High Titers of Circulating Maternal Antibodies Suppress Effector and Memory B-Cell Responses Induced by an Attenuated Rotavirus Priming and Rotavirus-Like Particle–Immunostimulating Complex Boosting Vaccine Regimen

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Received 7 November 2005/Returned for modification 27 December 2005/Accepted 30 January 2006

We investigated maternal antibody (MatAb) effects on protection and immune responses to rotavirus vaccines. Gnotobiotic pigs were injected intraperitoneally at birth with pooled serum from sows hyperimmunized with human rotavirus (HRV); control pigs received no sow serum. Pigs with or without MatAbs received either sequential attenuated HRV (AttHRV) oral priming and intranasal boosting with VP2/VP6 virus-like particle (VLP)–immunostimulating complex (ISCOM) (AttHRV/VLP) or intranasal VLP-ISCOM prime/boost (VLP) vaccines at 3 to 5 days of age. Subsets of pigs were challenged at 28 or 42 days postinoculation with virulent Wa HRV to assess protection. Isotype-specific antibody-secreting cell (ASC) responses to HRV were quantitated by enzyme-linked immunosorbent assay to measure effector and memory B-cell responses in intestinal and systemic lymphoid tissues pre- and postchallenge. Protection rates against HRV challenge (contributed by active immunity and passive circulating MatAbs) were consistently (but not significantly) lower in the MatAb-AttHRV/VLP groups than in the corresponding groups without MatAbs. Intestinal B-cell responses in the MatAb-AttHRV/VLP group were most suppressed with significantly reduced or no intestinal immunoglobulin A (IgA) and IgG effector and memory B-cell responses or antibody titers pre- and postchallenge. This suppression was not alleviated but was enhanced after extending vaccination/challenge from 28 to 42 days. In pigs vaccinated with nonreplicating VLP alone that failed to induce protection, MatAb effects differed, with intestinal and systemic IgG ASCs and prechallenge memory B cells suppressed but the low intestinal IgA and IgM ASC responses unaffected. Thus, we demonstrate that MatAbs differentially affect both replicating and nonreplicating HRV vaccines and suggest mechanisms of MatAb interference. This information should facilitate vaccine design to overcome MatAb suppression.

Vaccination of neonates faces many challenges due to the immaturity of the neonatal immune system and interference by maternal antibodies (MatAbs) present at vaccination. Various degrees of interference of vaccine-induced immune responses by MatAbs have been reported for live vaccines such as measles, diphtheria, Haemophilus influenzae, hepatitis A and B, classical swine fever virus, and measles virus vaccines (5, 6, 10, 12, 16, 34). The effects of MatAbs on DNA vaccines are variable. Mice immunized with a DNA vaccine coding for hepatitis B envelope protein S (HBs) in the presence of maternally derived HBs antibodies had lower antibody responses than those immunized with the vaccine in the absence of HBs antibodies, suggesting that MatAb interference also occurs for DNA vaccines (34). In contrast, a DNA vaccine coding for gB and gD of pseudorabies virus primed immune responses in the presence of MatAbs more efficiently than a conventional vaccine or in the absence of MatAbs (naïve pigs) (31). The effects of MatAb, whether positive (enhancing) or negative (suppressive), may depend on the ratio between the amount of MatAb and the antigen present (26, 28). Optimal amounts of MatAb can enhance B-cell responses by forming antigen-antibody complexes that induce complement deposition, in turn engaging the B-cell receptor and the complement receptor CD21, thereby producing costimulation of B cells via CD19/CD81. On the other hand, MatAbs can neutralize live vaccines and reduce the antigen mass available for immune response induction. The positive or negative influences of MatAbs cannot be reliably predicted; experimental models and field trials are required to study the MatAb effects on vaccines.

