Subunit Rotavirus Vaccine Administered Parenterally to Rabbits Induces Active Protective Immunity

MAX CIARLET,1 SUE E. CRAWFORD,1 CHRISTOPHER BARONE,1 ANDREA BERTOLOTTI-CIARLET,1 ROBERT F. RAMIG,1 MARY K. ESTES,1 AND MARGARET E. CONNER1,2*

Division of Molecular Virology, Baylor College of Medicine,1 and Veterans Affairs Medical Center,2 Houston, Texas 77030

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Virus-like particles (VLPs) are being evaluated as a candidate rotavirus vaccine. The immunogenicity and protective efficacy of different formulations of VLPs administered parenterally to rabbits were tested. Two doses of VLPs (2/6-, G3 2/6/7-, or P[2], G3 2/4/6/7-VLPs) or SA11 simian rotavirus in Freund's adjuvants, QS-21 (saponin adjuvant), or aluminum phosphate (AlP) were administered. Serological and mucosal immune responses were evaluated in all vaccinated and control rabbits before and after oral challenge with 10^4 50% infective doses of live P[14], G3 ALA lapine rotavirus. All VLP- and SA11-vaccinated rabbits developed high levels of rotavirus-specific serum and intestinal immunoglobulin G (IgG) antibodies but not intestinal IgA antibodies. SA11 and 2/4/6/7-VLPs afforded similar but much higher mean levels of protection than 2/6/7- or 2/6-VLPs in QS-21. The presence of neutralizing antibodies to VP4 correlated (P < 0.001, r = 0.55; Pearson's correlation coefficient) with enhanced protection rates, suggesting that these antibodies are important for protection. Although the inclusion of VP4 resulted in higher mean protection levels, high levels of protection (97 to 100%) from infection were observed in individual rabbits immunized with 2/6/7- or 2/6-VLPs in Freund's adjuvants. Therefore, neither VP7 nor VP4 was absolutely required to achieve protection from infection in the rabbit model when Freund's adjuvant was used. Our results show that VLPs are immunogenic when administered parenterally to rabbits and that Freund's adjuvant is a better adjuvant than QS-21. The use of the rabbit model may help further our understanding of the critical rotavirus proteins needed to induce active protection. VLPs are a promising candidate for a parenterally administered subunit rotavirus vaccine.

Rotaviruses are the most common etiologic agents of acute viral gastroenteritis in young children throughout the world, and a worldwide effort is under way to design an effective vaccination strategy. In the United States, rotavirus infection is primarily a problem of morbidity and associated health care costs (48, 52), whereas in developing countries, mortality is high, with >870,000 deaths per year attributed to rotavirus (39). Live attenuated rotavirus vaccine candidates consisting of human-animal (simian or bovine) rotavirus reassortants were tested in children but showed variable effectiveness in different settings (20, 74). In recent trials, these vaccines provided approximately 70% effectiveness against severe diarrhea (16, 43, 44, 61, 69, 75, 76).

Rotaviruses belong to the Reoviridae family and are composed of three protein layers surrounding 11 segments of double-stranded RNA (32). The innermost layer is composed of VP1, VP2, VP3, and the genome, the middle layer is composed of VP6; and the outer layer is composed of the glycoprotein VP7 and spikes of VP4 dimers (32, 64, 70). VP4 and VP7 possess distinct antigenic activities, defining P serotypes and G serotypes, respectively. VP4 and VP7 independently elicit antibodies capable of neutralizing rotavirus infectivity and inducing protective immunity (32).

Rotavirus genes encoding the rotavirus structural proteins VP2, VP6, VP4, and VP7 have been cloned in baculovirus, and the recombinant rotavirus proteins have been coexpressed in the baculovirus expression system (26, 31, 47). Stable virus-like particles (VLPs) self-assemble following expression of VP2 alone (47). Coexpression of VP2 and VP6 alone or with VP4 results in the production of double-layered 2/6- or 2/4/6-VLPs, respectively (26, 47). Coexpression of VP2, VP6, and VP7, with or without VP4, results in triple-layered 2/6/7- or 2/4/6/7-VLPs (26). All VLPs maintained the structural and functional characteristics of native particles (26, 63, 65, 69), including binding to and internalization of 2/4/6/7-VLPs into MA104 cells (26, 28).

Models of rotavirus infection, without disease, were developed in rabbits (14, 17–19, 38, 73) and in adult mice (58, 79, 80) to monitor the development of active serum and mucosal immunity as well as protection from infection following a live rotavirus challenge. We demonstrated that parenteral vaccination with live or inactivated rotavirus induces active immunity and protection in the rabbit model (19). Preliminary results for rabbits showed that VLPs administered parenterally in Freund’s adjuvants and aluminum phosphate (AlP) were immunogenic and induced active protection from homologous serotype G3 oral rotavirus challenge (21, 23). Here, we report the immunogenicity and protective efficacy of parenterally administered VLPs of different compositions (2/6, 2/6/7, and 2/4/6/7) in rabbits, using different adjuvants: Freund’s, AlP, and QS-21. QS-21 has the advantage that it may be licensed for use in humans (46) and has been tested with VLPs in mice (23, 24, 42, 50).

**MATERIALS AND METHODS**

**Cells and viruses.** The lapine rotavirus ALA (P[14], G3) strain (11), used for rotavirus challenge inoculations or enzyme-linked immunosorbent assays (ELISAs) and fluorescent-focus neutralization assays (FFNAs), was passaged in the presence of trypsin 10 times in fetal rhesus monkey kidney MA104 cells and viruses.

