An Oral versus Intranasal Prime/Boost Regimen Using Attenuated Human Rotavirus or VP2 and VP6 Virus-Like Particles with Immunostimulating Complexes Influences Protection and Antibody-Secreting Cell Responses to Rotavirus in a Neonatal Gnotobiotic Pig Model

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We determined the impact of mucosal prime/boost regimens and vaccine type (attenuated Wa human rotavirus [AttHRV] or nonreplicating Wa 2/6 rotavirus-like particles [VLP]) on protection and antibody-secreting cell (ASC) responses to HRV in a neonatal gnotobiotic pig disease model. Comparisons of delivery routes for AttHRV and evaluation of nonreplicating VLP vaccines are important as alternative vaccine approaches to overcome risks associated with live oral vaccines. Groups of neonatal gnotobiotic pigs were vaccinated using combinations of oral (PO) and intranasal (IN) inoculation routes as follows: (i) 3 oral doses of AttHRV (AttHRV3×PO); (ii) AttHRV3×IN; (iii) AttHRVP0, then 2/6VLP2×IN; (iv) AttHRVIN, then 2/6VLP2×IN; and (v) mock-inoculated controls. Subsets of pigs from each group were challenged with virulent Wa HRV [P1A(8) G1] (4 weeks post-primary inoculation) to assess protection. The AttHRVPO+2/6VLP2×IN pigs had the highest protection rates against virus shedding and diarrhea (71% each); however, these rates did not differ statistically among the vaccine groups, except for the AttHRVIN+2/6VLPIN group, which had a significantly lower protection rate (17%) against diarrhea. The isotype, magnitude, and tissue distribution of ASCs were analyzed by enzyme-linked immunospot assay. The highest mean numbers of virus-specific IgG and IgA ASCs were observed pre- and postchallenge in both intestinal and systemic lymphoid tissues of the AttHRVPO+2/6VLPIN group. Thus, the AttHRVPO+2/6VLPIN vaccine regimen using immunostimulating complexes (ISCOM) and multiple mucosal inductive sites, followed by AttHRV3×PO or IN regimens, were the most effective vaccine regimens, suggesting that either AttHRVPO+2/6VLPIN or AttHRV3×IN may be an alternative approach to AttHRV3×PO for inducing protective immunity against rotavirus diarrhea.

Safety is a major concern in the use of live vaccines due to potential adverse effects. However, immune responses at mucosal sites are usually most effectively induced in naïve hosts by the administration of living microorganisms. In contrast, non-replicating vaccines administered orally (PO) require an effective mucosal adjuvant and delivery system to enhance the immunogenicity of the vaccines and to avoid oral tolerance.

Rotavirus is the most common cause of infantile gastroenteritis worldwide. Each year, rotavirus causes approximately 25 million clinic visits, 2 million hospitalizations, and 500,000 deaths in children <5 years of age (29). Although two new live attenuated rotavirus (RV) vaccines are licensed, there is an urgent need to develop rotavirus vaccines that are more efficacious in developing countries than the existing vaccines (17, 37). The association of an increased risk of intussusception with the rhesus × human rotavirus reassortant vaccine Rotashield prompted concerns related to possible side effects of live rotavirus vaccines, including excessive virus shedding, fever, diarrhea, vomiting, and irritability, prompting the development of alternative vaccine strategies. Virus-like particles (VLP) composed of rotavirus inner capsid proteins VP2 and VP6 (2/6VLPs) constructed by coexpressing rotavirus gene 2 and gene 6 in a baculovirus expression system using Spodoptera frugiperda (Sf9) insect cells are one strategy (11, 15, 39). However, these 2/6VLP vaccines do not induce rotavirus-neutralizing antibodies. In our previous studies, a vaccine using intra-
nasal (IN) 2/6VLP (2/6VLPIN) (RF VP2, Wa VP6) with mutant Escherichia coli heat-labile toxin (mLT) adjuvant was immunogenic but not protective in gnotobiotic pigs (39). Also, three oral doses of a vaccine of 2/6VLP (2/6VLP3×PO) with immunostimulating complexes (ISCOM) were less immunogenic than three doses of 2/6VLPIN vaccine, but both failed to induce protection against rotavirus diarrhea or shedding (18). However, partial protection against diarrhea and virus shedding was induced when 2/6VLPIN+mLT or 2/6VLPPO+ISCOM was used as a booster vaccine in pigs that were previously primed orally with live attenuated Wa HRV (AttHRVPO+2/6VLPIN+mLT or AttHRVPO+2/6VLPPO+ISCOM) (18, 40). The efficacies of these two vaccine regimens against rotavirus diarrhea (44% and 50%, respectively) were similar or slightly lower than that induced by three oral doses of live attenuated Wa HRV vaccine in pigs (50 to 67%) (18, 40, 41, 43). These results suggest that the prime/boost vaccine regimens may be a more effective approach than multiple doses of either live or VLP vaccines alone.

