Protective Immunity and Antibody-Secreting Cell Responses Elicited by Combined Oral Attenuated Wa Human Rotavirus and Intranasal Wa 2/6-VLPs with Mutant Escherichia coli Heat-Labile Toxin in Gnotobiotic Pigs

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Rotavirus infections are the most important cause of severe infantile gastroenteritis (19), accounting for more than 125 million cases of diarrhea and an estimated 600,000 to 870,000 deaths annually worldwide (7). In the United States, rotavirus infections cause 500,000 physician visits and 50,000 hospitalizations each year (16). Although the worldwide impact of this virus on public health has led to major efforts to develop vaccines to control rotaviral disease, many difficulties have been encountered. All rotavirus vaccines assessed in human infants were live oral vaccines targeting induction of protective intestinal immunity against severe diarrhea, but their efficacy was variable (7). A licensed live oral reassortant rotavirus vaccine was withdrawn due to an association with cases of intussusception after the first dose (1). For these reasons, alternative vaccines and vaccination approaches are being evaluated, e.g., nonreplicating vaccines and extraintestinal immunization routes, by using adult mouse and rabbit models of rotavirus infection and a neonatal gnotobiotic pig model of rotavirus infection and diarrhea. The protective efficacy against rotavirus shedding of alternative vaccines tested in adult mice or rabbits (inactivated virus [27] and 2/6-VLPs [10, 31, 32]) did not predict the protective efficacy against disease observed in gnotobiotic pigs (50, 52) in which minimal or no protection against infection and disease were obtained. Differences in the pathogenicity of rotavirus infections in adult mice or rabbits (inactivated virus [27] and 2/6-VLPs [10, 31, 32]) did not predict the protective efficacy against disease observed in gnotobiotic pigs (50, 52) in which minimal or no protection against virus shedding and diarrhea occurred. Differences in the pathogenicity of rotavirus infections in adult mice (virus shedding, but no intestinal lesions or disease) (11, 21, 48) compared to neonatal pigs (virus shedding, intestinal lesions, and diar-
rhea induced by HRV) (38, 39, 40) may have contributed to the different results observed in mice versus pigs.

Rotaviruses replicate in the small intestinal enterocytes of infants and neonatal pigs causing villous atrophy and diarrhea (39). Fecal or intestinal immunoglobulin A (IgA) antibody responses have been most consistently associated with protective immunity in naturally infected humans and in gnotobiotic pigs experimentally infected with HRV (12, 22, 45, 51, 53). For enteric viral vaccines such as rotavirus, oral immunization with live virus appears to be the most effective way to induce intestinal IgA antibody responses, because it mimics the natural route of infection and viral replication amplifies the magnitude of antigen-stimulation in the intestine. In our previous studies, oral inoculation of gnotobiotic pigs with 2 or 3 doses of attenuated Wa human rotavirus (HRV) conferred partial protection against virus shedding (19 and 67%, respectively) and diarrhea (34 and 63%, respectively) upon virulent Wa HRV challenge (51, 53). The protection rates of the three-dose-attenuated Wa HRVs in gnotobiotic pigs concur with the protection rates against mild to severe rotavirus diarrhea conferred in children by three doses of oral tetravalent rotavirus vaccines in the United States (50 to 69%) (7, 51).

The effects of a combined oral and intraperitoneal vaccination regimen were examined in mice (26). Oral inoculation of mice with live heterologous rhesus rotavirus, followed by intraperitoneal inoculation with inactivated homologous murine rotavirus (EDIM), elicited a small but significant enhancement of serum IgG and IgA and fecal IgA antibody responses upon challenge with EDIM rotavirus (postinoculation day [PID] 49) and a reduction in virus shedding after challenge. The authors of that study suggested that these enhanced antibody responses and partial protection observed were additive (26). For non-replicating vaccines such as inactivated rotavirus or virus-like particles (2/6-VLPs), intranasal (i.n.) immunization with effective mucosal adjuvants induces higher intestinal IgA antibody responses in adult mice than immunization by the oral or parenteral routes (17, 27, 32). The use of combined oral and i.n. vaccination routes stimulates multiple mucosal inductive sites, i.e., both gut-associated lymphoid tissues (GALT) and nasal-associated lymphoid tissues (NALT) in contrast to the individual oral or i.n. vaccination routes alone. This combined approach has not been reported previously in rotavirus vaccine studies. Our hypothesis was that by exploiting the advantages of multiple mucosal immunization routes (i.e., oral and i.n.) and vaccine types (replicating virus and nonreplicating VLPs), a combined vaccination regimen may optimally stimulate the mucosal immune system and increase the protective efficacy of rotavirus vaccines.