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rotavirus reassortant viruses R-N33 (P[8][11], G3) and R-A32 (P[2], G10) used in FFNAs were propagated in MA104 cells with trypsin and plaque purified two or three times as described elsewhere (29). Original derivation and characterization of the SA11 Cl3 × B223 rotavirus reassortant viruses R-N33 and R-A32 were described previously (R-N33 [P[11], G3] contains genome segments 1 and 4 from B223 and the remainder of its genes from SA11 Cl3, and R-A32 [P[2], G10] contains genome segments 2 and 4 from SA11 Cl3 and all other genome segments from B223 (66, 67).

Based on the encoded full-length genes of bovine RVFV (V.26[2],4), simian SA11 Cl3 VP6 (pAc461/SA11-6 [30]), and simian SA11 segments from B223 (66, 67).

Preparation of rotavirus SA11 Cl3 virus. Live or formalin-inactivated SA11 Cl3-infected cell lysates were prepared as described elsewhere (19). Following formalin inactivation, SA11 Cl3 preparations were characterized by ELISA as described previously (26) with a panel of VP4- and VP7-specific neutralizing and nonneutralizing monoclonal antibodies (MAbs) to confirm that virus inactivation did not result in loss of epitope reactivity. SA11 Cl3 triple-layered particles (TLPs) were purified by 4-aminomethyltriioxalin-hydrochloride (psoralen; Lee Biomolecular Research Laboratories, Inc., San Diego, Calif.) (37). Before and after psoralen inactivation, SA11 Cl3 TLPs were (i) examined by electron microscopy to confirm that the TLPs resembled polyacylamide gels with silver nitrate following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) to determine purity; and (iii) following SDS-PAGE, analyzed by Western blotting (immunoblotting) using a mouse hyperimmune serum prepared against one of the parent viruses to determine protein composition as described previously (5, 26). Psoralen-inactivated CScl-purified SA11 Cl3 TLPs were not analyzed by ELISA with the panel of MAbs for epitope reactivity since psoralen inactivation of rotavirus results in antigenically intact particles (37). To determine whether the virus inactivation of both formalin- and psoralen-treated viruses was passage three times in cell culture; no cytopathic effect or plaques were seen, indicating that inactivation was complete. Complete virus inactivation was more readily obtained with psoralen than with formalin inactivation; in the later studies with CScl-purified virus, psoralen inactivation was used.

Production and characterization of VLPs. S9 cells were coinfected with the baculovirus recombinants as described previously (26) to produce 2/6, (G3) 2/6/7, and (P[2], G3) 2/6/7-VLPs. Therefore, 2/67- or 2/6/7-VLPs contained a VP7 of serotype G3 specificity, while 2/4/6/7-VLPs contained a VP7 of serotype G3 specificity, when 2/4/6/7-VLPs were examined by Western blotting using a mouse hyperimmune serum prepared by immunoneutralization with total VP7. Coated discs of polyclonal antibodies against SA11 polypeptides were used as the standard for the assay to be acceptable. Neutralization endpoints were expressed as the reciprocal of the highest serum dilution for each vaccine group.

Detection of infectious virus from fecal samples by FFA. The titers of infectious virus from selected fecal samples collected from 17 rabbits following ALA rotavirus challenge were measured by fluorescent-focus assay (FFA) (10, 12). The mean fecalidal suspension (10−4) was inoculated into MA104 cells and treated with psoralen than with formalin; therefore, in the later studies with CScl-purified virus, psoralen inactivation was used.

Production and characterization of VLPs. S9 cells were coinfected with the baculovirus recombinants as described previously (26) to produce 2/6, (G3) 2/6/7, and (P[2], G3) 2/6/7-VLPs. Therefore, 2/67- or 2/6/7-VLPs contained a VP7 of serotype G3 specificity, while 2/4/6/7-VLPs contained a VP7 of serotype G3 specificity, when 2/4/6/7-VLPs were examined by Western blotting using a mouse hyperimmune serum prepared by immunoneutralization with total VP7. Coated discs of polyclonal antibodies against SA11 polypeptides were used as the standard for the assay to be acceptable. Neutralization endpoints were expressed as the reciprocal of the highest serum dilution for each vaccine group.
RESULTS

Preparation and characterization of VLPs used for inoculation of rabbits. Coinfection of SF9 cells with the respective baculovirus recombinants resulted in the formation of 2/6-, 2/6/7-, and 2/4/6/7-VLPs. 2/6-VLPs resembled double-layered particles and 2/6/7- and 2/4/6/7-VLPs resembled rotavirus TLPs (data not shown). EM analysis revealed that the structural integrity of all VLP types was high (>90% [data not shown]). The total protein concentration of each VLP type was determined by the Bio-Rad protein assay. The endotoxin levels in the VLP preparations used for rabbit inoculations were quantitated at 0.037 EU/10 μg, 0.016 EU/20 μg, and 0.015 to 0.05 EU/50 μg. The purity and protein composition of all VLPs used in this study were confirmed by electrophoretic analysis and staining of gels with silver nitrate and by Western blot analysis (data not shown).

Immune response and protection from challenge of rabbits parenterally immunized with 10 or 20 μg of P[2], G3 2/4/6/7-VLPs in Freund’s adjuvants or absorbed to AlP. Two doses of live or formalin-inactivated simian rotavirus SA11 was previously shown to induce protection in rabbits (19). We next examined whether VLPs would afford the same protection and whether the adjuvant used would influence the immunogenicity or protective efficacy of the VLPs. In an initial experiment, rabbits were immunized twice parenterally (0 and 21 dpv) with either 10 or 20 μg of 2/4/6/7-VLPs either in Freund’s adjuvants (n = 5) or absorbed to AlP (n = 5). Control animals were immunized with either live or formalin-inactivated SA11-infected cell lysates (1.4 × 10⁷ PFU) either in Freund’s adjuvants (n = 3) or absorbed to AlP (n = 2). Negative control rabbits were immunized with MA104 cell lysates in either adjuvant.