Respiratory symptoms and rotavirus shedding in nasopharyngeal secretions of children have been reported (26, 31), and we previously demonstrated shedding of AttHRV in the respiratory tract of gnotobiotic pigs. Because lymphocytes sensitized in the nasal lymphoid tissue (NALT) can relocate to distant effector sites through the common mucosal immune system (6), the respiratory tract should be explored further as a possible route to improve rotavirus vaccine efficacy. In this study, we first evaluated the dose response to 2/6VLPIN boosting after priming with AttHRVPO. We then assessed the effect of PO versus IN priming with AttHRV using the optimized 2/6VLPIN booster dose. We further compared the impact of IN versus PO priming and boosting using AttHRV alone. Such vaccine regimens, although requiring priming with live AttHRV, may at least reduce the risk associated with live virus boosters or, by using IN delivery, avoid side effects or interference (i.e., intestinal parasites, maternal antibodies, etc.) more prominent in infants in developing countries when live oral rotavirus vaccines are used. Accordingly, the use of 2/6VLP boosters given IN might overcome some of the suppressive effects of maternal antibodies on live oral RV vaccines (19, 25). Bertolotti-Ciarlet et al. (4) showed that oral administration of homologous or heterologous 2/6VLPs to CD-1 mice with or without adjuvant induced low protection rates against rotavirus challenge (10 and 39%), whereas intranasal administration induced higher protection rates (85 and 84%). Their findings together with the findings of others (5) suggest that the harsh environment of the gastrointestinal (GI) tract (3, 34), including the low pH and presence of digestive enzymes which influence the degradation of protein antigens, may account for the differences observed between immunizations using these two routes. Thus, less degradation of VLPs occurs when they are given IN compared to when they are given PO, which may permit lowering of the VLP dosage. The use of an effective and age-appropriate mucosal adjuvant such as ISCOM may also increase the efficacy of the nonreplicating 2/6VLP rotavirus vaccine. Intrarectal immunization was also used to avoid GI degradation. In adult mice, intrarectal immunization using 2/6VLP induced protection against rotavirus shedding and higher intestinal immune responses (1). Similarly, intrarectal inoculation of 8-2/6/7VLP induced complete protection against rotavirus shedding in the same model when administered with LT or cholera toxin (CT) adjuvants (30).

The gnotobiotic pig is the only animal model susceptible to HRV diarrhea for at least the first 8 weeks of age, the time necessary to assess protective immunity against disease upon challenge (44), whereas other animal models are susceptible to rotavirus diarrhea only up to 14 to 21 days of age (9, 10). Studies of our selected candidate rotavirus vaccines and new vaccine strategies in neonatal gnotobiotic pigs that mimic immune responses of infants (33) should generate data potentially applicable for the development of similar rotavirus vaccines for infants. Our findings should further improve our understanding of effective prime/boost strategies comparing live versus nonreplicating vaccines and PO versus IN routes to induce intestinal immunity in neonates.

(Materials and Methods)

Virus.

The attenuated cell culture-adapted strain of Wa HRV [PIA(6) G1], derived from the 26th passage in African green monkey kidney cells (MA104), was used for inoculation of the gnotobiotic pigs at a dose of 5×10⁷ fluorescent focus-forming units (FFU) and was also used for the immune assays (44). The virulent Wa HRV from pooled intestinal contents of gnotobiotic pigs was used as a challenge virus at a dose of 10⁶ 50% infectious doses (ID₅₀). The ID₅₀ of the virulent Wa HRV inoculum for gnotobiotic pigs was previously determined to be at least 1 FFU (36). 2/6VLPs and ISCOM. The recombinant baculoviruses expressing the individual rotavirus proteins VP2 from the bovine RF strain (21) and VP6 from the Wa HRV strain were constructed using the pBlueBac 4.5 system and used to express 2/6VLPs by coinfection of Sf9 cells (13). The 2/6VLPs were purified and characterized as previously described, including examination of the 2/6VLP-ISCOM vaccine by immunoelectron microscopy (IEM) before inoculation (18).