The objectives of the present study were (i) to assess whether immunization of gnotobiotic pigs with sequential oral-attenuated Wa HRV and i.n. 2/6-VLPs + mLT vaccines confers greater protection against rotavirus infection and diarrhea compared to the individual vaccines; (ii) to determine whether the sequence of immunization by oral priming with live attenuated HRV, followed by i.n. boosting with recombinant 2/6-VLPs versus i.n. priming with 2/6-VLPs followed by oral boosting with live attenuated HRV, plays a role in the induction of protection and intestinal and systemic antibody-secreting cell (ASC) responses to Wa HRV; and (iii) to examine how the combination of oral and i.n. mucosal immunization routes and vaccine types influences the magnitude, isotype, and tissue distribution of virus-specific ASCs in gnotobiotic pigs.

**MATERIALS AND METHODS**

**Virus.** The attenuated (cell culture-adapted) Wa strain HRV propagated and titered in monkey kidney (MA104) cells was used for the inoculation of pigs and the ELISPOT assay (50, 51–53). The virulent Wa HRV (intestinal contents from infected gnotobiotic pigs) used for challenge contained 10^5 50% infectious doses (ID₅₀) (50, 52, 53). To prepare the double-layered inactivated Wa HRV inoculum, the attenuated Wa HRV was treated twice with 50 mM EDTA (25), purified by ultracentrifugation on CsCl gradients (9), and inactivated by using 0.01 M binary ethylenimine as previously described (2, 15). The inactivated Wa HRV preparation was examined using immunoelectron microscopy to verify the size and morphologic integrity of the rotavirus particles (36). A plaque assay and a cell culture immunofluorescence infectivity (CCIF) assay (3, 49) were performed to assure complete inactivation. Western blot using hyperimmune serum against Wa HRV and monoclonal antibodies against homotypic VP4 and VP7 antigens in the double-layered inactivated HRV preparation as previously described (13).

**VLPs and adjuvant.** The 2/6-VLPs (VP2 from RF bovine rotavirus and VP6 from virulent Wa HRV) were produced by coexpression of recombinant baculoviruses containing the VP2 and VP6 genes in Spodoptera frugiperda 9 insect cells (13, 50). The 2/6-VLPs were purified by using CsCl gradient ultracentrifugation (13). The protein concentration and integrity of the 2/6-VLPs were determined as previously described (50). The mLT adjuvant was provided by J. Clements (Tulane University Medical Center, New Orleans, La.) and used at 5 μg per dose in gnotobiotic pigs as described in a previous study (50).

**Inoculation of gnotobiotic pigs.** Near-term pigs were derived by hysterectomy and maintained in isolation units as described previously (28). Pigs were assigned to nine groups as follows: (i) one oral dose of attenuated Wa HRV, followed by 2 i.n. doses of 2/6-VLPs + mLT (AttHRV/VP2/L2VLP); (ii) two i.n. doses of 2/6-VLPs + mLT, followed by one oral dose of attenuated Wa HRV (VLP2/AttHRV); (iii) three oral doses of attenuated Wa HRV (AttHRV3/L); (iv) one oral dose of attenuated Wa HRV, followed by two i.n. doses of mLT alone (AttHRV1/L); (v) two i.n. doses of mLT alone, followed by one oral dose of attenuated Wa HRV (Mock2/AttHRV1); (vi) three i.n. doses of purified double-layered inactivated Wa HRV plus mLT (InactHRV3/L); (vii) three i.n. doses of 2/6-VLPs plus mLT (VLP3/L); (viii) three i.n. doses of mLT alone (mLT); and (ix) mock-inoculated controls (Mock).