Doses of 10 or 20 μg of 2/4/6/7-VLPs in Freund’s adjuvants or AlP were immunogenic (Fig. 1). VLPs in Freund’s adjuvants induced high levels of rotavirus-specific total Ig (IgM, IgG, and IgA) in serum (Fig. 1A). Compared to responses with Freund’s adjuvants, SA11 rotavirus or VLPs in AlP induced significantly lower titers of serum antirotavirus antibody (P < 0.049) (Fig. 1A). A dosage of 10 or 20 μg VLPs in both Freund’s adjuvants and AlP induced equivalent high levels of serum neutralizing antibodies (N-Abs) against homologous SA11 serotype G3 (geometric mean titers [GMTs] of 6,400 and 5,572, respectively [Table 1]). Only one rabbit, vaccinated with SA11 with Freund’s adjuvants, developed low levels of rotavirus-specific intestinal IgA following vaccination (Fig. 1B). IgG antirotavirus antibody was detected in the intestines of all rabbits after parenteral vaccination with VLPs or SA11 (Fig. 1C). The intestinal IgG titers were at least two- to eightfold lower in rabbits immunized with SA11 or VLPs in AlP than in rabbits immunized with VLPs in Freund’s adjuvants, although the titers were not statistically different (P = 0.063 and P = 0.059, respectively).

Since there was no significant difference (P = 0.623) in mean protective efficacy between rabbits vaccinated with either live (n = 2; 81%) or formalin-inactivated (n = 1; 100%) SA11-
infected cell lysates in Freund’s adjuvants and the number of animals was small, the two groups were combined (mean protective efficacy, 87%) to perform statistical analyses (Fig. 2). The mean protective efficacy of the rabbits (n = 2) vaccinated with formalin-inactivated SA11-infected cell lysates absorbed to AlP was 76% (Fig. 2) and was not significantly different from the protected animals in Freund’s adjuvants (71%). Although the mean protection achieved with VLPs in Freund’s adjuvants (36%) (Fig. 2), the difference was not significant (P < 0.05 Mann-Whitney U test). Bold face titers indicate that a significant (P < 0.05, Mann-Whitney U test) increase in rotavirus antibody GMTs was induced following challenge. ND, not done.

VLPs

- **VLPs**: 2/4/6/7
  - 10–20 μg AIP 5 25,600**A** 6**A** 2.5**A** 5,572**A** ND 36**AHDM**
  - 10–20 μg Freund’s 5 77,605**AE** 17**A** 2.5**A** 77,605**B** 16,890**B** 89**NO**
  - 50 μg Freund’s 5 713,550**EFG** 211**D** 2.5**A** 75,250**B** 10,973**B** 55**BC**
  - 2/6 50 μg Freund’s 5 378,929**BHF** 118**B** 2.5**A** 2,500**A** ND 83**AE**
  - 2/6 50 μg Freund’s 5 1,080,994**I** 366**D** 2.5**C** 50**C** 50**C** 41**BDEHIMNOP**
  - 2/6 50 μg QS-21 10 145,096**VE** 35**A** 2.5**A** 4,222**A** 1,213**A** 56**BH**
  - 2/6 50 μg QS-21 10 102,400**G** 25**A** 2.5**A** 4,222**A** 1,493**A** 26**GMKL**
  - 2/6 50 μg QS-21 5 155,200**VEH** 53**C** 2.5**A** 50**C** 50**C** 26**FM**
  - 200 μg QS-21 5 178,289**AIFH** 61**C** 2.5**A** 50**C** 50**C** 18**FJKM**
  - 50 μg QS-21 5 117,627**ME** 53**C** 2.5**A** 50**C** 50**C** 18**FJKM**

- **MA104 cells**
  - 0.5 ml AIP 1 255 2.5**E** 2.5**A** 50**C** 50**C** 0*KL*
  - 0.5 ml Freund’s 2 255 2.5**E** 2.5**A** 50**C** 50**C** 0*KL*

- **TNC buffer**
  - 0.5 ml QS-21 11 255 2.5**E** 2.5**A** 50**C** 50**C** 0*KL*

* GMTs of total serum, total intestinal, intestinal IgG, or intestinal IgA of rabbits vaccinated with SA11 rotavirus or 10 or 20 μg of VLPs in Freund’s adjuvants (0.109 ≤ P ≤ 0.593). In contrast, following challenge of rabbits immunized with VLPs in AIP, GMTs of total serum, total intestinal, intestinal IgG, or intestinal IgA were significantly higher (0.038 ≤ P ≤ 0.042). The levels of serum N-Abs against SA11 serotype G3 in rabbits vaccinated with 10 or 20 μg of VLPs in either Freund’s adjuvant or AlP did not increase significantly following challenge (P ≤ 0.180).

**Immune response and protection from challenge of rabbits parenterally immunized twice with 50 μg of P[2], G3 2/4/6/7- or G3 2/6/7-VLPs in Freund’s adjuvants or QS-21.**

We next examined whether increasing the dose of 2/4/6/7-VLPs would increase protective efficacy from ALA rotavirus challenge (103 ID50). Since the response with AlP was weaker than with Freund’s adjuvants or AlP did not increase significantly following challenge (P ≤ 0.180). We also examined whether QS-21, a saponin adjuvant, would be able to induce higher antibody responses and protection in rabbits. Following challenge, we also examined whether QS-21, a saponin adjuvant, would be able to induce higher antibody responses and protection in rabbits. Following challenge, we also examined whether QS-21, a saponin adjuvant, would be able to induce higher antibody responses and protection in rabbits. Following challenge, we also examined whether QS-21, a saponin adjuvant, would be able to induce higher antibody responses and protection in rabbits. Following challenge, we also examined whether QS-21, a saponin adjuvant, would be able to induce higher antibody responses and protection in rabbits.
ing formulation in QS-21 (Fig. 3). No significant difference in mean protection values among the groups that received either SA11’s adjuvants, QS-21, or AlP were not statistically different (P = 0.462 to 1.000) (Fig. 2 and 4). Therefore, choice of adjuvant was not critical when rabbits were vaccinated with SA11 virus, although significant differences might have been observed with larger group sizes.

