Rotavirus Virus-Like Particles Administered Mucosally Induce Protective Immunity

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We have evaluated the immunogenicity and protective efficacy of rotavirus subunit vaccines administered by mucosal routes. Virus-like particles (VLPs) produced by self-assembly of individual rotavirus structural proteins coexpressed by baculovirus recombinants in insect cells were the subunit vaccine tested. We first compared the immunogenicities and protective efficacies of VLPs containing VP2 and VP6 (2/6-VLPs) and G3 2/6/7-VLPs mixed with cholera toxin and administered by oral and intranasal routes in the adult mouse model of rotavirus infection. VLPs administered orally induced serum antibody and intestinal immunoglobulin A (IgA) and IgG. The highest oral dose (100 μg) of VLPs induced protection from rotavirus challenge (>50% reduction in virus shedding) in 50% of the mice. VLPs administered intranasally induced higher serum and intestinal antibody responses than VLPs administered orally. All mice receiving VLPs intranasally were protected from challenge; no virus was shed after challenge. Since there was no difference in immunogenicity or protective efficacy between 2/6- and 2/6/7-VLPs, protection was achieved without inclusion of the neutralization antigens VP7 and VP4. We also tested the immunogenicities and protective efficacies of 2/6-VLPs administered intranasally without the addition of cholera toxin. 2/6-VLPs administered intranasally without cholera toxin induced lower serum and intestinal antibody titers than 2/6-VLPs administered with cholera toxin. The highest dose (100 μg) of 2/6-VLPs administered intranasally without cholera toxin resulted in a mean reduction in shedding of 38%. When cholera toxin was added, higher levels of protection were achieved with 10-fold less immunogen. VLPs administered mucosally offer a promising, safe, nonreplicating vaccine for rotavirus.

Rotaviruses are the leading cause of severe gastroenteritis in young children worldwide. Rotavirus infections lead to more than 500,000 deaths each year in developing countries (38). In the United States, rotavirus infections are estimated to cost $1 billion a year in health care costs (40). Intense efforts have been under way for 15 years to develop an effective rotavirus vaccine. Recent Centers for Disease Control and Prevention estimates concluded that a rotavirus vaccine would be cost-effective, and a vaccine with 50% efficacy against severe diarrhea is predicted to save $79 million a year in health care and $466 million in general savings (60).

Our laboratory is working on the development of a subunit vaccine for rotavirus. Virus-like particles (VLPs) are made by coinfected insect cells with baculovirus recombinants expressing rotavirus structural proteins; these proteins self-assemble into VLPs (22). Rotavirus particles consist of three capsid layers: the inner layer composed of VP2, the intermediate VP6 layer, and the outer VP7 layer, with VP4 spikes emanating from the outer surface layer. VP4 and VP7 independently induce neutralizing antibodies. Antibodies to VP4 and VP7 can passively protect mice against rotavirus challenge (1, 5, 43, 44, 53). Antibodies to VP6 are not neutralizing in vitro but can protect against rotavirus infection in mice when administered by injection of hybridoma cells into the backs of immunodeficient (nude) mice (backpack model) (9). However, the role of neutralizing antibody in active protection remains controversial.

Previously, we have shown that VLPs containing VP2, VP4, VP6, and VP7 (2/4/6/7-VLPs) administered parenterally are immunogenic in mice (21, 22) and can protect rabbits from a homologous rotavirus challenge (12, 20). While parenteral immunization is effective, it may be advantageous to administer VLPs mucosally. Potential advantages to mucosal administration of VLPs include ease of delivery, reduced vaccination cost, and induction of local secretory immunoglobulin A (IgA). However, oral administration of VLPs may not be the most effective route, because large amounts of antigen may be needed, due to antigen degradation in the stomach and intestine. Alternate sites of mucosal immunization may be able to induce immunity in the intestine through the common mucosal immune system (46, 58, 69). Intranasal administration of other immunogens has been shown to induce intestinal antibody (32, 71).

In the present study, we used the adult mouse model of rotavirus infection, originally described by Ward et al. (67), to compare the immunogenicity and protective efficacy of VLPs administered orally and intranasally, with and without the addition of cholera toxin (CT). CT has been shown to be an effective adjuvant when administered orally and intranasally (42, 63, 64). VLPs are also a useful tool for dissecting the components of a protective immune response to rotavirus. VLPs allow us to define which rotavirus proteins are necessary for active protection, as well as to clarify the role of neutralizing antibody in protection. Mice were inoculated with 2/6- and 2/6/7-VLPs to determine the necessity of neutralizing antibody in protection from rotavirus challenge.