(i) **Oral inoculation with attenuated Wa HRV followed by i.n. boosting with 2/6-VLPs + mLT (AttHRV/VP2/L2VLP).** At 3 to 5 days of age, pigs were given 5 ml of 100 mM NaHCO₃ to reduce gastric acidity and then inoculated orally with ~5 x 10^7 fluorescent focus-forming units (FFU) of attenuated Wa HRV (AttHRV/VP2× and AttHRV1× pigs) or 5 ml of minimal essential medium (MEM) diluent (mLT and Mock control pigs). At PIDs 10 and 20, the AttHRV/VP2× pigs were inoculated i.n. with 250 μg of 2/6-VLPs plus 5 μg of mLT. The VLPs and mLT were diluted in 0.5 ml of sterile TNC buffer (10 mM Tris-HCl, pH 7.5; 140 mM NaCl; 10 mM CaCl₂) and slowly administered by drops into the pig’s nostrils. The AttHRV1× and mLT control pigs were similarly inoculated i.n. twice or three times, respectively, with 5 μg of mLT alone. Mock control pigs were given an equal volume of TNC buffer.

(ii) **i.n. inoculation with 2/6-VLPs + mLT, followed by oral boosting with attenuated Wa HRV (VLP2×/AttHRV).** At 3 to 5 days of age and 10 days later, pigs were inoculated i.n. with 250 μg of 2/6-VLPs plus 5 μg of mLT in 0.5 ml of TNC buffer. The Mock2×/AttHRV pigs were given an equal volume of TNC buffer. At PID 21 (post-first inoculation), the VLP2×/AttHRV and Mock2×/AttHRV pigs were inoculated orally with ~5 x 10^7 FFU of the cell-culture-adapted attenuated Wa HRV in 5 ml of MEM (Gibco/Life Technologies, Grand Island, N.Y.). Pigs were given 5 ml of 100 mM NaHCO₃ orally to reduce gastric acidity 10 min prior to administration of the virus.

(iii) **Other vaccine groups.** Three oral doses of attenuated Wa HRV, three i.n. doses of 2/6-VLPs + mLT, or three i.n. doses of purified double-layered inactivated Wa HRV + mLT were given to pigs in the same manner and dosages for the AttHRV/VP2× or VLP2×/AttHRV pigs. Because of the small numbers of pigs in these groups, protection data and immune responses from the AttHRV3× and VLP3× pigs were pooled from the data from the pigs treated with the same vaccine regimens in our previous studies (50, 51).

**Assessment of protection.** At PID 28, 7 days after the last inoculation, subsets of pigs from AttHRV/VP2×, VLP2×/AttHRV, AttHRV3×, AttHRV1×, VP1×, and InactHRV groups and all pigs from the Mock2×/AttHRV and mLT and Mock control groups were challenged orally with ~10^6 ID₅₀ of virulent Wa HRV (47). This challenge dose was previously determined (47) to cause
**RESULTS**

Protection against virulent Wa HRV challenge conferred by the combined vaccination regimens. Protection levels (assessed by the reduction of virus shedding and diarrhea) conferred by the two combined vaccination regimens were compared with controls and the other individual vaccines. Protection data were pooled between AttHRV× and Mock2×/AttHRV groups and mLT and Mock control groups, respectively, for statistical analysis because there were no significant differences between the corresponding groups (Table 1). All pigs in the mLT and Mock control groups shed virus, and 89% of the pigs developed diarrhea upon challenge with virulent Wa HRV. The vaccination regimens of both AttHRV/VLP2× and VLP2×/AttHRV induced partial protection against virus shedding and diarrhea upon challenge with virulent Wa HRV (Table 1). Pigs in the AttHRV/VLP2× group had significantly reduced virus shedding (42%) and diarrhea (50%) rates compared to the mLT and Mock controls. The pigs that developed virus shedding and diarrhea in the AttHRV/VLP2× group had a significantly shorter mean duration of virus shedding, significantly reduced mean cumulative fecal scores (indicating reduced severity of disease), and lower mean peak titers of virus shed with a shorter mean duration of diarrhea (half as long) compared to the mLT and Mock controls. The protection rates conferred by the AttHRV/VLP2× regimen were comparable with, although slightly lower than, the protection rates conferred by the AttHRV3× regimen (protection rates of 58 versus 67% for shedding and 44 versus 63% for diarrhea, respectively) (Table 1). However, among the AttHRV/VLP2× pigs that shed virus after challenge, the mean duration of virus shedding was 1 day only, which was shorter than the mean for the AttHRV3× pigs that shed virus (Table 1).