All rabbits except those (n = 5) that were 100% protected from ALA challenge developed an intestinal IgA antiviral response after challenge (Fig. 3C and F). Following challenge, GMTs of total serum, serum N-Abs, total intestinal, intestinal IgG, and intestinal IgA antibodies of animals vaccinated with SA11, TNC buffer, 2/4/6/7- or 2/6/7-VLPs in QS-21 and 2/6/7-VLPs in Freund’s adjuvants (except serum N-Abs) increased significantly (P = 0.042) (Table 1). The titers of rabbits immunized with SA11 in Freund’s adjuvants or AlP or with 10, 20, or 50 µg of 2/4/6/7-VLPs in Freund’s adjuvant did not increase significantly (P > 0.109). These results correspond with the levels of protection from infection (Fig. 4), since the level of protection was higher in the latter groups.

Immune response and protection from challenge of rabbits parenterally immunized twice with 50 µg of 2/6-VLPs in Freund’s adjuvants or QS-21. We recently showed that 2/6-VLPs administered parenterally, orally, or intranasally to mice provide protection (42 to 100%) from homologous E or murine rotavirus challenge (2, 33, 42, 58, 59). To determine if either of the outer capsid proteins, VP7 or VP4, is required to achieve protection from infection in the rabbit model, 50 µg of 2/6-VLPs in Freund’s adjuvants (n = 5) and QS-21 (n = 5) was administered parenterally to rabbits.

In Freund’s adjuvants, the total serum antibody responses induced by 2/6-VLPs were not significantly different (P = 0.278) from those induced by 2/4/6/7-VLPs; however, they were significantly higher (P = 0.012) and lower (P = 0.042) than those induced by 2/6/7-VLPs and SA11, respectively (Fig. 3A). Similarly, rotavirus-specific IgG intestinal antibodies induced by 2/6-VLPs were not significantly different from those induced by 2/4/6/7-VLPs (P = 0.118) but were significantly higher (P = 0.024) than those induced by 2/6/7-VLPs and SA11, respectively (Fig. 3B).

Rabbits immunized with 2/6-VLPs in QS-21 developed lower serum (P = 0.008) and intestinal (P = 0.011) GMTs than the corresponding formulation in Freund’s adjuvants. The total serum antibody responses induced by 2/6-VLPs in QS-21
FIG. 3. Serum and intestinal antibody responses following two parenteral immunizations (0 and 21 or 28 dpv) of rabbits with formalin-inactivated SA11 rotavirus, MA104 cell lysate, or 50 μg of G3 2/4/6/7-, G3 2/6/7-, or 2/6-VLPs in Freund’s adjuvants (A to C) and with 50 μg of live-psoralen-inactivated SA11 rotavirus, TNC buffer, or 50 μg of G3 2/4/6/7-, G3 2/6/7-, or 2/6-VLPs in QS-21 (D to F). Total (IgM, IgG, and IgA) serum (A and D), intestinal IgG (B and E), and intestinal IgA (C and F) antirotavirus antibodies were measured by ELISA before (56 or 69 dpv) and after (84 or 97 dpv) oral challenge with lapine ALA rotavirus. Antibody titers were measured for individual rabbits, and results are plotted as GMTs of the groups. The GMT of intestinal IgG antibody titers for both 0 and 28 dpc of the rabbits vaccinated with 2/6-VLPs in Freund’s adjuvants is 368. Therefore, for this group, only one box appears on the graph (B). Error bars represent 1 standard error of the mean. For titers of <50 and <25, 25 and 2.5, respectively, were used to calculate the GMT. A GMT of 25 or 2.5, for serum or intestinal antibodies, respectively, was considered negative. A significant (P < 0.05, Wilcoxon signed ranks test) increase in rotavirus antibody GMTs following challenge is indicated by an asterisk.
were not significantly different ($P = 0.204$) from those of any other VLP formulations in QS-21 but were lower ($P \leq 0.004$) than SA11-vaccinated rabbits (Fig. 3D). Titers of rotavirus-specific IgG intestinal antibodies induced by 2/6-VLPs were not significantly different from those induced by any VLP formulation in QS-21 ($P \geq 0.125$) or from those induced by SA11 in QS-21 ($P = 0.093$) (Fig. 3E). As expected, none of the rabbits vaccinated with 2/6-VLPs in either Freund’s adjuvants or QS-21 developed serum or intestinal N-Abs against SA11 and ALA rotavirus (Table 1).

The protective efficacies of 2/6-VLPs in the two adjuvants were equivalent ($P = 0.917$). In Freund’s adjuvants, the mean protective efficacy afforded by 2/6-VLPs (41%) was not significantly different ($P = 0.071$) from that afforded by SA11 (87%), 2/4/6/7-VLPs (89%), or 2/6-VLPs (58%) (Fig. 4A). The inability to detect significant differences in protective efficacy between 2/6-VLPs and any other VLP formulation or SA11 may have been due to the number of animals in the 2/6-VLP group ($n = 5$). 2/6-VLPs ($n = 9$), which afforded a similar level of mean protection as 2/6-VLPs, provided significantly lower protection ($P = 0.034$) than 2/4/6/7-VLPs ($n = 5$). Trend analysis of protective efficacy of 2/6-, 2/6/7-, and 2/6/7-VLPs in Freund’s adjuvants revealed that the protective efficacy increased as the number of proteins in the VLPs increased ($P = 0.009$, linear regression). Therefore, the level of protection of VLPs in Freund’s adjuvants was dependent on VLP formulation. In QS-21, protection afforded by 2/6-VLPs (26%) was significantly lower than the protection afforded by 2/4/6/7-VLPs (56%, $P = 0.014$) or SA11 (83%, $P = 0.004$) (Fig. 4B). However, 2/6-VLPs provided the same levels ($P = 1.000$) of protection (26%) against challenge as 2/6/7-VLPs in QS-21 (Fig. 4B).