MATERIALS AND METHODS

Virus. Wild-type murine rotavirus EC<sub>wt</sub> and tissue culture-adapted EC<sub>tc</sub> (serotypes [16], G3) were obtained from Harry Greenberg (Stanford University Medical School, Palo Alto, Calif.) (27). A stock of the wild-type virus was prepared by orally inoculating 5-day-old CD-1 suckling mice with 400 50% diarrhea doses of EC<sub>wt</sub> in a volume of 50 μl. Forty-eight hours following inoculation, the mice were euthanized and their entire intestinal tracts were removed, combined, and freeze-thawed once. Then, medium 199 supplemented with 1-glutamine, penicillin, and streptomycin was added to make a 20% (wt/vol) prep-
aration, and the intestines were homogenized with a tissue homogenizer, aliquoted, and stored at −80°C until use. The 50% shedding dose (SD50) of ECastro in adult mice was determined as described elsewhere (27). The titer of the ECastro stock used for all mouse inoculations was 4 × 107 SD50/ml (data not shown). A stock of 2/6-VLPs was prepared by passage in toto in Spodoptera frugiperda (Sf9) cells in the presence of tretinoin, and the virus titer was determined by the fluorescence focus assay (FFA). Plaque-purified SA11 clone 3 (SA11 cl 3; serotypes [P2], G3) rotavirus was cultivated in MA104 cells in the presence of tretinoin as previously described (17, 26) and was used for the enzyme-linked immunosorbent assay (ELISA) and the fluorescence focus neutralization assay (FFNA).

**Animals.** Virus antibody-negative adult female CD-1 mice (22 to 24 g) were obtained from Charles River Laboratories (Portage, Mich.). The mice were confirmed to be antibody free by ELISA prior to vaccination. All mice were housed in microisolator cages throughout the experiment. For the rotavirus challenge, the mice were moved to a physically separated animal facility to avoid any possible contamination of rotavirus-negative mice.

**VLPs.** 2/6- and 2/6/7-VLPs were prepared in insect cells and purified as previously described (22), except that a full-length VP7 gene was used (21). VLPs were composed of bovine RV VP2 with SA11 VP6 and VP7(G3). VLP protein composition was confirmed by Western blot and electron microscopy (EM) analysis as previously described (22). Endotoxin levels were determined by the Limulus amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, Mass.). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad, Hercules, Calif.) with bovine IgG as the standard.

**Vaccination.** All mice were inoculated s.c. with 50 μl of 2/6-VLPs (0.19 mg/mouse), and acepromazine (0.037 mg/mouse). All mice were administered Blotto (5% [wt/vol] Carnation powdered milk in PBS) for 2 h at 37°C (8).

**Oral vaccination.** Mice were gavaged with a final volume of 100 μl. Control mice were gavaged with endotoxin-free phosphate-buffered saline (PBS), were mixed with 10 μg of CT (Sigma, St. Louis, Mo.) prior to administration to the mice. Each mouse was gavaged with a final volume of 100 μl. Control mice were gavaged with endotoxin-free PBS mixed with 10 μg of CT in a volume of 100 μl. No clinical changes were observed in mice immunized with CT or PBS.

**Intranasal vaccination.** Prior to inoculation, mice were anesthetized by intraperitoneal administration of a mixture of ketamine (3.75 mg/mouse), xylazine (0.19 mg/mouse), and acepromazine (0.037 mg/mouse). All mice were administered 0.05% benzamidine, 10 μl of 2/6-VLPs mixed with 5 μg of CT (Sigma, St. Louis, Mo.) prior to administration to the mice. Each mouse was monitored until consciousness was regained. No adverse signs were noted in any mice receiving intranasal inoculation.

**i) Three vaccinations.** Mice received 10 μg of VLPs mixed with 5 μg of CT per dose. Control mice received endotoxin-free PBS mixed with 5 μg of CT. No clinical changes were observed in mice immunized with CT or PBS.

**ii) Two vaccinations.** Mice were inoculated with either 10, 50, or 100 μg of 2/6-VLPs without CT or 10 μg of 2/6-VLPs mixed with 5 μg of CT. Control mice received PBS mixed with 5 μg of CT.

**Sample collection and processing.** Serum and fecal antibody samples were collected from each mouse on 0, 14, and 28 DPI, as well as 22 days postchallenge (DPC). Mice receiving three vaccinations had additional serum and fecal antibody samples collected 137 DPI. Serum was collected by tail bleeding on the day of challenge (DPC). Mice receiving three vaccinations had additional serum and fecal antibody samples collected 137 DPI. Serum was collected by tail bleeding on the day of challenge (DPC). Mice receiving three vaccinations had additional serum and fecal antibody samples collected 137 DPI. Serum was collected by tail bleeding on the day of challenge (DPC). Mice receiving three vaccinations had additional serum and fecal antibody samples collected 137 DPI. Serum was collected by tail bleeding on the day of challenge (DPC).

**Measurement of total antirotavirus antibody in serum.** Measurement of total antirotavirus antibody in serum was performed as previously described (19), with the following modifications: (i) plates were coated with hyperimmune guinea pig anti-SA11 serum, (ii) the antigen was a lysate of SA11-infected cells with 0.02 M EDTA, (iii) the initial dilution of serum samples was 1:50, and (iv) HRP-conjugated goat anti-mouse IgA, IgG, and IgM (Kirkegaard and Perry Laboratories) diluted in 0.5% BSA containing 5% normal guinea pig was used as the conjugate. Antibody titers were defined as the reciprocal of the highest dilution giving a net OD value (OD with SA11 virus minus OD with 0.5% BSA) of 2.5 μl. Mice were inoculated with 2/6/7-VLPs were produced by coexpression of baculovirus recombinant proteins. Western blotting was performed as described elsewhere (5) with the following modifications: (i) plates were coated with 0.2 M EDTA, (ii) initial dilution of serum samples was 1:50, and (iii) HRP-conjugated goat anti-mouse IgA, IgG, and IgM (Kirkegaard and Perry Laboratories) diluted in 0.5% BSA containing 5% normal guinea pig was used as the conjugate.