In contrast, the VLP2×/AttHRV regimen conferred lower protection rates against virus shedding (17%) and diarrhea (25%) (Table 1). A single oral dose of attenuated Wa HRV, given at 3 to 5 days of age or at 7 days before challenge, induced a 33% protection rate against diarrhea; however, all of the pigs in this group shed virus after challenge. Pigs in the VLP2×/AttHRV and one-oral-dose-attenuated Wa HRV groups had significantly lower mean cumulative fecal scores, shorter mean duration of virus shedding and diarrhea, and lower mean peak virus shedding titers, although not significantly so for the latter two parameters, compared to the mLT and Mock controls. Three i.n. doses of 2/6-VLPs+mLT or double-layered inactivated Wa HRV conferred no protection against either virus shedding or diarrhea. The severity of virus shedding and diarrhea in the pigs from these two groups were comparable to those of the mLT and Mock controls (Table 1).

**Rotavirus-specific ASC responses.** The rotavirus-specific ASC responses in the intestinal and systemic lymphoid tissues of pigs inoculated with the two combined vaccine regimens (AttHRV/VLP2× and VLP2×/AttHRV), one and three oral doses of attenuated Wa HRV (AttHRV×, Mock2×/AttHRV, and AttHRV3×), three i.n. doses of 2/6-VLPs (VLP3×), and
mLT alone (mLT control) are depicted in Fig. 1 and 2. The rotavirus-specific ASCs are presented as mean numbers of ASC per 5 × 10^5 MNC. To assess whether the combined vaccine regimens elicited significantly higher ASC responses in the intestinal and systemic lymphoid tissues compared to the oral attenuated or i.n. VLP vaccines, statistical comparisons were made pairwise between the AttHRV/VLP2/H11003 or VLP2/H11003/AttHRV groups and the other vaccine groups. Table 2 highlights the ASC numbers for the combined vaccine groups AttHRV/VLP2/H11003 and VLP2/H11003/AttHRV.

(i) Prechallenge. Before challenge, no virus-specific ASCs were detected in the mLT control group (data not shown). The combined vaccine regimens (AttHRV/VLP2/H11003 or VLP2/H11003/AttHRV) induced higher virus-specific IgM and IgA ASC responses in all lymphoid tissues, but especially in the intestinal tissues, compared to the individual vaccine regimens VLP3, AttHRV1/H11003, and AttHRV3/H11003; the AttHRV/VLP2/H11003 regimen induced higher IgM, IgA (significantly higher), and IgG ASC responses in the intestinal lymphoid tissues than the VLP2/H11003/AttHRV regimen at PID 28 and PCD 0 (Fig. 1). The numbers of IgG ASCs in the duodenum, ileum, and MLN of the AttHRV/VLP2/H11003 pigs were significantly higher than those of the VLP3/H11003 (18- to 23-fold), AttHRV1/H11003 (18- to 57-fold), and AttHRV3/H11003 (25- to 47-fold) pigs (Fig. 1A). The numbers of IgA ASCs in the duodenum, ileum, and MLN of the AttHRV/VLP2/H11003 pigs were also higher or significantly higher than those of the AttHRV1/H11003 (12- to 54-fold), AttHRV3/H11003 (7- to 36-fold), and VLP2/H11003/AttHRV (3- to 19-fold) pigs. The numbers of IgG ASCs in the AttHRV/VLP2/H11003 pigs were comparable to those of the VLP3/H11003 group in the duodenum and ileum, but they were significantly higher in the MLN (7-fold), spleen (3.5-fold), and peripheral blood (at least 3-fold) than those of the VLP3/H11003 pigs at PID 28 and PCD 0 (Fig. 1). In contrast to the higher or significantly higher numbers of IgM, IgA, and IgG ASCs in the intestinal lymphoid tissues induced by the AttHRV/VLP2/H11003 regimen compared to the VLP2/H11003/AttHRV regimen, the numbers of ASCs induced by the AttHRV/VLP2/H11003 regimen in the spleen and peripheral blood were similar to or lower than those induced by the VLP2/H11003/AttHRV regimen at PID 28 and PCD 0 (Table 2).