Inoculation and challenge of gnotobiotic pigs. Near-term pigs were surgically obtained and maintained in gnotobiotic isolator units as described elsewhere (23). At 3 to 5 days of age, pigs were assigned to a dose-response study or subsequently to a study comparing different prime/boost strategies and routes of vaccination.

(i) Dose-response study of 2/6VLP booster vaccine. To assess the effect of the 2/6VLP booster doses in enhancing protection and ASC responses after PO AttHRV priming, a dose-response study was conducted. Pigs in three different groups received 2/6VLP booster doses with ISCOM adjuvant (1,250 µg) as follows: (i) 25 µg (AttHRV2/6VLP25), (ii) 100 µg (AttHRV2/6VLP100), and (iii) 250 µg (AttHRV2/6VLP250). At postinoculation day (PID) 28, subsets of pigs were challenged with virulent Wa HRV and observed daily for diarrhea and virus shedding (44). Pigs from each group were euthanized at PID28/postchallenge day (PCD) 0 and PID35/PCD7. All procedures were conducted in accordance with protocols reviewed and approved by the Ohio State University’s Institutional Laboratory Animal Care and Use Committee.

(ii) Comparison of different vaccine inoculation routes, vaccine types (attenuated RV versus nonreplicating 2/6VLPs), and prime/boost strategies. Based on the highest protection rate and numbers of intestinal antibody-secreting cells (ASC) in the dose/response study for the 2/6VLP booster vaccine, 2/6VLPIN was selected for the subsequent studies. Pigs were assigned to one of four vaccination groups or to a medium or ISCOM matrix control group and inoculated at 10-day intervals between doses, as follows: (i) three oral doses of attenuated (Att) Wa HRV (AttHRV3×PO), (ii) three IN doses of attenuated Wa HRV (AttHRV3×IN), (iii) one oral dose of Wa AttHRV followed by two IN doses of 250 µg of 2/6VLP + 1,250 µg ISCOM (AttHRVPO+2/6VLPIN), (iv) one IN dose of attenuated Wa HRV followed by two IN doses of 250 µg of 2/6VLP + 1,250 µg ISCOM (AttHRV+2/6VLPIN), and (v) three IN doses of ISCOM matrix or oral minimal essential medium (MEM) (controls). Pigs were challenged and protection was assessed as described in the above dose-response study.

Isolation of MNC and ELISPOT assay. For the isolation of mononuclear cells (MNC), the small intestines (duodenum and ileum), mesenteric lymph nodes
The numbers of ASC were fixed MA104 cell enzyme-linked immunospot (ELISPOT) assay for quantitating PID35/PCD7 and processed as previously described (35, 44). An HRV-infected, (MLN), spleen, and blood were collected from pigs euthanized at PID28 and challenged with virulent Wa HRV at a dose of 10⁶ ID₅₀, which causes diarrhea in nearly 100% of control pigs upon challenge as previously determined (36).

Viral shedding was detected by antigen capture enzyme-linked immunosorbent assay (ELISA) and cell culture immunofluorescence infectivity assay. Determined by ELISA and cell culture immunofluorescence infectivity assay.

Statistical analysis. The proportions of pigs with diarrhea and virus shedding were determined using Fisher’s exact test. One-way analysis of variance (ANOVA) was used to compare mean cumulative diarrhea scores, mean duration of virus shedding and diarrhea, and mean peak titers of virus shed. The ASC numbers were compared among and within the groups on PID28/PCD0 and PID35/PCD7 using the Kruskal-Wallis rank sum test (nonparametric). Statistical significance was assessed at P < 0.05 throughout.

RESULTS

(i) 2/6VLPIN booster dose/response study. Different booster doses of 2/6VLP induced a dose-response effect on the protection rates against virus shedding in pigs primed PO with AttHRV. Protection against diarrhea was observed only with doses of 2/6VLP ≥ 100 μg, with the highest protection rate against diarrhea (71%) also associated with the highest dose of 2/6VLP (250 μg) tested (Table 1). The protection rates against virus shedding were statistically similar (P > 0.05) in all three groups receiving different IN doses of 2/6VLP as boosters; however, the highest rate of protection (71%) was associated with the highest dose (250 μg) of 2/6VLP booster tested (Table 1). The duration of virus shedding was significantly decreased in the group receiving the 250-μg dose of 2/6VLP.