**FNA to measure neutralizing antibody in serum.** The FNA was performed as previously described (13).

**Western blot analysis of serum.** Western blot analysis of serum samples was performed as previously described (8). Briefly, the proteins in an SA11 cl 3-infected cell lysate were separated by electrophoresis on a 10% polyacrylamide gel, transferred to nitrocellulose, and blocked with 5% Blotto and developed by using ECL Western Blotting Detection Reagents (Amersham Life Science, Arlington Heights, Ill.) according to the directions of the manufacturers.

**Statistical analysis.** Statistical analyses were performed with SPSS version 7.0 for Windows (SPSS, Inc., Chicago, Ill.). Percent reductions in shedding were compared by the Mann-Whitney U test. Antibody titers following the second inoculation, the third inoculation, and challenge within a given group 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. Trend analysis was done by linear regressions antibody (IgA, IgG, and IgM) in serum.

**RESULTS**

**Characterization of VLPs used for inoculations.** 2/6- and 2/6/7-VLPs were produced by coexpression of baculovirus recombinants containing rotavirus genes. EM analysis of the particles purified from the media of infected Sf9 cells showed the 2/6- and 2/6/7-VLPs were intact and properly formed (data not shown). Silver staining and Western blot analysis of the proteins in the VLPs confirmed the presence in the expressed particles of each of the expected structural proteins; no additional proteins were present (data not shown). Endotoxin levels of 2/6- and 2/6/7-VLPs were 0.0015 EU/μg and 0.004 EU/μg, respectively.
Rotavirus VLPs administered mucosally with CT are immunogenic. Mice were confirmed to be rotavirus antibody negative (<1:50) by screening serum samples collected prior to immunization at a 1:50 dilution for total rotavirus antibody by ELISA. Rotavirus antibody-negative mice were inoculated three times, orally or intranasally, with 2/6- or 2/6/7-VLPs mixed with CT. To confirm that the mice had an immune response only to the proteins present in the VLPs, Western blot analysis with SA11 cl 3 was performed on serum collected after the third inoculation with VLPs and before virus challenge (Fig. 1, lanes 2, 4, 6, and 8). Results from representative samples are shown. Although not every mouse developed serum antibody to all of the proteins present in the VLP vaccines, no mice developed serum antibody to proteins not present in the VLP inoculum.

VLPs induce serologic antibody responses. We initially compared the effects of dosage, the number of inoculations (two or three), and protein composition (2/6- or 2/6/7-VLPs) of orally administered VLPs on immunogenicity and protective efficacy. Serologic antibody responses in serum collected 1 month following the second and third vaccinations were evaluated (Fig. 2). We have examined isotype-specific rotavirus serum antibody (data not shown), but we do not routinely detect antirotavirus serum IgA, and serum antirotavirus IgG titers are equivalent to total antirotavirus serum antibody titers. Therefore, total (IgA, IgG, and IgM) antibody to rotavirus in serum is reported. Control mice inoculated with PBS remained rotavirus antibody negative following both the second and the third inoculations.

We first determined the effect of the number of inoculations and the dose of VLPs administered on the serologic rotavirus-specific total antibody responses following oral administration of 2/6-VLPs (Fig. 2A). Although serum geometric mean titers (GMT) in serum increased following a third inoculation or with a fourfold increase in dose (25 to 100 μg), the increases were not significant (P > 0.05 [Wilcoxon Signed Ranks test]). Similar results were seen in animals inoculated with the 2/6/7-VLPs (Fig. 2B), except that increasing the dose by fourfold caused a significant increase in serum GMT (P = 0.034 [Kruskal-Wallis and then Mann Whitney U tests]). Both 2/6- and 2/6/7-VLPs induced serologic immune responses when administered orally, although not all mice developed antibody responses (Fig. 2A and B). Orally administered 2/6- and 2/6/7-VLPs induced similar serum antibody GMTs (P > 0.05 [Kruskal-Wallis]).

Intranasal inoculations of both 2/6- and 2/6/7-VLPs induced serum antibody responses in all mice (Fig. 2A and B). As was seen following oral administration of VLPs, increasing the number of intranasal inoculations or varying the composition of VLPs did not significantly enhance the serum antibody titers (P > 0.05 [Wilcoxon Signed Ranks test]). Comparison of the two routes of mucosal administration, intranasal and oral, on immunogenicity showed that intranasal inoculation with 10 μg of VLPs induced significantly higher titers of serologic antibody than oral inoculation with 25 μg of VLPs (P = 0.009 [Mann Whitney U test]). Although intranasal administration of 10 μg of VLPs induced higher serum antibody GMTs than those induced by oral administration of 100 μg of VLPs, the difference was not significant (P > 0.05 [Kruskal-Wallis test]). However, the immune response was achieved with 10-fold less antigen and 2-fold less adjuvant by the intranasal route.