Since 2/6-VLPs did not induce significantly lower total serum or intestinal IgG titers than 2/4/6/7-VLPs ($P = 0.445$) or SA11 virus ($P = 0.093$) in QS-21 (Fig. 3D and E), these results suggest that the presence of antirotavirus IgG in the intestine alone is not sufficient to protect rabbits from challenge. Intestinal VP4- but not VP7-specific antibodies may be required to induce higher levels of protection. Nevertheless, although our results indicate that an increase in VP4 in the VLP formulation, the correlation is not absolute, as individual rabbits (4 of 14) immunized with VLPs that lacked VP4 in Freund’s adjuvants showed high levels (>80%) of protection (Fig. 4A).

Following challenge, total serum and intestinal IgG and IgA antibody titers increased significantly ($P \leq 0.043$) in 2/6-VLP-vaccinated rabbits in QS-21 (Fig. 3D to F). However, in rabbits vaccinated with 2/6-VLPs in Freund’s adjuvants, only total serum and intestinal IgA antibody titers increased significantly after challenge ($P = 0.043$) (Fig. 3A to C). Following challenge, all 2/6-VLP-vaccinated rabbits developed serum N-Abs to the serotype G3 simian SA11 and lapine ALA viruses. The serum N-Ab GMT to SA11 (800) observed after challenge was significantly lower ($p = 0.008$) than that to ALA (11,143), suggesting that N-Abs were preferentially made to the challenge ALA virus, although SA11 and ALA rotaviruses have the same VP7 type (G3) (12, 13, 72).

Immune response and protection from challenge of rabbits parenterally immunized twice with different concentrations of 2/6-VLPs and QS-21. Experiments in mice showed that parenteral administration of 2/6-VLPs in QS-21 induced high levels of protection from challenge (2, 33, 42). However, 2/6-VLPs in QS-21 administered parenterally to rabbits failed to induce high levels of protection, and 2/6-VLPs in Freund’s adjuvants afforded high levels of protection in only two of five rabbits (Fig. 4). To determine if either an increase of VLP or QS-21 dose would afford higher levels of protection in rabbits, 200 μg of 2/6-VLPs in 20 μg of QS-21 ($n = 5$) or 50 μg of 2/6-VLPs in 50 μg of QS-21 ($n = 5$) was administered parenterally to rabbits. Increases in the dose of either 2/6-VLPs (200 μg) or QS-21 (50 μg) failed to increase the levels of total serum...
antibodies ($P \geq 0.072$), intestinal IgG antibodies ($P \geq 0.228$), or mean protective efficacy (5 and 18%, respectively) ($P \geq 0.141$) compared to 50 mg of 2/6-VLPs in 20 mg of QS-21 (data not shown). As observed with lower doses of VLPs or QS-21, the total serum and intestinal IgG antibody levels or mean protective efficacy with either the increased dose of VLPs or adjuvant were significantly lower ($P \leq 0.048$) than those induced by 2/6-VLPs in Freund’s adjuvants (data not shown).

Analyses of the immune response to individual rotavirus proteins and correlation of protective efficacy and immune responses detected by Western blotting and FFNA. Table 1 summarizes the rabbit immune responses (as measured by ELISA and FFNA), percent protective efficacy, and the corresponding statistical analyses among all vaccine groups. Immunization with virus or with the inclusion of VP4 in 2/4/6/7-VLP vaccines resulted in significantly higher ($P < 0.05, \text{Mann-Whitney U test}$) mean protective efficacy than in the 2/6/7-VLP vaccines. Therefore, we investigated whether rabbits vaccinated with 50 μg of live or psoralen-inactivated SA11 rotavirus developed antibodies only to structural rotavirus proteins, indicating that no rotavirus replication occurred. The predominant antibody responses was directed to VP6 irrespective of virus or VLP vaccine administered to rabbits. Antibodies to VP2 were observed seldom with Freund’s adjuvants and not at all with QS-21. Antibodies to VP7 were detected in about 50% of the samples of rabbits vaccinated with VP7-containing formulations in Freund’s adjuvants or QS-21. VP4-specific antibodies were not detected in any prechallenge serum samples from rabbits immunized with SA11 or 2/4/6/7-VLPs in either adjuvant. Following ALA challenge, rabbits that were 98 to 100% protected from virus antigen shedding did not develop antibodies to additional rotavirus structural or nonstructural proteins. Conversely, rabbits that were not protected from challenge developed antibodies to most rotavirus proteins, including nonstructural proteins.

The failure to detect antibody to VP4 by Western blotting does not indicate that rabbits did not develop serum antibodies to VP4 but rather suggests that this method may not be sensitive enough to detect antibodies to individual proteins or the induced antibodies do not react with denatured proteins because the immunodominant epitopes are conformational. To determine if antibodies to VP4 play a role in protection, the prechallenge serum samples of all rabbits vaccinated with 2/4/6/7-VLPs in Freund’s adjuvants or QS-21 or with
live or psoralen-inactivated SA11 rotavirus in QS-21 were tested by FFNA. SA11 C13 × B223 reassortant rotavirus strains R-N33 (P8[11], G3) and R-A32 (P2, G10), and their parental rotavirus strains, were used in FFNAs to measure directly SA11 VP4-specific serum N-Abs.