Different booster doses of 2/6VLP induced a dose-response effect on the antibody-secreting cell (ASC) numbers in pigs primed PO with AttHRV. Intestinal (duodenum, ileum, and mesenteric lymph nodes) and systemic (spleen and peripheral blood) ASC responses at PID28/PCD0 and PID35/PCD7 are depicted in Fig. 1. The IgA ASC responses in the duodenum and ileum increased proportionally with increasing 2/6VLP booster doses, with significantly higher numbers of IgA and IgG ASC at the 250-μg dose level than at the 25- and 100-μg dose levels (Fig. 1). In systemic lymphoid tissues, only IgA ASC numbers in the spleen were significantly increased at the highest dose of 2/6VLP (250 μg). Thus, intestinal ASC immune responses required higher 2/6VLP booster doses to be optimally stimulated, whereas for systemic ASC responses, a plateau was reached with lower doses.

(ii) Comparison of different vaccine inoculation routes, vaccine types, and prime/boost strategies. AttHRVPO or IN induced primarily nasal shedding, and ISCOM was a safe IN mucosal adjuvant in neonatal pigs. After IN or PO priming or boosting with AttHRV, no clinical signs were observed. Moreover, nasal and rectal shedding of AttHRV was detected in 95% and 5% of the AttHRVIN pigs and 79% and 17% of the AttHRVPO pigs after the priming dose, respectively. Details of nasal and fecal virus shedding data after AttHRVPO or IN inoculation were reported elsewhere (2). No adverse effects were observed in neonatal pigs after receiving 2/6VLPIN+ISCOM (or ISCOM matrix alone in the control pigs) in the booster vaccines.

The highest protection rates against virus shedding and diarrhea were induced by the live virus priming PO followed by 2/6VLPIN booster vaccine. Clinical signs and virus shedding data are summarized in Table 2. Protection against diarrhea and virus shedding after challenge with virulent Wa HRV was compared among vaccine groups and between vaccinated and control groups. Virus shedding and diarrhea, respectively, after challenge were observed in 29% and 29% of the AttHRVPO+2/6VLPIN pigs, 33% and 56% of the AttHRV3×PO pigs, 33% and 50% of the AttHRV3×IN pigs, 50% and 83% of the AttHRVIN+2/6VLPIN pigs, and 100% and 100% of the control group. Protection rates against virus shedding and diarrhea were highest in the AttHRVPO+2/6VLPIN group but did not differ significantly from those of the AttHRV3×PO or IN groups. The mean durations of virus shedding differed significantly among the vaccine groups and compared to those of controls: the AttHRVPO+2/6VLPIN and AttHRV3×IN groups had the shortest mean dura-

### TABLE 1. Dose-response study of 2/6VLPIN booster vaccine and impact on virus shedding and diarrhea in gnotobiotic pigs after challenge with virulent Wa HRV

| Treatment                  | Virus shedding | Diarrhea | Protection rate (% against: | % with diarrhea | Mean duration in days | Mean cumulative score |
|----------------------------|----------------|----------|-----------------------------|-----------------|---------------------|----------------------|
| AttHRV-2/6VLP (25 μg)      |                |          |                             |                 |                     |                      |
| Controls (diluent or ISCOM)|                |          |                             |                 |                     |                      |

**Note:** Mean days to onset of shedding, Mean duration in days, and mean cumulative score were determined using Fisher’s exact test. One-way analysis of variance (CCIF) assay of processed rectal swab fluids as described previously (32, 44).

**Statistical significance was assessed at P < 0.05 throughout.**
tions, followed by those of the AttHRV3/H11003 and AttHRVIN/H11001 groups, all of which were significantly shorter than those of the controls. Similar results were observed for the mean cumulative scores of diarrhea, the lowest of which were detected in the AttHRV3/H11003 IN group, followed by the AttHRVPO-2/6VLPIN group, but both groups had significantly lower scores than the challenged controls.

In summary, although statistically the protection rates did not differ significantly among groups, numerically the highest protection rates against virus shedding were observed in the AttHRVPO-2/6VLPIN group vaccinated using the combined mucosal routes (PO/IN) and vaccine types (live AttHRV/H11001 nonreplicating VLP), followed by the AttHRV groups vaccinated using single routes (PO/PO or IN/IN) of administration. Notably, boosting with a live attenuated virus or a nonreplicating VLP vaccine did not alter the protection rates against virus shedding among the groups. For protection against diarrhea, the combined PO/IN route and vaccine types (live/H11001 nonreplicating) induced the highest protection rates against diarrhea, compared to the single