2/6/7-VLPs administered mucosally with CT induce serologic but not intestinal neutralizing antibody. We compared the effects of doses of VLPs, compositions of VLPs, and routes of VLP administration on induction of neutralizing antibody to both SA11 cl 3 (the virus used to make the VLPs) and EC (the challenge virus) (Fig. 3). None of the mice inoculated with 2/6-VLPs either orally or intranasally had detectable serum or intestinal neutralizing antibody to SA11 cl 3 or EC virus (Fig. 3A). Serum-neutralizing antibodies to both SA11 cl 3 and EC viruses were present in mice inoculated with 2/6/7-VLPs (Fig. 3B). Immunization with 100 μg orally or 10 μg intranasally induced an immune response to SA11 cl 3 in more mice and to significantly higher titers compared to 25 μg orally (P = 0.03 and 0.031, respectively). Neutralizing antibody to EC virus was determined with pooled sera; therefore, statistical comparisons of titers were not performed. Intestinal neutralizing antibody was not detected in any mice.

VLPs administered mucosally with CT induce rotavirus-specific intestinal IgA and IgG. The mice were assayed for intestinal IgA and IgG following the second and third inoculations (Fig. 4). Intestinal antibody titers were determined by measuring antibody by ELISA in processed fecal samples. Although measurement of intestinal antibody in intestinal lavage samples may be more sensitive, the collection of lavages is very time-intensive and requires anesthesia of multiple mice, pre-
FIG. 2. Serum antibody responses following mucosal immunization with VLPs. Total (IgA, IgG, and IgM) serum antirotavirus antibodies were measured in each group of mice after two (45 DPI [left panels]) and three (137 DPI [middle panels]) inoculations with 2/6-VLPs (A) or 2/6/7-VLPs (B) administered orally or intranasally (IN) with CT. Mice were challenged 1 month following the third inoculation, and antibody titers 23 DPC with EC<sub>50</sub> are shown (right panels). The numbers of mice that responded over the numbers of mice in the group are indicated along the x axis. The mice were inoculated on days 0, 14, and 107 at the doses indicated along the x axis. The numbers of mice having a greater than 50% reduction in virus shedding following challenge over the numbers of mice in the group are also indicated along the x axis. Antibody titers were measured for individual mice, and the results are plotted as the GMTs of the groups (n = 4 or 5 per group). Error bars represent one standard error of the mean. Significant differences in GMTs within a panel are indicated by the symbols.
Two intranasal inoculations of 2/6-VLPs did not induce intestinal IgG in any mice (Fig. 4B), while three of five mice developed IgG following two intranasal inoculations with 2/6/7-VLPs (Fig. 4D). All mice inoculated intranasally with either 2/6- or 2/6/7-VLPs had intestinal IgG following three inoculations. There was no difference in intestinal IgG GMT induced by 2/6- and 2/6/7-VLPs given intranasally (P > 0.05 [Mann-Whitney U test]). The effects on induction of intestinal IgG for the two routes of mucosal administration, intranasal and oral, were compared. Intranasal inoculation with 10 μg of 2/6-VLPs induced significantly higher titers of intestinal IgG than oral inoculation with 25 or 100 μg of 2/6-VLPs (P = 0.01 and 0.019, respectively [Kruskal-Wallis followed by Mann-Whitney U test]). Intranasal inoculation of 2/6/7-VLPs induced significantly higher titers of intestinal IgG than oral inoculation with 25 μg of 2/6/7-VLPs (P = 0.005 [Mann-Whitney U test]).

**Rotavirus VLPs administered mucosally with CT induce protection against rotavirus challenge.** Mice were challenged with G3 homotypic EC wt 1 month following the third inoculation, and protection from infection was determined (Fig. 5). Only one mouse inoculated orally with 25 μg of 2/6- or 2/6/7-VLPs was protected (had a ≥50% reduction in virus shedding). Although 100 μg of 2/6-VLPs induced a slightly lower mean reduction in shedding (39%) compared to 2/6/7-VLPs (53%), this difference was not significant (P > 0.05 [Mann-Whitney U test]). Intranasal administration of VLPs induced the highest mean reductions in shedding, i.e., 92% for 2/6-VLPs and 100% for 2/6/7-VLPs. When the results with both types of VLPs were combined, intranasal administration of 10 μg of VLPs induced a significantly higher mean reduction in shedding (96%) than 25 μg of VLPs administered orally (4%) (P = 0.0009 [Mann-Whitney U test]) and 100 μg of VLPs administered orally (50%) (P = 0.006 [Mann-Whitney U test]). The higher levels of protection induced by intranasal administration were achieved with 10-fold less immunogen and 2-fold less adjuvant. 2/6-VLPs induced protection without the production of neutralizing antibody.

Following challenge, all mice developed antibody in serum (Fig. 2). Following challenge, significant increases in serum antibody GMTs were detected only in mice previously inoculated with PBS (P = 0.038 [Wilcoxon Signed Ranks test]). The serum antibody GMTs following challenge were significantly lower in mice inoculated orally with PBS or 25 μg of 2/6/7-VLPs than in mice inoculated orally with 100 μg of 2/6/7-VLPs (P = 0.002 [Mann-Whitney U test]) or intranasally with 10 μg of 2/6/7-VLPs (P = 0.0002 [Mann-Whitney U test]) and 10 μg of 2/6-VLPs (P = 0.012 [Mann-Whitney U test]).