The N-Ab GMTs induced to SA11 and R-N33 (both G3) by SA11 rotavirus in QS-21 or 2/4/6/7- or 2/6-VLPs in Freund’s adjuvants were equivalent (P ≥ 0.643), although the N-Ab titers to the reassortant R-N33 were slightly lower than those obtained to the parental strain SA11 (Fig. 6A). Rabbits vaccinated with 2/4/6/7- and 2/6-VLPs in Freund’s adjuvants developed low-level N-Abs to the heterologous B223 rotavirus

FIG. 6. Correlation of higher protection rates with presence of serum N-Abs to VP4. (A) Serum N-Ab responses following two parenteral immunizations (0 and 28 dpv) of rabbits with 50 μg of P[2], G3 2/4/6/7-, G3 2/6/7-, or 2/6-VLPs in Freund’s adjuvants or with 50 μg of live or psoralen-inactivated SA11, TNC buffer, or 50 μg of P[2], G3 2/4/6/7-, G3 2/6/7-, or 2/6-VLPs in QS-21 were measured by FFNA against rotavirus strains SA11 (P[2], G3) and B223 (P8[11], G10) and against reassortant virus strains R-N33 (P8[11], G3) and R-A32 (P2, G10) prior to (56 or 69 dpv) oral challenge with lapine ALA rotavirus. Serum N-Ab titers were measured for individual rabbits, and results are plotted as the GMTs of the groups. Error bars represent 1 standard error of the mean. For titers of <100, 50 was used to calculate the GMT. A GMT of 50 and its log were considered negative. A significant difference (P ≤ 0.021, Mann-Whitney U test) in VP4-specific N-Abs induced by 2/4/6/7-VLPs vaccines measured by comparing serum N-Abs measured against B223 and R-A32 is indicated by an asterisk. (B) Scatterplot of the log of the SA11 VP4-specific-NAb titers and the percent reduction of virus antigen shedding for individual rabbits (n = 58). Results were graphed to indicate whether rabbits were immunized with both VP4 and VP7 (■; SA11 or 2/4/6/7-VLPs in either adjuvant), absence of VP4 but presence of VP7 (○; 2/6/7-VLPs in either adjuvant), or absence of both VP4 and VP7 (▲; 2/6-VLPs in either adjuvant or TNC buffer in QS-21). Correlation coefficient was calculated by Pearson’s correlation coefficient.
strain (GMTs = 528 and 147, respectively) that were significantly lower (P < 0.023) than those obtained with the homologous G3 VP7 type (Fig. 6A). Rabbits vaccinated with live or psoralen-inactivated SA11 virus or with 2/4/6/7- or 2/6/7-VLPs in QS-21 developed low N-Abs to B223 (Fig. 6A). The ability of 2/4/6/7- and 2/6/7-VLPs to induce heterotypic N-Abs to other additional strains of different serotypes is described elsewhere (27). VP4-specific N-Abs induced by SA11 rotavirus or 2/4/6/7-VLP vaccines were directly measured by comparing the N-Ab responses obtained against strain B223 and the reassortant rotavirus strain R-A32, since the latter possesses an SA11 VP4 type (P2) and a B223 VP7 type (G10). The serum N-Ab GMTs of rabbits vaccinated with 2/4/6/7-VLPs in Freund's adjuvants measured with R-A32 were significantly higher (P = 0.021) than those measured by B223 alone (Fig. 6A). N-Ab GMTs of serum samples of rabbits vaccinated with 2/6/7-VLPs measured with B223 and R-A32 were equivalent (P = 0.864), indicating that the difference in N-Ab GMT observed with R-A32 and the serum of rabbits vaccinated with 2/4/6/7-VLPs in Freund's adjuvants was due to N-Abs to VP4 present in the serum samples (Fig. 6A). Similar significant results (P < 0.001) were observed when N-Abs were measured in the serum samples of rabbits vaccinated with 2/4/6/7-VLPs in QS-21 against B223 and R-A32. The N-Ab GMTs of serum samples of rabbits vaccinated with 2/6/7-VLPs in QS-21 were similar (P = 0.909) against both viruses tested, as were those of rabbits vaccinated with SA11 rotavirus in QS-21 (P = 0.197) (Fig. 6A). As expected, no N-Abs to the reassortant or parental rotavirus strains were detected in any of the rabbits vaccinated with 2/6-VLPs in either adjuvant (Fig. 6A).

Although significant differences were observed in SA11 VP4-specific N-Ab GMTs, not all rabbits that were immunized with, and protected by, a vaccine that included VP4 in the formulation developed a detectable SA11 VP4-specific neutralizing immune response. To determine whether higher protection from challenge correlated with an specific immune response to VP4, the Pearson's correlation coefficient between the protective efficacy and the log of SA11 VP4-specific serum N-Ab titers for all rabbits was calculated. Our results showed that higher protection levels correlated with the presence of N-Abs to VP4 (P < 0.001, r = 0.55, Pearson's correlation coefficient) (Fig. 6B). Therefore, generally the inclusion of VP4 significantly increases the protective efficacy against infection in rabbits parenterally immunized with VLPs, but antibodies to VP4 alone may not explain the enhanced protection levels in some animals. Unfortunately, we were unable to isolate the reassortants necessary to measure VP4-specific N-Abs to ALA, the challenge rotavirus strain (68).

Detection of infectious virus in fecal samples collected 0 to 10 dpc by FFA. To determine if protective efficacy measured by percent reduction in virus antigen shedding by ELISA predicts the amount of infectious virus shed, the infectivity titers of the ALA virus shed following challenge were measured by FFA (Fig. 7). For comparison, a subset of rabbits (n = 17) were chosen to cover the whole range of protection, independent of vaccine formulation. To establish a correlation, Pearson's correlation coefficient was calculated by plotting the areas under the curve for the virus antigen shedding curves and the virus titer curves. A significant (P < 0.001, r = 0.979) correlation coefficient was found (Fig. 7A).

