a G3 virus challenge. Because preliminary data suggested that VLPs containing a human G1 VP7 induced heterotypic neutralizing antibody, the immunogenicity and protective efficacy of G1 2/4/6/7-VLPs administered with QS-21 were compared to those of G3 2/4/6/7-VLPs, G3/G1 2/4/6/7-VLPs, and virus in the rabbit model. Similar levels of protection were seen in the VLP-immunized rabbits, indicating that G1 2/4/6/7-VLPs induced protection from a heterologous live rotavirus challenge. Protection induced by VLPs was assessed by measuring virus antigen shedding detected by ELISA following challenge. We have previously shown a strong correlation between the amount of virus antigen shedding and infectious virus shedding (7). Based on this correlation, the level of protection induced by the G1 VLPs with QS-21 indicates that the amount of infectious virus shed by these rabbits was approximately 2 logs lower than that for control rabbits. It is difficult to predict how the reduced shedding relates to protection from rotavirus diarrhea because comparable measurements of the amount of virus shedding have not been reported for naturally infected children or for any disease animal model. Determination of whether the reduction of rotavirus shedding induced by VLPs in QS-21 will be sufficient to decrease the severity of rotavirus disease in humans awaits clinical trials.

The rabbits immunized with the G1 VLPs had serum neutralizing antibodies against both G1 and G3 viruses. The induction of cross-reactive neutralizing antibody may be common among human G1 rotavirus strains since primary infections with G1 human rotaviruses frequently induce heterotypic neutralizing antibody responses to both G1 and G3 viruses, whereas G3 human rotaviruses induce a homotypic anti-G3 response (15, 35). Whether the protection observed in the rabbits immunized with G1 VLPs was due to the induction of neutralizing antibodies to G3 virus or heterotypic neutralizing antibody is unclear. Heterotypic protection has been observed in a subset of rabbits infected with non-G3 viruses and challenged with G3 ALA virus in the absence of detectable neutralizing antibody to G3 viruses, SA11, or ALA (8). For VLPs, assessment of heterotypic protection based on non-G3 neutralizing antibodies will require production of and immunization with VLPs that do not induce G3 cross-reactive neutralizing antibody, followed by challenge with G3 virus.

When we examined immune correlates of protection in rabbits immunized with VLPs or SA11 in QS-21 or Freund's adjuvant separately or together (this report and reference 7), the degree of protection from ALA challenge correlated significantly with the level of total serum antibody, fecal IgG antibody and serum neutralizing antibody against SA11 or SA11 VP4. In this and previous studies in rabbits, the highest levels of protection were obtained with VLPs containing VP4 (7). While VP4 and VP7 play a role in protection in rabbits, the induction of serum neutralizing antibody to these two outer capsid proteins is not the sole determinant of protection. High levels of protection (80 to 100% in Freund's adjuvant) were observed in four or two rabbits immunized with VLPs lacking VP4 or lacking VP4 and VP7, respectively (7). Studies with VLPs in mice and rabbits indicate that multiple rotavirus proteins, both neutralization and nonneutralization antigens, induce protection (7, 26, 33, 37). Therefore, the conflicting reports for the need of neutralizing antibody in numerous studies in humans and in animal models may represent biological differences in how the immune response is stimulated. Understanding these differences will require further study.

Correlates of protection from rotavirus infections have not been completely defined, but neutralizing antibody responses have been implicated in many studies, including this report. We report for the first time the induction of neutralizing antibodies in mice and rabbits by VLPs to a number of hetero-