Following challenge, significant increases in intestinal IgA GMTs were detected in mice previously inoculated with PBS, 25 μg of 2/6-VLPs, and 100 μg of 2/6/7-VLPs given orally (P = 0.013, 0.041, and 0.042, respectively [Wilcoxon Signed Ranks test]) (Fig. 4A and C). No significant changes in intestinal IgA titers were seen in any other groups. The groups with increases in intestinal IgA had no or low levels of protection, resulting in virus replication leading to increases in intestinal IgA. Mice inoculated with PBS or 25 or 100 μg of 2/6/7-VLPs orally had significantly higher titers of intestinal IgA following challenge than mice inoculated intranasally with 2/6/7-VLPs (P = 0.001, 0.008, and 0.008 [Mann-Whitney U test]). Following challenge, intestinal IgG GMTs remained relatively stable, except for a significant decrease in IgG GMT in mice inoculated intranasally with 2/6/7-VLPs (P = 0.019 [Mann-Whitney U test]). This decrease in intestinal IgG following challenge may be due to isotype interference in the ELISA due to the significant increase in IgA in these mice (Fig. 4A). Isotype interference may occur when one isotype in a given sample is more concentrated...
than another, with both isotypes being antigen specific. Interference in the detection of serum IgM due to high levels of IgG has been reported elsewhere (34, 35).

To confirm that virus replication did not occur after challenge in mice that showed no virus shedding, serum collected after virus challenge was assessed by Western blot analysis for antibody to nonstructural proteins (Fig. 1, lanes 3, 5, 7, and 9). After challenge with live ECwt virus, an antibody response would be induced to rotavirus nonstructural proteins only when virus replication occurred. In mice that were totally protected (no virus shedding) (Fig. 1, lanes 3 and 5), there was no antibody response to rotavirus nonstructural proteins, confirming that virus replication did not occur. In mice that were not protected or were partially protected from challenge (50 to 90% reduction in shedding) (Fig. 1, lanes 7 and 9), serum antibody to at least one nonstructural protein was detected, confirming that virus replication did occur.

2/6-VLPs administered intranasally without CT are immunogenic. Since CT may not be approved for use as an adjuvant in humans, we tested the immunogenicities and protective efficacies of VLPs administered intranasally without CT. Only intranasal administration was tested, since intranasal administration of VLPs with CT had yielded higher antibody titers and levels of protection than oral administration. Various doses of 2/6-VLPs were administered intranasally twice without CT. 2/6-VLPs were the only particles used, since they were shown to be sufficient for protection. As a control, one group of mice was immunized with 10 \( \mu \)g 2/6-VLPs mixed with 5 \( \mu \)g of CT.

Following the second inoculation, mice receiving PBS were seronegative (Fig. 6A). All mice inoculated with 2/6-VLPs had a serum antibody response regardless of the dose of VLP administered or the inclusion of CT. The inclusion of CT significantly enhanced the serum antibody GMT compared to an equivalent 10- \( \mu \)g dose or higher doses of 50 and 100 \( \mu \)g of VLPs without CT (\( P < 0.017, 0.012, \) and 0.043, respectively [Mann-Whitney U test]).

Vaccinated mice were tested for rotavirus-specific intestinal IgA (Fig. 6B). Following the second VLP inoculation, none of the mice inoculated with PBS and only one mouse inoculated with 10 \( \mu \)g of 2/6-VLPs without CT had intestinal IgA. All mice inoculated with 50 or 100 \( \mu \)g of 2/6-VLPs without CT had intestinal IgA to significantly higher titers than mice inoculated with 10 \( \mu \)g of 2/6-VLPs without CT (\( P = 0.012 \) and 0.012, respectively [Mann-Whitney U test]). However, mice inoculated with 50 or 100 \( \mu \)g of 2/6-VLPs without CT had the same level of intestinal IgA (\( P > 0.05 \) [Mann-Whitney U test]).
Addition of CT significantly increased the immunogenicity of VLPs. Two intranasal inoculations with 10 μg of 2/6-VLPs mixed with CT induced an IgA GMT response that was significantly higher than that seen for mice inoculated with 10 or 50 μg of 2/6-VLPs without CT (P = 0.015 and 0.029, respectively [Mann-Whitney U test]), but was not higher than that seen for mice inoculated intranasally with 100 μg of 2/6-VLPs without CT (P > 0.05).

Mice were also tested for rotavirus-specific intestinal IgG (Fig. 6C). Following the second inoculation, none of the mice inoculated with PBS or 10 or 50 μg of 2/6-VLPs without CT developed intestinal IgG. All mice inoculated with 100 μg of 2/6-VLPs without CT had an intestinal IgG response. Therefore, the intestinal IgG immunogenic dose (≥50 to ≤100 μg) was greater than the intestinal IgA immunogenic dose (≥10 to <50 μg). Addition of CT significantly decreased the immunogenic dose of VLPs. Two intranasal inoculations with 10 μg of 2/6-VLPs mixed with CT induced intestinal IgG in all mice. No significant differences in IgG GMTs in mice inoculated with 10 μg of VLPs with CT and 100 μg of VLPs without CT were observed.