Rotavirus infections can occur in infants under 3 months of age, even when the levels of MatAb in the circulation (acquired from placental transfer) and intestine (acquired from breast milk) remain high. It has been suggested that the variable efficacies and seroconversion rates in many rotavirus vaccine trials in human infants may be associated with these preexisting antibodies (likely maternally derived in children less than 6 months of age) (22). However, controlled studies to define the influence of MatAbs on rotavirus vaccines are lacking. Using gnotobiotic pigs, Hodgins et al. (9) previously demonstrated that induction of antibody-secreting cell (ASC) responses after virulent Wa human rotavirus (HRV) (VirHRV) primary infection and challenge was suppressed in the
presence of high titers of circulating MatAbs and that milk anti-body added to the suppression. In addition, the intestinal immu-noglobulin A (IgA) responses were also suppressed (19). At a low MatAb titer, only IgG ASC and not IgA ASC numbers in duodenum and mesenteric lymph nodes were significantly reduced after challenge (9). Thus, the effect of different levels of MatAbs on different antibody isotypes varies. Similarly, calves fed with colostrum before inoculation with bovine rotavirus showed an inverse relationship between the IgG titers in colostrum and the ASC responses (18). These studies, demonstrating the effects of different titers of MatAbs (circulating or local) on infection with virulent rotavirus, established the basis for our current study to develop rotavirus vaccine regimens that can overcome the suppressive effects of MatAbs.

Oral priming and intranasal (i.n.) boosting strategies used in rotavirus vaccine studies of gnotobiotic pigs were highly effective in inducing antibody responses, presumably due to exploiting multiple inductive mucosal sites. This strategy of avoiding mucosal sites where booster vaccines may be neutralized by local antibodies induced by the priming vaccine assures that booster vaccination will evoke effective antigenic stimulation. A vaccine regimen (attenuated HRV [AttHRV]/virus-like particle [VLP]) consisting of one oral priming dose of AttHRV and 2 i.n. booster doses of 2/6-VLP associated with immunostimulating complexes (ISCOMs) induced high protection rates against viral shedding and diarrhea and comparable or higher IgA antibody responses than that with 3 oral doses of AttHRV (8). However, it is unknown whether this vaccine scheme (AttHRV/VLP) induces similar immune responses and protection in the presence of high titers of rotavirus-specific MatAbs. The effect of low titers of MatAbs on this vaccine has recently been reported elsewhere (17). For other disease models such as measles and influenza vaccines in macaques and foals/yearlings, respectively, ISCOMs were successfully used to overcome the inhibition of immune responses by MatAbs (4, 30).

The overall goal of our study was to elucidate the mechanisms of MatAb inhibition of immune responses to HRV and to develop vaccine strategies to overcome their suppressive effects. In particular, we explored the effects of high titers of passive circu-lating MatAbs on the immune responses induced by the replicat-ing AttHRV for priming and VLP boosting vaccine regimen and by the nonreplicating 2/6-VLP-ISCOM vaccine alone. The antibody responses, ASCs, and memory B cells induced by both types of vaccines were assessed to determine if high titers of MatAbs interfere with the development of B-cell responses after rotavirus vaccination or impact the levels of protection against VirHRV challenge. We also evaluated the effect of declining MatAbs by comparing the B-cell responses and protection in pigs given the AttHRV/VLP regimen and challenged with VirHRV at a longer interval (post inoculation day [PID] 42) than in pigs vaccinated and challenged at PID 28, the same time frame as that used in our previous study (8).

MATERIALS AND METHODS

Viruses. Tissue culture-adapted Wa AttHRV derived from the 27th passage in African green monkey kidney cells (Ma104) was used for vaccination and sow immunization. VirHRV derived from stools of an infected infant was maintained by serial passage in gnotobiotic pigs (35, 39). Pooled intestinal contents of the VirHRV-infected gnotobiotic pigs were used for challenge at 10^6 median infectious doses (ID_{50}). The ID_{50} of the VirHRV inoculum for gnotobiotic pigs was previously determined to be at least 1 fluorescent focus-forming unit (FFU) (23, 32, 33). The titers of both AttHRV and VirHRV were determined by cell culture immunofluorescence (CCIF) assay (23, 24).

Preparation of maternal serum pools. Serum with high HRV antibody titers (MatAb) was produced by intramuscularly immunizing rotavirus-seropositive sows (n = 3) with 5 doses of Wa AttHRV inactivated with 0.01 M binary ethylenimine (Aldrich Chemical Co., St. Louis, Mo.) and mixed with Freund’s adjuvant (25). The preactivation titer of the virus was 1 × 10^9 FFU/dose. Serum was collected and pooled after the last immunization, heat inactivated at 56°C for 30 min, and filtered through Seitz Micromedia filter pads (Ertel/Aisop, Kingston, N.Y.) followed by 0.22-μm membrane filters (Millipore, Bedford, Mass.). The IgG and virus-neutralizing (VN) antibody titers to Wa HRV were measured by enzyme-linked immunosorbent assay (ELISA) and a plaque reduc-tion assay, respectively, as described previously (24). The IgM, IgG, IgA, and VN antibody titers of the pooled hyperimmune sow serum were 16,384, 1,024, 1,024, 1,000,000, and 16,384, respectively.