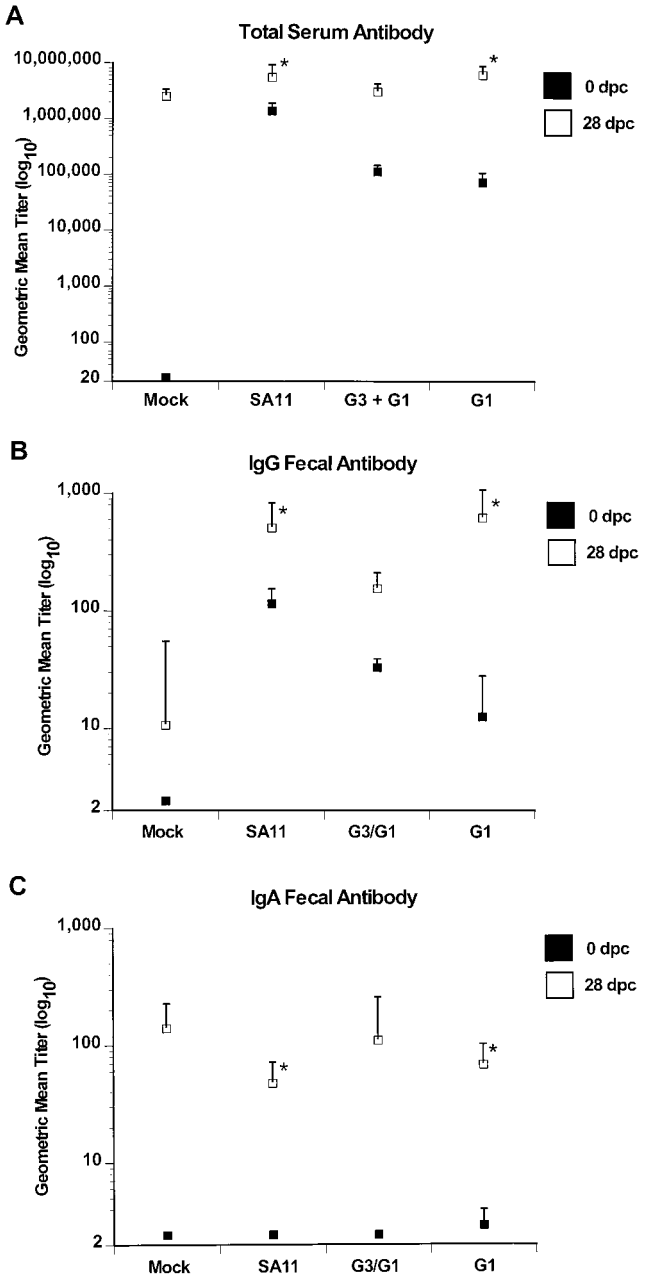

FIG. 4. Serum and fecal antibody responses in rabbits immunized twice parenterally with buffer (mock), SA11, G3 and G1 2/4/6/7-VLPs sequentially, or G1 2/4/6/7-VLPs and challenged orally with ALA (G3). Prechallenge (■) and postchallenge (□) serum and fecal samples were assayed by ELISA for total serum antirotavirus antibody (A), rotavirus-specific IgG fecal antibody (B), or rotavirus-specific IgA fecal antibody (C). The error bars represent 1 standard error of the mean. The GMTs of the rabbits immunized sequentially with the G3 and G1 or the G1 2/4/6/7-VLPs are compared to the GMTs for mock- and SA11-immunized animals as previously reported (7). Significant increases in GMT postchallenge are indicated by * ($P < 0.05$).

typic viruses. Generation of heterotypic neutralizing antibody following oral or parenteral immunization with a subset of rotavirus strains has been previously reported in several animal species or following natural infection in children (2, 13, 15, 44). The magnitude of the heterotypic neutralizing antibody responses induced by VLPs are similar to the titers induced by natural or experimental virus infection (13, 35, 44). Development of heterotypic neutralizing immune responses following primary infection in children may be a mechanism by which subsequent infections with different serotypes are less severe; after a second rotavirus infection, children generally do not suffer moderate to severe diarrhea (45). Similarly, the ability of VLPs to induce neutralizing antibody to a broad range of heterotypic viruses may provide sufficient priming of the immune system to induce an anamnestic immune response capable of reducing the severity of infection upon subsequent rotavirus exposure.

In mice, two doses of G1 VLPs induced neutralizing antibodies to G1 and G3 viruses, whereas two doses of G3 VLPs induced only neutralizing antibodies to G3 virus. However, a broad heterotypic neutralizing antibody response was induced following a third immunization with either G1 or G3 VLPs. Two doses of chimeric VLPs induced heterotypic neutralizing antibody, but the heterotypic response was delayed compared to homotypic neutralizing responses. These results suggest that heterotypic neutralizing antibody responses develop more slowly and are not absolutely dependent on more than two immunizations.

Heterotypic fecal neutralizing antibody was induced in mice following a third immunization with G1 VLPs. Although the data are limited in this report, the neutralizing antibody titers in pooled fecal samples from mice were similar in magnitude to the ELISA fecal antibody titers. Similar results have been observed in rabbits either orally infected with lapine ALA rotavirus or immunized parenterally with live or inactivated SA11 rotavirus; fold differences between fecal ELISA and neutralizing antibody titers are low (two- to fourfold) (11, 13, 14). In some rabbits, neutralizing antibody titers exceeded ELISA antibody titers. In contrast, in the same rabbits, serum ELISA titers exceeded neutralizing antibody titers greatly (10- to 60-fold). The reason for the differences in the ratios of neutralizing and ELISA antibody titers between serum and fecal samples is not known, but the results in rabbits indicate that the majority of antibodies in fecal samples are neutralizing. The data for mice reported in this paper are limited but support this novel finding that the majority of antibodies in fecal samples are neutralizing. This relationship in comparative ELISA and neutralizing antibody titers in fecal samples may not be well recognized because most prior reports have not measured fecal neutralizing antibody titers.