(ii) Postchallenge. After challenge, although the mean numbers of IgA and IgG ASCs in the AttHRV3/H11003 pigs were highest in most of the tissues, the mean numbers of IgA ASCs in all tissues of the AttHRV/VLP2/H11003 pigs remained higher (1.4- to 9-fold) than those of the VLP3/H11003, AttHRV1/H11003, and VLP2/H11003/AttHRV pigs, except in the MLN and PBL of the VLP3/H11003 pigs, in which the IgA ASC numbers were similar to those of the AttHRV/VLP2/H11003 pigs at PID 35 and PCD 7 (Fig. 2). The
numbers of IgA ASCs of the AttHRV/VLP2× pigs were significantly higher (12- to 31-fold) in the duodenum and ileum compared to those of the Mock2/AttHRV and mLT control pigs. Similarly, the mean numbers of IgG ASCs of the AttHRV/VLP2× pigs were significantly higher (8- to 95-fold) in the duodenum, ileum, and MLN compared to these two control groups. The numbers of IgG ASCs of the AttHRV/VLP2× pigs were also higher (in the ileum) or significantly higher (in the duodenum) than those of the VLP3×, AttHRV1×, and VLP2×/AttHRV pigs.

Pigs that received three i.n. doses of double-layered inactivated Wa HRV (InactHRV3×/H11003) developed none or low numbers of IgM, IgA, and IgG ASCs in the intestinal and systemic lymphoid tissues (0 to 2.5 ASC per 5×10^5 MNC) at the time of challenge and lower numbers of IgA and IgG ASCs after challenge (6 to 18 per 5×10^5 MNC) (data not shown) compared to the other vaccine groups.

**VN antibody responses in serum.** The virus-neutralizing (VN) geometric mean titer (GMT) in the serum of gnotobiotic pigs after inoculation and challenge are summarized in Table 3. At PIDs 10 and 21, the VN GMTs of the AttHRV/VLP2× pigs were statistically similar to those of the virulent Wa HRV-inoculated pigs (VRHRV1×) and the AttHRV3× and AttHRV1× pigs but were significantly higher than those of InactHRV3×, VLP2×/AttHRV, Mock2×/AttHRV, VLP3×, and control pigs. The VN GMT in the AttHRV/VLP2× group was about 2.3-fold higher (but not significantly) than that of the AttHRV1× pigs. After challenge, the AttHRV3× and AttHRV/VLP2× pigs developed significantly higher VN GMTs in the serum than all other groups; however, the VN GMTs in the VLP3× and InactHRV3× pigs were similar to the VN GMTs of mLT and Mock control pigs.