TABLE 2. Virus shedding and diarrhea in gnotobiotic pigs after challenge with virulent Wa HRV

| Vaccine regimen | n | % shed | Mean days to onset of shedding | Mean duration in days | % with diarrhea | Mean duration in days | Mean cumulative score | Protection rate (%) against: |
|-----------------|---|---------|-------------------------------|----------------------|----------------|----------------------|------------------------|-----------------------------|
| AttHRV3×PO     | 9 | 33 B    | 2.3 B                         | 0.7 B                | 56 B          | 1.4 B                | 7.4 BC                 | 67                          |
| AttHRV3×IN     | 6 | 33 B    | 2.0 B                         | 0.3 C                | 50 B          | 1.0 B                | 6.0 C                  | 67                          |
| AttHRVPO-2/6VLPIN | 7 | 29 B    | 2.0 B                         | 0.4 C                | 29 B          | 0.7 C                | 6.4 C                  | 71                          |
| AttHRVIN-2/6VLPIN | 6 | 50 B    | 2.3 B                         | 0.7 B                | 83 A          | 1.2 B                | 7.5 BC                 | 50                          |
| Controls       | 12| 100 A   | 1.6 A                         | 2.5 A                | 100 A         | 2.8 A                | 9.4 AB                 | 0                           |

*a* Determined by ELISA and cell culture immunofluorescence infectivity assay.

*b* Protection rate = [1 – (percentage of vaccinated pigs in each group with diarrhea/percentage of control pigs with diarrhea)] × 100. Protection data were partially presented previously by Gonzalez et al. (16). Gonzalez et al. reported the protection rates against diarrhea and virus shedding in pigs primed with AttHRVPO and boosted with 25 μg or 250 μg of 2/6VLP-ISCOM IN and their correlation with virus neutralizing antibody titers in serum and serum and fecal antibody isotypes and titers.

*c* Duration of diarrhea determined by number of days with fecal scores greater than or equal to 2. Feces were scored as follows: 0, normal; 1, pasty; 2, semiliquid; 3, liquid.

*d* Mean cumulative score = (sum of fecal consistency scores for 6 days postchallenge)/n.

*e* n, number of pigs/group.

*f* Proportions in the same column followed by different letters differ significantly (Fisher’s exact test). A, statistically different from B at $P < 0.05$.

*g* Means in the same column followed by different letters differ significantly (One way ANOVA). A, statistically different from B and C; B, statistically different from C at $P < 0.05$. 

FIG. 1. Prechallenge antibody-secreting cell responses in gnotobiotic pigs primed with AttHRVPO and boosted IN with various doses (25, 100, 250 μg) of 2/6VLP in a dose-response study. Different letters denote statistically significant differences at $P < 0.05$ in the same tissue for the same isotype, among vaccine groups. Statistical symbols: A, statistically different from B and C; B, statistically different from C at $P < 0.05$. 

FIG. 2. Postchallenge antibody-secreting cell responses in gnotobiotic pigs primed with AttHRVPO and boosted IN with various doses (25, 100, 250 μg) of 2/6VLP in a dose-response study. Different letters denote statistically significant differences at $P < 0.05$ in the same tissue for the same isotype, among vaccine groups. Statistical symbols: A, statistically different from B and C; B, statistically different from C at $P < 0.05$. 

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routes with AttHRV alone. Moreover, the use of the IN route for AttHRV priming followed by IN boosting with the nonreplicating 2/6VLP vaccine induced a significantly lower \( (P < 0.05) \) protection rate against diarrhea (Table 2).

The route of inoculation and type of vaccine booster influenced the prechallenge ASC responses to HRV vaccines. Intestinal (duodenum, ileum, and mesenteric lymph nodes) and systemic (spleen and peripheral blood) ASC responses at PID28/PCD0 and PID35/PCD7 are depicted in Fig. 2 and 3. Oral priming with AttHRV vaccine and IN boosting with the nonreplicating 2/6VLP vaccine induced the highest local and systemic IgA ASC responses prechallenge in the gnotobiotic pigs.