2/6-VLPs administered intranasally without CT induced low levels of protection against rotavirus challenge. Mice were challenged 1 month following the second vaccination with G3 homotypic ECwt virus and protection was determined (Fig. 7). Mice inoculated with 10, 50, or 100 μg of 2/6-VLPs without CT had low mean reductions in virus shedding (20, 29, and 38%, respectively). A dose response to the VLPs was seen (P = 0.006 [linear regression]). The highest reduction in shedding (70%) was seen in mice inoculated with 10 μg of 2/6-VLPs mixed with CT, which was significantly higher than that for mice inoculated with 10, 50, and 100 μg of 2/6-VLPs intranasally without CT (P = 0.02, 0.014, and 0.014, respectively [Mann-Whitney U test]). The level of protection in mice inoculated twice with 10 μg of 2/6-VLPs mixed with CT was lower (P = 0.042 [Mann-Whitney U test]) than that seen after three inoculations with 10 μg of 2/6-VLPs mixed with CT (92% [Fig. 5]).

Following challenge, all mice developed serum antibody (Fig. 6A). Significant increases in serum antibody GMTs were observed in mice inoculated with PBS or 10 μg of 2/6-VLPs without CT (P = 0.038 and 0.006 [Wilcoxon Signed Ranks test]). Serum antibody GMTs induced by oral challenge were significantly lower in mice inoculated with PBS than those in mice inoculated with 10, 50, or 100 μg of 2/6-VLPs without CT or with 10 μg with CT (P = 0.006, 0.022, 0.013, and 0.019, respectively [Mann-Whitney U test]). Following challenge, intestinal rotavirus-specific IgA GMTs increased significantly in PBS control mice (P = 0.013 [Wilcoxon Signed Ranks test]) (Fig. 6B). Rotavirus-specific intestinal IgG GMTs did not change significantly in any group (P > 0.05 [Wilcoxon Signed Ranks test]) (Fig. 6C).

DISCUSSION

We assessed the immunogenicities and protective efficacies of rotavirus VLPs administered orally and intranasally. VLPs administered by both routes induced serum and intestinal antibody and partial to total protection from rotavirus infection. Although oral administration of some immunogens leads to tolerance (10, 45, 61), oral administration of rotavirus VLPs induced protective immunity, not tolerance. We obtained similar results following oral administration of Norwalk virus-like particles (rNV VLPs) (2). The nontolerogenic properties of VLPs may be due to their particulate nature, since particulate antigens induce tolerance less often than soluble antigens (3).

We used the adult mouse model of rotavirus infection to assess the immunogenicities and protective efficacies of VLPs administered orally and intranasally. Protective efficacy in mice was measured based on decreased antigen, not infectious virus shedding, because of the use of non-tissue culture-adapted ECwt virus as the challenge virus. As measured by FFA, the infectivity of ECwt virus in MA104 cells is extremely limited; the SD50 titer of the ECwt inoculum in mice exceeds the virus titer in MA104 cells by at least three logs. We attempted but were unable to detect by FFA infectious virus in mouse fecal samples (data not shown). Therefore, we could not determine how reductions in antigen shedding correlated with reductions of infectious virus shedding. However, similar studies of rabbits immunized parenterally with VLPs and challenged with tissue culture-adapted Alabama virus have shown a correlation between the level of rotavirus antigen excretion measured by the magnitude of OD readings in ELISA and reductions in virus infectivity measured by FFA (12).

The adult mouse model is an infection rather than a disease model. Adult mice do not develop diarrhea following rotavirus infection. Protection from infection may be a more stringent measure of protection than protection from disease, since infection can occur in the absence of disease (16). In our study, VLPs induced total to partial protection. Although our results are encouraging, it is not yet clear how the protection from infection induced by the VLPs in the mouse model will predict protective efficacy in a rotavirus disease model, such as piglets or humans. Partial protection from infection may be sufficient to reduce the severity of disease in a child. Although our mouse challenge dose was 10-fold higher than the infectious dose, additional studies are needed to determine whether immunization with VLPs will provide protection from higher challenge doses. Based on the efficacy of VLPs in the mouse...
model, further testing of mucosal administration of VLPs in piglets is warranted.