Gnotobiotic pigs injected with maternal serum to mimic infants with passive circulating MatAbs. The heterocytometry-derived near-term pigs were obtained and maintained in isolation units, as described previously (15), under an approved animal use protocol. Newborn suckled pigs are devoid of MatAbs due to the impervious nature of the sow placenta to immunoglobulins (11). The MatAb administered via the intraperitoneal (i.p.) route is transferred to lymphatic ves-sels and enters the circulation (9) to mimic the effect of circulating passively derived MatAb. Pigs were given 30 ml of the MatAbs twice i.p. within the first 24 h after birth as determined by previous studies (9, 17, 19).

Experimental groups. The vaccination schemes are summarized in Fig. 1. (i) Experiment (Exp) I. Pigs in the groups designated MatAb-AttHRV/VLP, MatAb-VLP, and MatAb-ISCOM were given maternal serum; pigs in AttHRV/ VLP, VLP, and ISCOM groups did not receive maternal serum (n = 10 to 12 pigs/group). At 3 to 5 days of age, pigs in MatAb-AttHRV/VLP and MatAb-VLP groups were orally inoculated with VirHRV (5 × 10^8 FFU/dose) followed by 2 i.n. doses of 2/6-VLP-ISCOM (250 μg of 2/6-VLP associated with 1,250 μg of ISCOM) 10 days apart at PID 10 and 21. Pigs in MatAb-VLP or VLP groups were inoculated with 5 doses of 2/6-VLP-ISCOM 10 days apart, also starting from 3 to 5 days of age. Pigs in MatAb-ISCOM and ISCOM groups were inoculated with diluent and ISCOM matrix (ISCOM) i.n. as controls within the same time frame as the vaccinees. Subsets of pigs (5 to 7 pigs/group) from each group were challenged with VirHRV at PID 28.

(ii) Exp II. To study the longer-term (LT) effect of the maternal serum (LTMatAb), in a subsequent experiment, the same combined vaccine, AttHRV/ VLP, and control ISCOM were administered over a longer time frame, and the pigs were challenged with VirHRV at PID 42 (instead of PID 28), when the titer of MatAbs had declined further. This challenge time point falls within the 8-week interval during which the gnotobiotic pigs are susceptible to infection and disease (12). Pigs in LTMatAb-AttHRV/VLP, LTMatAb-VLP, and LTMatAb-ISCOM groups did not receive maternal serum (i.e., the letters “LT” preceding the group name indicate the longer-term groups in Exp II) were inoculated with one oral dose of AttHRV at 3 to 5 days of age and boosted with 2/6-VLP at PID 14 and 28, instead of PID 10 and 21. Pigs in LTMatAb-ISCOM and LT-ISCOM groups were inoculated with diluent and ISCOM matrix (ISCOM) i.n. as controls within the same time frame as the vaccine groups.

Assessment of protection. Challenge and protection studies were done as described previously (38). At PID 20 (for Exp I) and PID 42 (for Exp II), five to seven pigs per group were challenged orally with 10^8 ID_{50} of VirHRV. Rectal swabs were collected, and diarrhea scores were observed for 6 days after challenge for assessment of viral shedding and diarrhea (fecal scores were as follows: 0, normal; 1, pasty; 2, semiliquid; 3, liquid). Scores of greater than or equal to 2 were considered diarrhea. Susceptible age-matched mock-vaccinated control pigs (ISCOM group) were included in each experiment as a reference to evaluate the challenge inoculum and the susceptibility of pigs to rotavirus-induced diarrheal illness. The pigs were detected in the rectal swab fluids using CCIF and antigen capture ELISA, respectively, as previously described (3, 24). The pigs were considered completely protected against shedding or diarrhea upon challenge with VirHRV only when they did not shed virus or have diarrhea, respectively, during the entire observation period.

Plaque reduction assays for VN antibodies. Plaque reduction assays for VN antibodies were performed on serum samples of piglets and sows, as described previously, and the VN antibody titers were expressed as the reciprocal of the serum dilution that reduced the plaque numbers by >80% (24).

Isotype-specific antibody ELISA. The Wa HRV-specific IgM, IgA, and IgG antibody titers in sows’ sera and piglets’ sera and small intestinal contents (SIC) were determined by an indirect isotype-specific antibody ELISA as previously described (29).