(i) Intestinal ASC responses. The use of a single route of inoculation (PO) induced significantly higher ileal IgM ASC responses, whereas the use of combined routes (PO priming and IN boosting) induced significantly higher duodenal and ileal IgA ASC responses prechallenge. Only in ileum were numbers of IgM ASC significantly different among the vaccine groups, with the highest IgM ASC counts in the AttHRV3×PO group (Fig. 2). However, for IgA ASC, significantly higher numbers were detected in the duodenum and ileum of the AttHRVPO-2/6VLPIN group than in the other vaccine groups and controls. Significantly higher numbers of IgG ASC were also detected in duodenum and in MLN of the AttHRVPO-2/6VLPIN group than in all other groups. The impact of the 2/6VLPIN versus AttHRVPO or IN boosters on ASC responses was reflected by the higher mean numbers of IgA (2.9- to 11-fold, respectively) and IgG (4.2- to 12-fold, respectively) ASC in the AttHRVPO-2/6VLPIN group than in the AttHRV3×PO and AttHRV3×IN groups prechallenge.

(ii) Systemic ASC responses. Systemic ASC responses were lower overall for all vaccine groups and lowest for peripheral blood mononuclear cells (PBMC) but with no significant differences among groups. Significantly higher numbers of IgA ASC were detected in the spleens of the AttHRVPO-2/6VLPIN and AttHRVIN+2/6VLPIN groups, whereas significantly higher IgG ASC numbers were detected in spleens of the AttHRVPO+2/6VLPIN group than in the other groups. No significant differences were observed for IgM ASC in any systemic lymphoid tissues among the vaccine groups (Fig. 2).

Thus, comparing the groups primed with live AttHRVPO or IN and boosted with 2/6-VLP versus live virus, significantly higher IgA and IgG ASC numbers were consistently observed in the 2/6VLPIN booster groups than after the oral booster route with live virus alone (AttHRV3×PO). Similarly, in ileum and spleen, the AttHRVIN priming and 2/6VLPIN booster vaccine induced significantly higher IgA ASC than AttHRV×3IN.

The route of inoculation and type of vaccine booster influence the postchallenge ASC responses to HRV vaccines. (i) Intestinal ASC responses. The use of a single route of inoculation (PO or IN) induced significantly higher ileal IgM ASC
responses, whereas the use of combined routes (PO priming and IN boosting) induced significantly higher duodenal and higher ileal IgA ASC responses postchallenge. Significantly higher IgM ASC numbers were observed in the duodenum for both groups using only IN routes (AttHRVIN+2/6VLPIN, AttHRV3×IN) and in the ileum for all single-route (PO or IN) vaccine groups than in the controls (Fig. 3).

The combination of PO AttHRV priming and IN 2/6VLP boosting routes (AttHRVPO+2/6VLPIN) induced significantly higher IgA ASC numbers in the duodenum and MLN and significantly higher numbers of IgG ASC in the ileum and MLN of this group. The numbers of IgG ASC increased significantly in the duodenum and ileum postchallenge compared to prechallenge (4- and 5-fold, respectively) of the same groups (Fig. 2 and 3). The use of routes other than oral for boosting may be important to induce more effective gut and systemic immune responses, with higher gut immune responses being induced by the use of oral priming.

**DISCUSSION**

In our previous studies, Gonzalez at al. (16) reported the protection rates against diarrhea and virus shedding of pigs primed with AttHRVPO and boosted with 25 μg or 250 μg of 2/6VLPIN-ISCOM and their correlation with virus-neutralizing antibody titers in serum and serum and fecal antibody isotypes and titers, compared to three oral doses of AttHRV. Pigs receiving 3 doses of AttHRV or AttHRV-2/6VLP250 showed the highest protection rates against diarrhea upon challenge. Intestinal IgA antibody titers were significantly higher in the AttHRV-2/6VLP250 group than in all other groups. Iosef et al. (18) examined protection rates and antibody-secreting cell responses in pigs primed orally with AttHRV and boosted orally with 250 μg of 2/6VLP-ISCOM. In that study, using the oral route to prime and boost, the highest
mean numbers of IgA ASC in intestinal lymphoid tissues were in pigs receiving AttHRV-2/6VLP or 3 doses of AttHRV and were associated with the highest protection rates (75% protection against virus shedding and 50% protection against diarrhea). In the present study, unlike in the prior pig studies, we examined and compared the intranasal route to prime and boost with AttHRV only or AttHRV-2/6VLP vaccines using the AttHRV3×PO vaccine as a positive control to mimic the current live attenuated rotavirus vaccines.