In rabbits, two doses of all VLP formulations induced heterotypic neutralizing antibody responses. Of interest, following live virus challenge, the heterotypic neutralizing antibody responses significantly (≥4-fold) increased and broadened in VLP-immunized rabbits; this response was not seen in SA11-immunized rabbits. Therefore, VLPs prime for more broadly heterotypic immune responses than either live or inactivated SA11 virus. The structure of VLPs is similar to the native virus conformation, but subtle differences between virus and VLPs may be responsible for the observed different abilities of these immunogens to induce heterotypic neutralizing antibody. Differences in conformation between VLPs and virus may (i) alter antigen processing, which may occur differently or more efficiently with VLPs than with virus or (ii) expose broadly cross-reactive neutralization epitopes on VLPs but not virus. In either case, the induction of neutralizing antibody against a broad range of heterotypic viruses is more efficient with VLPs than with virus. In support of this hypothesis, the induction of broadly heterotypic neutralizing antibodies to human immunodeficiency virus (HIV) has been suggested to result by exposure of epitopes on HIV gp41 or gp120 following fusion with cells compared to immunization with native virus (31). An alternative possibility for the failure of virus challenge to boost

heterotypic neutralizing antibody in the SA11-inoculated rabbits is that prior SA11 immunization prevented replication of the challenge virus, effectively blocking antigen presentation. This possibility seems unlikely because virus replication was not totally blocked (65 to 77% protection) in several SA11-immunized rabbits, yet these rabbits failed to develop broadly reactive neutralizing antibody responses. In contrast, a rabbit that was immunized with G3 2/4/6/7-VLPs and had little virus replication after challenge (98% protection) developed broadly heterotypic neutralizing (>8-fold increases) antibody responses to all seven test viruses.

Our results for both mice and rabbits immunized with VLPs containing different protein formulations suggest the following. (i) VP7 alone can induce heterotypic neutralizing antibody. (ii) VP4 can influence the response. (iii) The combination of different VP4s and VP7s may affect induction of heterotypic neutralizing antibody. (iv) The induction of heterotypic neutralizing antibody may be variable in different animal species. (v) Neutralizing antibody to VP4 or VP7 is one, but not the only, correlate of protection. (vi) The protective efficacy of VLPs may depend on the adjuvant. (vii) VLPs induce homotypic and heterotypic protection from virus challenge.

The goals of a rotavirus vaccine are to target all children in the world to reduce severe diarrhea associated with rotavirus (6). Although three doses of the live, attenuated tetravalent vaccine have been shown to induce 48 to 80% efficacy against all rotavirus disease and 70 to 95% efficacy against severe disease in the United States, Finland, and Venezuela, improved second-generation vaccines may be needed for other countries (6). In less developed countries, there is greater virus strain diversity, infections with multiple rotavirus strains (up to 20 to 30% of infections) occur commonly, and mixed enteric infections occur that may act synergistically. Recent reports indicate that the common worldwide serotypes (G1 to G4) that are included in the tetravalent vaccine are underrepresented in India, Indonesia, and Brazil, where G10, G9, or G5 strains comprise a significant percentage of the circulating rotaviruses (22, 40). In the United States, G9 rotavirus comprise 6 to 45% of isolates from multiple cities (41). In such settings, the ability to create VLPs with different G or P types and to induce broadly heterotypic immune responses by a VLP vaccine may be particularly advantageous, especially if heterotypic anamnestic immune responses induced by subsequent rotavirus infection are capable of reducing the severity of disease. The SA11 P5B VP4 used in the VLPs in our studies is the same P serotype as the VP4 in the live, attenuated tetravalent vaccine (24). A more relevant P type for VLP vaccine formulation for humans might be P1A because many human rotaviruses (serotypes G1, G3, G4, G5, and G9) possess this VP4 serotype.

Rotavirus vaccines are targeted for use in young children, so safety issues are paramount. VLP vaccines offer several safety advantages. First, the inability of VLPs to replicate and reassort is an important consideration since the live, attenuated tetravalent vaccine viruses were detected in stools of both vaccinated children and placebo recipients with diarrhea illnesses in Venezuela during recent vaccine trials (39). Even though the attenuated vaccine virus was detected only by PCR and was present in low concentrations, its presence in conjunction with other rotaviruses highlights the potential for vaccine transmission that could lead to reassortment and possibly an increase in virulence. Second, in an immunocompromised host, a nonreplicating VLP vaccine administered parenterally could provide an immediate immunogenic stimulus that might not be subjected to interference by infection with HIV or other virulent viruses to which infants are exposed in developing countries. This has been the rationale for the combined parenteral-oral poliovirus vaccination protocol now recommended for use in the United States and may serve as a model for strategies to increase protection obtained with the oral, live, attenuated tetravalent vaccine alone. Alternatively, VLPs administered intranasally induce high serum and intestinal immune responses and require low immunizing doses with adjuvants to obtain protection (37). VLPs may be a good primary vaccine since they are stable for at least 5 years, are highly immunogenic, and induce homotypic and prime for heterotypic neutralizing antibodies, and parenteral or mucosal administration in adjuvants being tested in humans stimulates mucosal antibody responses that are protective from live rotavirus challenge.

Both VLPs and inactivated virus can induce high levels of immunogenicity and protective efficacy in rabbits and mice and are potentially excellent vaccine candidates (7, 26, 33, 37). VLPs offer several advantages over inactivated or live rotavirus vaccines, including (i) the ability to create VLPs with different G or P types and only relevant antigens, (ii) no need to use toxic inactivating agents which may alter immunogenic epitopes, (iii) the ability to prime for neutralizing antibody to more heterotypic viruses than virus, (iv) no possibility of transmission or reassortment, (v) immediate early immunogenic stimulus not subjected to interfering or inhibiting factors, and (vi) safety. The efficacy of VLPs or inactivated virus to protect children from severe disease awaits clinical testing.

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