Based on the scatterplot (Fig. 7A), two distinct groups were readily evident, controls (n = 3) that were not protected (0%) and rabbits (n = 3) with that were completely protected (100%). Rabbits (n = 11) with intermediate protection rates (20 to 98%) cluster in the middle of the plot and were subdivided into two groups, one with rabbits exhibiting relatively high protection levels (66 to 98%) and another with those exhibiting lower protection levels (20 to 65%). These results show a strong association between percent protection from infection to both virus antigen shedding and infectious virus titers.
The amount of infectious virus shed by rabbits with 20 to 65% protection was approximately 1 log lower than that for control rabbits (0%) (Fig. 7B). In contrast, there was a 2- to 3-log difference in the titer of virus shed in the group that was 68 to 98% protected with respect to the control group. Also, the duration of shedding of infectious virus was 2 to 3 days shorter for the protected group than for the control group. Rabbits that were 100% protected from challenge did not shed detectable infectious ALA virus and showed at least a 4-log decrease in the amount of infectious virus shed compared to the 0% control group. Therefore, protection as measured by reduction in antigen virus shedding (68 to 100%) corresponded with 2- to 4-log reductions of infectious virus shedding.

**DISCUSSION**

We examined the immunogenicity and protective efficacy of different formulations of parenterally administered candidate rotavirus VLP subunit vaccines in the rabbit model of rotavirus infection. Previously, we showed that parenteral vaccination with live or inactivated rotavirus induces complete active protective immunity in rabbits against virus challenge. This protection correlated with the presence of rotavirus-specific IgG, but not IgA, in the intestine (19). In this study, we showed that VLPs administered parenterally are immunogenic and can induce active protective immunity in rabbits. Vaccinated rabbits developed high levels of total (IgM, IgG, and IgA) rotavirus-specific antibody in the serum. Rabbits vaccinated with VLPs or with live or inactivated SA11 virus developed antirotavirus intestinal antibody of the IgG, but not IgA, isotype. Protective efficacy varied with both VLP formulation and adjuvant. With all adjuvants tested, 2/4/6/7-VLPs consistently induced the highest levels of protection from homotypic (G3) ALA rotavirus challenge.

There was no significant difference in the mean protective efficacy afforded by live or formalin-inactivated SA11 in either Freund's adjuvants (87%) or AlP (76%) or purified live or psoralen-inactivated SA11 in QS-21 (83%) (P = 0.462). Although the results are similar to our previous work comparing live or inactivated SA11 virus administered in Freund's adjuvants and AlP (19), in the present study, unlike the previous study, total protection from rotavirus challenge was not achieved in rabbits vaccinated with SA11 in either adjuvant. The live and formalin-inactivated SA11 vaccine preparations were the same vaccine preparations used previously (19), but antigen ELISA results indicated that limited degradation of the immunogen occurred in this study (data not shown). Therefore, the lower protective efficacy in this study is likely due to immunization with a lower dose of antigen.

The difference in virus inactivation procedures probably did not influence the immune responses because (i) we did not observe any significant difference in either immunogenicity or protective efficacy between live, formalin-inactivated, and psoralen-inactivated SA11 virus, (ii) we had previously shown (19) that following formalin treatment of virus, the reactivity levels of MAbS for several viral epitopes remain unchanged on formalin-inactivated virus compared to live virus, and (iii) likewise, others have shown that psoralen-inactivated virus retains epitope reactivity to the same panel of MAbS (37). Therefore, the effect of the differences in inactivation procedures on the immune responses elicited was not a major concern.

Live or inactivated SA11 rotavirus elicited the highest antibody responses and levels of protection in all three adjuvants tested. The high levels of antibody were not due to replication of the SA11 rotavirus administered intramuscularly to rabbits because prior to challenge none of the SA11-vaccinated rabbits developed serum antibodies to nonstructural proteins (Fig. 5 and data not shown), and fecal samples collected 2 to 14 dpv from rabbits vaccinated intramuscularly with live or formalin-inactivated SA11 rotavirus did not contain rotavirus antigen, indicating that virus replication did not occur (19). It is probable that the higher responses induced by SA11 compared to VLPs were due to differences in the immunizing dose. The doses of SA11 and VLPs administered to rabbits were based on total protein concentration determined by the Bio-Rad protein assay. Other studies have determined that if vaccine dose is based on the amount of VP7 in VLP and SA11 preparations, then SA11 has to be administered at a lower total protein concentration to yield equivalent doses of VP7 (50). Therefore, SA11 may have provided better responses in all adjuvants in this study compared to VLPs because of slightly higher doses, not differences in inherent properties between SA11 and VLPs.

Since the rabbit is an infection, rather than a disease, model, we determined protective efficacy of the VLPs by calculating percent reduction in antigen virus shedding measured between vaccinated and mock-vaccinated rabbits. Prevention of virus antigen shedding may be a more stringent measure of protection than protection from clinical disease, since detectable levels of virus antigen shedding can occur in the absence of clinical signs (21). Based on the strong correlation (r = 0.979, P < 0.001, Pearson’s correlation coefficient) between the amount of virus antigen and infectious virus shedding, calculation of percent protection from infection reflects both the efficacy and the duration of both infectious virus and virus antigen shedding. The significant reduction of infectious virus shedding in vaccinated rabbits that had >68% protection from virus antigen shedding indicates that substantially less virus replication occurred in the intestines of vaccinated rabbits. A decrease in the amount of virus shedding could lead to a considerable decrease in the amount of infectious virus disseminated among the population. Although VLPs administered parenterally can effect a significant reduction in rotavirus shedding, it is difficult to predict how the reduced shedding relates to protection from any rotavirus diarrhea or severe rotavirus diarrhea because comparable measurements of these parameters have not been reported for naturally infected children or for any disease animal model.