Although rotavirus VLPs alone were immunogenic and induced partial protection, the coadministration of CT increased the immunogenicity and lowered the protective dose of VLPs administered intranasally. 2/6-VLPs administered intranasally without CT induced significantly lower levels of serum antibody and intestinal IgA than 2/6-VLPs administered intranasally with CT. No intestinal IgG was induced with doses of 10 or 50 μg of VLPs. These significantly reduced titers of antibody induced without CT could account for the lower level of protection seen in mice immunized with VLPs in the absence of CT. To achieve high levels of protection against rotavirus with a mucosally administered VLP vaccine, doses at or exceeding 100 μg of VLPs or a mucosal adjuvant will be needed. Immunogenicity studies in the absence of CT with orally administered rNV VLPs have shown that VLP doses at or exceeding 200 μg are required to achieve antibody responses in all animals (2). Since toxigenic CT is unlikely to be approved for use in humans, other mucosal adjuvants, such as Escherichia coli labile toxin (LT) or reduced or nontoxic mutant forms of CT or LT, are being assessed (24, 25). Recent results in our lab have shown that 2/6-VLPs administered intranasally with LT or a nontoxic mutant of LT (LT-R192G) induce levels of antibody and protection equivalent to or higher than those of VLPs with CT (54). These results show promise for use of VLPs in humans, since LT-R192G or other mutant toxins may be approved for use in humans. Alternatively, other formulations of VLPs, such as microencapsulation, to increase the targeting or uptake of VLPs to Peyer’s patches may be effective without the need for an adjuvant, as recently shown with live or inactivated rotavirus (7, 39, 52).

Mucosal administration of VLPs offers advantages over parenteral immunization, including ease of delivery and elimination of the cost and need of needles. In addition, our results suggest that intranasal administration of rotavirus VLPs may be advantageous in children by reducing the vaccine dose, thereby reducing the cost of each dose. The increased immu-

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**FIG. 6.** Serum and intestinal antibody responses to 2/6-VLPs administered intranasally; Total serum (A) and intestinal IgA (B) and intestinal IgG (C) antirotavirus antibody in mice receiving two intranasal inoculations of 2/6-VLPs at the doses indicated along the x axis. Addition and dose of CT are also indicated along the x axis. Samples were collected 45 DPI and 23 DPC. Antibody titers were measured for individual mice, and the results are plotted as the GMTs of the groups. The numbers of mice having a greater than 50% reduction in virus shedding following challenge are indicated. Significant differences in GMTs within a panel are indicated by the symbols.

**FIG. 7.** Protection from rotavirus challenge in mice receiving two intranasal inoculations of 2/6-VLPs with and without CT. Mice were orally challenged on day 45 with 10 SD50 of ECwt. Stool samples were collected daily and quantitated for levels of rotavirus antigen by ELISA. Virus shedding curves were plotted for individual mice, and the mean area under the curve for each group was calculated. Results are plotted as percent reduction in shedding for each individual animal (n = 4 or 5 per group), as well as the mean for each vaccine group ( ), which was calculated by comparing the mean area under the curve of each vaccine group to the mean area under the curve of the control group. Significant differences in reduction in shedding are indicated by the symbols.
nogeneity and protection that we observed following intranasal administration of VLPs may be due to (i) less antigen degradation in the respiratory tract because of differences in the proteolytic environment compared to the intestine, (ii) increased antigen retention and interaction with lymphoid cells in the respiratory tract compared to the intestine, or (iii) inherent differences in uptake and processing of antigen administered orally and intranasally.

Although rotavirus VLPs were potent inducers of antibody and protection following intranasal administration, similar responses have not been observed with all immunogens. Induction of intestinal antibody following intranasal inoculation of an immunogen occurs through the common mucosal immune system (46, 58, 69), but it appears that induction of intestinal antibody following intranasal inoculation may be antigen specific or regulated by other mechanisms that are not yet understood. Intranasal inoculation of humans with CT B, or of mice with dextran-CT B conjugates or a human immunodeficiency virus gp120 peptide mixed with CT, induced little to no intestinal antibody (4, 57, 62), while intranasal inoculation of mice with influenza virus hemagglutinin combined with CT B or streptococcal surface protein with CT B or with diphtheria toxoid all induced significant levels of intestinal antibody (32, 36, 71). It is not clear why only a subset of immunogens are able to induce intestinal antibody following intranasal inoculation, but intranasally administered rotavirus VLPs are potent inducers of intestinal antibody.

The role of neutralizing antibody in protection from rotavirus is controversial (for a review, see reference 18). A role for neutralizing antibody in protection from rotavirus infection is supported by the passive protection of suckling mice from rotavirus diarrhea due to (i) oral administration of neutralizing anti-VP4 or VP7 monoclonal antibodies, but not anti-VP6 antibodies, or (ii) lactogenic antibodies induced in dams to baculovirus-expressed VP4 or VP7 or adenovirus- or vaccinia virus-expressed VP7 (1, 5, 28, 43, 44, 53). However, the role of neutralizing antibody in active immunity is not clear. Neutralizing antibody has correlated with protection from infection or disease in humans and piglets in some studies (11, 37, 55, 66), while protection in calves, humans, piglets, and mice has not correlated with serum or intestinal neutralizing antibody in other studies (6, 14, 68, 70, 72). The failure of monovalent animal or animal × human reassortant live virus vaccines to induce significant protection in children is attributed to failure to induce heterotypic protective antibody to multiple serotypes of virus and has resulted in the belief that a successful rotavirus vaccine will need to induce neutralizing antibody to multiple serotypes of rotavirus (29, 56).