In comparing three different doses of 2/6VLP booster vaccine given IN after AttHRVPO priming, the level of intestinal ASC increased proportionally with increasing 2/6VLP booster doses, whereas the systemic ASC were stimulated even with lower 2/6VLP doses. Because pig reagents are not available to characterize the homing markers on B cells, we were unable to clarify if IgA ASC present in spleen and PBMC were rotavirus-specific IgA ASC trafficking from the gut-associated lymphoid tissue through the circulation or if they were induced by antigen-presenting cells (APC) from the mucosal lymphoid tissues that entered systemically for antigen presentation. Yuan et al. showed that in vitro stimulation of memory B cells required a larger amount of antigen to stimulate MNC from ileum and duodenum than from spleen and MLN, suggesting that memory B cells in the intestinal tissues had a lower affinity to recall antigen or a higher threshold for reactivation than the memory B cells in the systemic tissues (40).

Using the optimal 250-μg 2/6VLP dose for the subsequent vaccine regimens, we then demonstrated that the ASC immune responses and protection rates induced by the AttHRV+2/6VLP vaccine regimen were higher when combined mucosal prime/boost (PO/IN) routes and vaccine types (AttHRV+2/6VLP) were used, but protection rates did not differ significantly from those of the PO/PO or IN/IN route.

The mechanism for the higher ASC responses and higher protection rate against rotavirus diarrhea observed for the PO/IN (AttHRVPO+2/6VLPIP) regimen in this study (71%) compared to the PO/PO AttHRV3×regimen (44%) (18) can be explained by the fact that the IN route avoids the destruction of PO-administered VLPs by low pH and enzymes in the gastrointestinal tract, providing more efficient antigen uptake at the mucosal surface of the respiratory tract after IN immunization. However, oral priming with attenuated live HRV is also important to obtain adequate levels of protection, since the use of the IN route for AttHRV priming followed by IN AttHRV or 2/6VLP boosting induced lower (17 to 50%) protection rates against diarrhea. Similar observations were reported for 2/6VLP administered to adult mice. Virus-like-particles administered IN induced higher serum and intestinal antibody responses than VLPs administered orally, suggesting that less antigen degradation occurs via the IN route than via the gastrointestinal tract and that there may be increased VLP antigen retention and interaction with lymphoid cells in the respiratory tract compared to those in the intestine (28).

Generally, the strongest intestinal and systemic ASC responses were induced by the AttHRVPO+2/6VLPIP regimen, with the highest intestinal and systemic IgA ASC responses before and after challenge compared to all other vaccine regimens evaluated in the gnotobiotic pigs. These high IgA ASC responses corresponded to higher protection rates. The AttHRVPO+2/6VLPIP regimen also induced higher intestinal and systemic IgA antibody responses than the AttHRV3×PO (16). These data agree with previous results showing a positive correlation between the magnitude of the intestinal IgA antibody responses and protection against diarrhea upon rotavirus challenge (12, 14, 44). The presence of rotavirus-specific IgA ASC in the circulation was shown to correlate with the IgA ASC in the human small intestinal lamina propria (8).

Although the importance of systemic immune responses in protection against rotavirus infection is unclear, the presence of both systemic and mucosal ASC responses may correlate with memory cells induced during vaccination. Youngman et al. (38) detected long-term IgA ASC in the Peyer’s patches and lamina propria but not in the spleen or MLN and concluded that Peyer’s patches are the secondary lymphoid organ where the majority of rotavirus-specific memory IgA ASC reside, whereas most IgG ASC were found in spleen and bone marrow. After systemic immunization, the predominant homing receptor on ASC is α4β7; after oral immunization, it is α4β7 integrin; and after nasal immunization, a large portion of ASC expressed both α4β7 and α4β7 integrin. Nasal immunization induced antibodies in a broader range of sites, such as saliva, the urogenital tract, and the gastrointestinal tract (22). Youngman et al. (38) also found rotavirus-specific memory B cells in spleen 4 months after rotavirus infection, and the majority of the cells were α4β7. In addition to the different homing receptors expressed according to the route of immunization, Yuan et al. (40) detected high numbers of IgG memory B cells in the spleen after oral inoculation of pigs with AttHRV, indicating that naïve B cells in systemic lymphoid tissues were primed by the viral antigen. Long-term IgG serum antibodies were also observed, suggesting that the spleen is a major site for long-term serum IgG antibody production. The authors also detected IgA memory B cells in the spleen at PID83 in pigs inoculated with virulent Wa HRV. They concluded that this finding could explain the success of the parenteral route to boost orally primed humans and pigs and most probably could apply to the IN booster route as well.

We have shown that use of 2/6VLP+ISCOM as an IN boost induced mucosal immune responses equivalent to or higher than those obtained with an IN booster of 2/6VLP+mLT, the latter a potent mucosal adjuvant.