To assess the immunogenicity and protective efficacy of different VLP formulations in the rabbit model, Freund’s adjuvants, AlP, and QS-21 were tested as adjuvants. Only AlP is licensed for use in humans. Although our results indicate that VLPs can induce homotypic protection from challenge by the parenteral route, our data combined with data obtained for mice (42, 50) indicate that AlP and aluminum hydroxide are not potent adjuvants for use in conjunction with VLPs. Nevertheless, in previous rabbit experiments (14), the antibody titers induced by SA11 virus in AlP was sufficient to provide protection. It is probable that higher doses of VLPs in AlP will be needed to induce complete protective immunity. QS-21 has been shown to be a powerful immunopotentiator with VLPs in mice (42, 50) and has been tested with soluble protein antigens in preclinical studies in humans (45, 46, 56). Although Freund’s adjuvant proved to be superior to QS-21 in our study, QS-21 may prove to be a better adjuvant than AlP.

Studies with children and animals have both supported and refuted protection based on the development of N-Abs (1, 15, 16, 40, 49, 53, 57, 77, 78, 81, 82). Recently, we showed that mucosally administered 2/6-VLPs, parenterally administered 2/6-VLPs, or inactivated double-layered murine EDIM rotavirus with QS-21 induced protection from rotavirus challenge in mice (2, 33, 55, 58, 59), indicating that neither VP7 nor VP4 is
absolutely required for protection. However, parenteral immunization of rabbits with 2/6/7- or 2/6-VLPs with QS-21 resulted in levels of protection lower than those achieved with the 2/4/6/7-VLPs. Increases in the dose of either 2/6-VLPs or QS-21 failed to improve the protective efficacy. The presence of neutralizing VP4 antibodies in VLP- or SA11-vaccinated rabbits correlated ($P < 0.001, r = 0.55; Pearson's correlation coefficient) with enhanced protection rates. Therefore, it appears that the presence of antibodies to VP4 plays an important role in protection of rabbits from infection. Although an absolute requirement for VP7 is not supported by our results with rabbits, VP7 may be needed to stabilize VP4 or to achieve proper expression of the epitopes on VP4 required for the induction of protective antibodies (26). It remains unclear whether N-Abs are required to induce high levels of protection; however, if N-Abs are needed for protection, then a limited number of VLP formulations may be sufficient to produce a broadly protective VLP subunit vaccine (27). A parenteral 2/4/6/7-VLP vaccine as opposed to an inactivated rotavirus vaccine will have the advantages of safety (i.e., it will be nonreplicating), no residual infectious virus or chemicals following inactivation, antigen stability (>6 years at 4°C), purity, and ability to alter protein content as needed (26, 27).

Taken together, our results using VLPs and QS-21 in rabbits suggest that both VP4 and VP7 neutralization antibodies may be required in a parenteral immunogen to induce protection from rotavirus challenge. Nevertheless, the correlation is not absolute because individual rabbits immunized with 2/6/7- or 2/6-VLPs in Freund's adjuvants achieved high levels of protection (87 to 100%). The widely variable protective efficacies obtained with the 2/6-VLPs in Freund's adjuvants may be due to variation in the immunodominance of epitopes or immunological regulatory mechanisms of the outbred rabbit, which may be important factors influencing the outcome of the immune response.

The dichotomy in results obtained in mice and rabbits may be influenced (i) by differences in the P types of the mouse (P[16]) and rabbit (P[14]) virus strains used for challenge relative to the immunizing P type (P[2]) or (ii) by inherent differences in antigen uptake, processing, and epitope recognition between rabbits and mice. Following oral infection of mice with a heterologous virus, the majority of the intestinal antibody-secreting cells were directed toward VP2 and VP6, and only about 1% of the total virus-specific response was directed to VP4 (71). It would be interesting to perform similar experiments in rabbits. Ultimately, clinical testing of VLPs in the gnotobiotic piglet rotavirus disease model and humans will be required to determine whether results from the rabbit or mouse model will be more predictive of protection against disease in children. Results from studies of children naturally infected with human rotaviruses or bovine-human virus reassortants have suggested that VP4 may be the immunodominant neutralization antigen in a homotypic response (9, 81), while data from another study of children suggested that VP7 is the immunodominant neutralization antigen (78). Currently, virus reassortants with different VP4-VP7 combinations are being applied to study immune responses to each neutralization antigen; however, if N-Abs are needed for protection, then a limited number of VLP formulations may be sufficient to produce a broadly protective VLP subunit vaccine (27). A parenteral 2/4/6/7-VLP vaccine as opposed to an inactivated rotavirus vaccine will have the advantages of safety (i.e., it will be nonreplicating), no residual infectious virus or chemicals following inactivation, antigen stability (>6 years at 4°C), purity, and ability to alter protein content as needed (26, 27).

Protection studies in rabbits and mice suggest that VLPs may provide a safe and efficacious alternative to live oral rotavirus vaccines. A parenteral vaccine might overcome two of the problems observed with oral live rotavirus vaccines: (i) interference by maternal antibodies and (ii) poor replication of the vaccine virus. Because a parenteral vaccine would not require replication of the rotavirus vaccine strains, formulation of the vaccine might be easier since differences in immunogenicity based on growth properties of individual viruses would not come into play. However, the need for multivalent parenteral vaccines to induce broad immunity against the various human rotavirus serotypes will need to be examined. The results of our studies support the further development of rotavirus subunit vaccines as well as evaluation of combined parenteral-oral vaccination regimens.

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