**DISCUSSION**

The risk of intussusception in infants using a live oral rotavirus vaccine prompted us to explore alternative vaccination routes and combined live and nonreplicating vaccines. The combined vaccine regimen with multiple vaccine types and routes is a new rotavirus vaccine approach that we explored in the present study. By taking advantage of both the oral-live and the i.n.-2/6-VLP vaccination approaches, the AttHRV/VLP2× vaccination regimen stimulated the highest intestinal IgA effector B-cell responses and a high protection rate against HRV infection and diarrhea in gnotobiotic pigs. Interestingly, of the combined immunization routes, oral priming followed by i.n. boosting, and vaccine types, live attenuated Wa HRV for priming followed by 2/6-VLPs+mLT for boosting, was more effective in stimulating intestinal IgA ASC responses than each
individual vaccine (AttHRV1, 2, or 3 or VLP3) or the converse combined VLP2/AttHRV regimen. The VLP2/AttHRV regimen conferred only a low protection rate (19%) against diarrhea, which was higher than that with VLP3 or InactHRV3 (0%) but lower than that with AttHRV3 (67%) and AttHRV/VLP2 (44%). In contrast, the AttHRV/VLP2 vaccine sequence induced the highest mean numbers of intestinal IgA ASCs compared to all other vaccination approaches tested in gnotobiotic pigs, including one to three oral doses of live attenuated Wa HRV, two or three intramuscular doses of inactivated Wa HRV with incomplete Freund adjuvant, and two or three i.n. doses of SA11 or Wa 2/6-VLPs with or without mLT (50, 51, 52, 53). One possible explanation for why the AttHRV/VLP2 regimen induced the highest intestinal IgA ASC responses may be the sequence of stimulation of the mucosal inductive sites, e.g., first the GALT and then NALT, in combination with the initial priming with intact live virus (which mimics natural infection and amplifies the virus dose), followed by boosting with 2/6-VLPs + mLT. After oral inoculation of pigs with attenuated Wa HRV, the rotaviral antigens are presumably taken up via GALT, e.g., ileal and jejunal Peyer's patches (8). The antigen-sensitized precursor B cells circulate and then enter and reside in mucosal effector sites (A. Anderson, Art Anderson's Immunology Lecture [http://www.geocities.com/CapeCanaveral/Hanger/1962/artnotes.html]). These sites would include the lamina propria of the intestine in particular, but, in addition, after trafficking via the common mucosal immune system, these sites could also include mucosal tissues in the respiratory tract (30, 35). The intestinal Peyers's patches have previously been reported to be efficient inductive sites for precursors of IgA effector B cells that populate distant mucosal lymphoid tissues, including the lamina propria of the upper respiratory tract (6). The rotavirus-sensitized precursor B cells in the upper respiratory tract (e.g., the lymphoid tissues in nasopharyngeal tonsils) may then be restimulated by the 2/6-VLPs + mLT from the i.n. boosting, undergoing clonal expansion and maturation into effector B cells. In addition, priming of naive B cells in the NALT may have occurred after the sequential i.n. inoculations. The effector cells may then migrate into the intestines via the blood circulation due to the gut-seeking properties (interaction of intestinal homing receptor α4β7 integrin on the ASCs and its ligand mucosal addressin cell adhesion molecule, MAECAM-1, on postcapillary high endothelial venules) of GALT-derived primed B cells and the homing heterogeneity of NALT-derived primed B cells (6, 33). Researchers (33) reported that a large majority of circulating ASCs induced by i.n. inoculation of humans with recombinant cholera toxin B subunit coexpressed L-selectin and α4β7. Thus, the rotavirus-specific ASCs detected in the intestinal lymphoid tissues after the sequential oral and i.n. immunizations could have been generated in the two mucosal inductive sites: GALT and NALT. The dual se-

FIG. 2. Isotype-specific ASCs to Wa HRV in gnotobiotic pigs inoculated with various vaccine regimens. MNC from the duodenum and ileum (A) and the MLN, spleen, and PBL (B) of pigs were collected and assayed by ELISPOT on PID 35 and PCD 7. The data represent the mean number of Wa HRV-specific ASCs per $5 \times 10^5$ MNC for 4 to 12 pigs at each time point. The error bars show the SEM. The asterisks denote significant differences (Kruskal-Wallis rank sum test, $P < 0.05$) in ASC numbers compared to AttHRV/VLP2 group for the same isotype at the same time point.
quential inductive sites may have resulted in the higher IgA, IgG, and especially higher IgM ASC numbers detected after the second boosting dose of 2/6-VLPs+mLT compared to ASC numbers in pigs inoculated with one to three oral doses of attenuated Wa HRV or three i.n doses of 2/6-VLPs alone.