VLPs provided a means to directly test the role of neutralizing antibody in active protection. The observation that 2/6- and 2/6/7-VLPs induce equivalent levels of protection in mice immunized by either of two mucosal routes indicates that neutralizing antibody is not an absolute requirement for protection from infection in mice. These results are consistent with the observation that nonneutralizing VP6-specific IgA can protect against rotavirus challenge when administered in a backpack model, but not when administered orally (9). It is not clear how nonneutralizing antibodies to 2/6-VLPs induce protection. It is possible that (i) the lack of neutralization by anti-VP6 or -VP2 antibodies in vitro does not faithfully reflect neutralization in vivo, (ii) intracellular neutralization may occur (9), or (iii) other immune mechanisms such as cytotoxic lymphocytes (CTLs) may play a role in the protection induced by active immunization with 2/6-VLPs. Although VP7 and VP4 are not absolutely required to induce protection, it is possible that the addition of VP4, the viral attachment protein, enhances the immune response to mucosally administered VLPs and increases protection by stimulating binding of the VLPs to rotavirus cell receptors (22).

It has recently been reported that long-term protection of mice from rotavirus infection is dependent on the presence of antibody (30, 31, 47) and that protection from infection or disease in mice and piglets correlates with intestinal IgA following inoculation with live virus (27, 72). We determined correlates of protection by calculating Pearson’s correlation coefficients of protection versus various antibody responses. Correlation coefficients were determined by including results from all animals receiving VLPs orally or all animals receiving VLPs intranasally regardless of the dose of VLPs or the presence of adjuvant. Combining results from different immunization groups was necessary, because correlation coefficients could be calculated only when a range of protective efficacy was achieved. In our experiments, the correlates of protection differed, depending on the route of administration of the VLPs. Protection following oral administration of VLPs correlated with the presence of either serum antibody (P = 0.001 [Pearson’s correlation coefficient]), intestinal IgA (P = 0.012), or intestinal IgG (P = 0.012). Following intranasal administration of VLPs, protection correlated with serum antibody (P < 0.001) and intestinal IgG (P < 0.001) but did not correlate with intestinal IgA (P = 0.575). The differences in immune correlates of protection induced by oral and intranasal administration of VLPs may reflect the predominance of IgG in the lung (48). Therefore, intranasal immunization may preferentially induce IgG responses to VLPs. Similar results were seen following infection with coronavirus in pigs. Following intranasal inoculation with live porcine respiratory coronavirus, enhanced numbers of IgG-antibody secreting cells were seen in the gut lamina propria and mesenteric lymph nodes compared to pigs inoculated orally with live transmissible gastroenteritis virus (65).

Although we found that VLPs-induced antibody and antibody titers correlated with protection, it is not known if VLPs can induce CTLs. CTLs can mediate passive and short-term active protection from rotavirus infection (23, 31, 51). Therefore, we cannot exclude the possibility that CTLs also play a role in protection following inoculation with VLPs. Studies are underway to determine if CD8 T cells play a role in the protection induced by VLPs. Overall, our new results reemphasize the need to identify immune correlates for protection from rotavirus infection which continue to be unclear.

Oral and intranasal administration of VLPs induced high GMTs of rotavirus-specific intestinal IgG and low GMTs of IgA. The ratio of intestinal IgA to IgG differs, depending on the route of administration of VLPs. Protection following oral administration of VLPs correlated with the presence of serum antibody (P = 0.001) whereas protection following intranasal administration of VLPs correlated with intestinal IgG (P < 0.001) but not with serum antibody (P = 0.575). The differences in immune correlates of protection induced by oral and intranasal administration of VLPs may reflect the predominance of IgG in the lung (48).

VLPs have been shown to induce antibody and protection against rotavirus infection in both mice and pigs. Oral and intranasal administration of VLPs have been shown to induce antibody and protection against rotavirus infection in both mice and pigs. Oral and intranasal administration of VLPs have been shown to induce antibody and protection against rotavirus infection in both mice and pigs.
2/6-VLPs had high levels of serum antibody, but no intestinal IgG (Fig. 7). Although this lack of correlation does not rule out that some IgG transudates from the serum, there is evidence for local production of IgG in the intestine. Oral inoculation of mice or piglets with live rotavirus (15, 72), intramuscular inoculation of mice with live or inactivated rotavirus (15), or intramuscular inoculation of rabbits with VLPs (41) induces IgG-specific antibody-secreting cells in the intestine. The mechanism of IgG-mediated protection in the intestine needs further investigation.

VLPs administered mucosally protect against rotavirus infection and are a useful model to probe induction of immune responses to particulate antigens given at mucosal sites. 2/6-VLPs can be produced in yields sufficient for use as a vaccine. The immune response to 2/6-VLPs is long lasting: 6 months after oral immunization, serum and intestinal antibody to rotavirus can still be detected (unpublished observation). VLPs administered mucosally offer a promising, safe, and non-replicating candidate vaccine for rotavirus.

ACKNOWLEDGMENTS

We thank Andrea Bertolotti-Ciarlet for production of the VLPs, Christopher Barone for assistance with inoculations and sampling of mice, Juan Alvarado for assistance in processing of fecal samples, and Robert Atmar for helpful discussions.

This work was supported by Public Health Service grant AI24998 from the National Institute of Allergy and Infectious Disease and Public Health Service training grant T32-DK07664 from the National Institute of Digestive Diseases.

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