The 2/6-VLP vaccine was effective as a booster vaccine for enhancing VN antibody responses in pigs previously primed with the live AttHRV and was more effective when boosted by the IN than the PO route (16). However, the 2/6VLP booster was effective only when administered orally or IN after PO priming and not after IN priming with live attenuated HRV. Moser and Offit (24) showed that in mice inoculated orally with Rhesus rotavirus, virus-specific T cells capable of stimulating virus-specific memory B cells might migrate slowly to and accumulate in the lamina propria. Antigen-presenting cells capable of inducing or stimulating virus-specific memory B or T cells might migrate to the lamina propria over time. In our study, oral priming was more effective than intranasal, which can be attributed to the largest mass of lymphoid tissue being located in the gastrointestinal tract, most of it in inductive sites, compared to more restricted lymphoid tissues in the respiratory tract. Furthermore, the gut mucosa contains at least 80% of the body’s plasma cells and 90% of these activated B cells...
produce secretory IgA (sIgA) (7), an important correlate of protection against rotavirus infection (43). Thus, the administration of AttHRVPO followed by 2/6VLP+ISCOM IN boosting is a promising approach to prevent HRV diarrhea in a gnotobiotic pig model and may be applicable to HRV vaccines for infants.

In conclusion, oral priming with live attenuated Wa HRV followed by IN boosting with 250 μg of 2/6VLP× induced intestinal and systemic ASC responses higher than and protection rates equivalent to those obtained with three PO or IN doses of live AttHRV alone or following IN priming with AttHRV and IN boosting with 2/6VLP. Consequently, VLPs can be used effectively as booster vaccines after priming with live attenuated virus; however, the route of immunization also needs to be considered. In our previous studies, PO priming with AttHRV followed by PO boosting with 2/6VLP+ISCOM induced 50% protection against diarrhea (18). But in our present study, the same vaccine administered PO/IN induced higher protection (71%) against diarrhea, whereas using IN/IN routes, the same vaccine induced only 17% protection against diarrhea. Both the type of booster and the route of vaccination may be important to overcome blockage induced by local antibodies formed after priming. Furthermore, we previously showed nasal HRV shedding (2). Vaccine targeting to both gut and nasal tissues may have contributed to the prevention of challenge virus replication nasally and intestinally, thus preventing nasal spread of the virulent HRV.

In another previous study, we determined the magnitude and distribution of rotavirus-specific gamma interferon (IFN-γ)-producing or -proliferating T-cell responses in intestinal and systemic lymphoid tissues of gnotobiotic pigs orally infected with a virulent HRV, AttHRV, or AttHRV-2/6VLP (oral/IN) (45). We found significant correlations between the magnitude of intestinal IFN-γ-producing CD4+, CD8+, or CD4+ CD8+ T-cell responses induced by virulent HRV infection and the rotavirus vaccines tested and protection rates against rotavirus diarrhea. Thus, multiple vaccine types and inoculation routes, when optimally combined, induced not only robust intestinal B-cell responses but also higher T-cell responses than single formulation/route vaccines (AttHRV3×PO) in a gnotobiotic pig model. Similarly, Blutt et al. (5), using a mouse model, demonstrated that host, viral, and vaccine factors are associated with the level of efficacy induced by VLP vaccines. Nevertheless, the combination of routes for the same vaccine preparation did not improve VLP vaccine efficacy.

We realize that a dose of 250 μg is relatively high; however, compared to the doses of VLP given to mice (10 μg) (27, 28) versus pigs (250 μg), the relative dose of VLPs used in mice (0.4 μg/g of body weight) is generally much higher than that used in pigs (0.1 and 0.073 μg/g of body weight) when given as the second and third doses, respectively. Also, the weight of the piglets more accurately reflects the weight of infants, suggesting that our data may be more applicable to possible doses needed for use in humans. However, it is possible that with the use of a more potent adjvant or a more efficient delivery system, these doses could be reduced. Intramuscular doses of 80 μg are used with the current commercial human papillomavirus vaccine (20). We also observed immune responses in pigs given similar doses of 100 μg VLPIN. Currently, there is no licensed oral adjunct that would fulfill these needs. Nevertheless, approaches currently under development are very promising, such as pH-responsive microspheres, which would protect proteins from degradation. Another promising approach would be the use of chemical modifications such as N-terminal methylation, acetylation, and C-terminal amida- tion. Such approaches have been used to modify vasopressin to demopressin, which is three times more active than vasopressin itself (34).

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