Another factor which may have contributed to the higher numbers of ASCs induced by AttHRV/VLP2× is that boosting via the i.n. route avoided the preexisting antibodies stimulated by the prior oral inoculation of attenuated Wa HRV in the intestines. i.n. boosting with two doses of 2/6-VLPs+mLT elicited higher ASC responses than oral boosting with one or two doses of attenuated Wa HRV in the two- or three-dose oral attenuated Wa HRV regimens (51, 53). In the latter regimens, the intestinal antibodies induced by primary oral inoculation with live attenuated Wa HRV may have partially neutralized the live virus in the booster doses, limiting replication and the amount of viral antigen available for stimulation. In contrast, boosting with nonreplicating VLPs via an extraintestinal inductive site, the NALT, could prevent interference by the higher levels of local antibodies in the intestine compared to the upper respiratory tract.

Although the intestinal IgA ASC responses were significantly higher at challenge, the protection rate conferred by AttHRV/VLP2× was slightly lower than that of the AttHRV3× regimen. This finding and our previous findings of the lack of protection of 2/6-VLP vaccines (50) indicate that protective immunity to rotavirus diarrhea in pigs is dependent not only on the location, the magnitude, and the isotypes of antibody but also on the protein specificity (VP4 and VP7 neutralizing antibodies) of the B-cell responses induced. The VLP3× vaccine induced low intestinal IgA and high IgG effector B-cell responses prechallenge. This antibody response, however, did not confer protection against virus shedding and diarrhea upon challenge with virulent Wa HRV. On the other hand, the AttHRV/VLP2× regimen conferred a higher protection rate compared to AttHRV1× or Mock2×/AttHRV, indicating that boosting with 2/6-VLPs did increase the protection rate. Boosting with 2/6-VLPs+mLT might have “nonspecifically” boosted the primary antibody responses to all of the viral proteins (including VP4 and VP7) induced by the live attenuated Wa HRV, possibly mediated by cross-reactive T helper cells. This idea is also supported by the observation that the VN GMT in the serum of AttHRV/VLP2× pigs was elevated (2.3-fold) compared to that of the AttHRV1× pigs at PID 28 and PCD 0. Similar boosting responses were observed in studies of hepatitis B virus (29) and influenza virus (41) in which T helper cells directed against internal viral capsid proteins can provide cognate help to B cells specific for external viral proteins. However, in contrast to the study of mice (14), which suggested that primary inoculation with VP6 enhanced the VN antibody responses following challenge, the VN GMTs in the VLP3× or InactHRV3× vaccine-immunized pigs were similar or lower compared to mLT or Mock controls at PCD 7.

Although the AttHRV/VLP2 regimen did not confer a higher rate of protection compared to the AttHRV3× vaccine,
the potential advantage of the combined AttHRV/VLP2 vaccine was indicated by the significantly higher ASC responses induced in the intestinal lymphoid tissues. If a 2/4/6/7-VLP vaccine were used for boosting in the combined regimen, a much higher protection rate might have been achieved based on the high numbers of IgA ASCs in the intestinal lymphoid tissues induced by this vaccine approach and based on the correlation between the numbers of IgA ASCs in the intestinal lymphoid tissues induced by intact rotavirus and the degree of protection (51, 53).

The lower level of ASC responses induced by the VLP2/H11003/AttHRV regimen compared to the AttHRV/VLP2 regimen was possibly due to functional compartmentalization of the mucosal inductive site (NALT) involved in these vaccination approaches (33). The mucosal immune system has been proposed to be compartmentalized such that lymphocytes preferentially migrate back into tissues where the cells were primed (5, 33, 42). Investigations of mucosal immunization or experimental infection of rodents and pigs (23, 43, 46) suggest that the migration of B cells induced in NALT and bronchial associated lymphoid tissues (BALT) to the intestinal lamina propria is much less efficient in terms of generating intestinal IgA antibody responses than the extensive mucosal dissemination of GALT-derived B cells (24). In addition, the much greater mass of GALT inductive sites compared to NALT or BALT in pigs favors the generation of greater B-cell responses (46).

Previous studies of the immunization of women against cholera, an enteric pathogen, suggest that women who received parenteral immunizations with cholera vaccine and who were previously naturally exposed to cholera had significant increases in IgA antibodies in breast milk compared to previously unexposed women (44). A study of a respiratory Sendai virus vaccine showed that protection was provided by oral immunization of mice only after an i.n. priming dose of the live virus was given (20). The results of our studies, the cholera natural infection and vaccination (44), and the respiratory Sendai virus vaccines (20) collectively suggest that priming of the mucosal inductive site at the portal of natural infection with a replicating vaccine may be important for induction of protective immunity when boosting doses are given via a different vaccination route.

Higher IgM, IgA, and especially IgG ASC responses were observed in the spleen and/or the blood of pigs immunized with the VLP2×/AttHRV regimen compared to the AttHRV/VLP2× regimen. This result is consistent with previous observations of pigs i.n. inoculated with a porcine respiratory coronavirus (PRCV) and challenged orally with transmissible gastroenteritis virus (TGEV) (46). In this study, immunization via BALT (PRCV infection) induced high systemic ASC responses with low numbers of ASCs in the intestine and provided partial protection against gastroenteritis after challenge with TGEV. In contrast, immunization via GALT (TGEV infection) induced high numbers of IgA ASCs in the intestine and provided complete protection against TGEV disease. It has been suggested that i.n. immunization, in general, is efficient for stimulating systemic immunity (4) and elicits an antibody response with later onset but of longer duration than the oral route (34).

In conclusion, the combined vaccination routes (oral followed by i.n., with the involvement of GALT and then NALT)
and vaccine types (replicating virus followed by nonreplicating VLPs) effectively stimulated the intestinal immune system as indicated by highest ASC responses compared to all other types of vaccines tested and conferred moderately high protection to pigs against rotavirus infection and disease. The protection rate of this regimen may be further improved by using triple-layered 2/4/6/7 VLPs to directly boost the ASC responses to VP4 and VP7 and neutralizing antibodies that were primed by the first dose of live AttHRV, likely achieving a higher protection rate as reported with intact rotavirus (51, 53). These findings have important implications for the development of new vaccination strategies for various mucosal pathogens. The use of a replicating vaccine at a second mucosal inductive site, followed by boosting with a nonreplicating vaccine at a second mucosal inductive site, may be a highly effective approach to stimulate the mucosal immune system and induce protective immunity.

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REFERENCES


TABLE 3. VN GMTs in serum of gnotobiotic pigs after inoculation and challenge

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<td>AttHRV1×</td>
<td>8</td>
<td>2aB</td>
<td>8</td>
<td>135A</td>
<td>5.4</td>
</tr>
<tr>
<td>Mock2×/AttHRV</td>
<td>5</td>
<td>2c</td>
<td>5</td>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>InactHRV3×</td>
<td>4</td>
<td>2c</td>
<td>4</td>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>VLP3×</td>
<td>10</td>
<td>2c</td>
<td>10</td>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>mLT</td>
<td>12</td>
<td>2c</td>
<td>12</td>
<td>2b</td>
<td>1</td>
</tr>
<tr>
<td>Mock</td>
<td>7</td>
<td>2c</td>
<td>7</td>
<td>2b</td>
<td>1</td>
</tr>
</tbody>
</table>

* VRHRV1× = virulent HRV, one dose.
* n, number of pigs tested at the time point in each group. The GMTs in the same column with different superscript letters are significantly different (one-way ANOVA [general linear models procedure], followed by Duncan grouping on log10 transformed titers). GMTs were determined by a plaque reduction assay. All pigs had GMT of <4 (negative) at PID 0. Titers of <4 were recorded as 2 for data analysis.

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