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ELISPOT assay for rotavirus-specific ASCs. Pigs were euthanized at PID 28 (postchallenge day (PCD) 0 (prechallenge) and PID 35/PCD 7 (postchallenge) in Exp I and at PID 42/PCD 0 and PID 49/PCD 7 in Exp II (n = 5 to 7 pigs/group/time point). The small intestine (duodenum and ileum), spleen, and peripheral blood lymphocytes (PBLs) were collected for the isolation of mononuclear cells (MNCs) as previously described (39). The enzyme-linked immunospot (ELISPOT) assays to enumerate rotavirus-specific ASCs of different iso- types (IgM, IgA, and IgG) were conducted based on previously published methods (39). The assays were performed for the freshly isolated lymphocytes (effector ELISPOT) to measure the effector B-cell responses and for the cells stimulated in culture with AttHRV antigen (memory ELISPOT) to measure the short-term memory B-cell responses prechallenge (17).

Statistical analyses. For all the parameters, statistical comparisons were made (i) between pig groups with and without MatAbs for the same vaccine, (ii) between Exp I and Exp II for the same vaccine and control groups pre- and postchallenge, (iii) between AttHRV/VLP and VLP vaccine groups with and without MatAbs within Exp I, and (iv) between pre- and postchallenge time points for the same iso- type ASCs and intestinal antibody responses. For antibody titers (isotype-specific ELISA and VN antibodies), statistical analyses were performed on log10-transformed titers. One-way analysis of variance (ANOVA) (SAS Institute Inc., Cary, N.C.) followed by Duncan’s multiple-range test was used to reveal significant differences (marked by different capital letters: A, B, and C) among groups at each time point. The mean number of ASCs was calculated for each treatment group at PID 28/PCD 0 and PID 35/PCD 7 (Exp I) and at PID 42/PCD 0 and PID 49/PCD 7 (Exp II). Kruskal-Wallis rank sum (nonparametric) tests were first used to compare the ASC and memory B-cell numbers among groups at each time point. If significant differences were detected among groups, differences between particular group pairs were further tested using Kruskal-Wallis rank sum tests. Differences in proportions of pigs with diarrhea and virus shedding were determined by chi-square test; when significant differences were present among groups, pairwise comparisons were made by Fisher’s exact test. Unless the $P$ value is specified, a $P$ value of <0.05 was used to denote statistical significance.

RESULTS

Intraperitoneal injection of newborn pigs with maternal hyperimmunep serum to HRV mimics the passive circulating MatAb titers to HRV in infants in developing countries. The VN and rotavirus IgG antibody titers in the sera of MatAbs in pigs (2,000 to 2,300 and 65,000 to 110,000, respectively) at PID 0 (3 to 5 days after maternal serum injection) (Table 1) represented the range of high titers of rotavirus-specific antibodies in children in developing countries. The rotavirus-specific antibody titers in infants/young children in developing countries are 50 to 3,000 (for rotavirus VN antibody titers) and 160 to 80,000 for rotavirus-specific IgG antibody titers (1, 21, 40). In a prior study, we focused on the effect of low titers of MatAbs to mimic the passive circulating MatAb titers in infants from developed countries (17).

The moderate protection against viral shedding and diarrhoea in the MatAb-AttHRV/VLP group was contributed by both passive antibodies and active immune responses. In Exp I, protection rates were highest in the AttHRV/VLP group (71% protection against viral shedding and diarrhea) without MatAbs but decreased to 50% for both viral shedding and diarrhea in the corresponding vaccine group with MatAbs, illustrating a trend for MatAb interference with vaccine efficacy (Table 1). Protection in the MatAb-AttHRV/VLP group was likely partially due to the remaining level of MatAbs at challenge and the active immunity induced by vaccination, because neither the MatAb-VLP vaccine nor the MatAb-ISCOM control groups were protected against virus shedding, and protection rates against diarrhea were lower (29% and 33%, respectively). The subclinical infections seen in some pigs in the MatAb-VLP and MatAb-ISCOM groups were probably due to the protective effect of the MatAbs, which remained high at challenge (VN titers of 1,621 and 1,015, respectively). The duration of viral shedding and diarrhea did not differ significantly between the same vaccine group with or without MatAbs (data not shown). In the MatAb-VLP and MatAb-ISCOM groups that were not protected against viral shedding, the mean virus-shedding titer was 22- to 24-fold lower than those in the respective VLP and ISCOM groups (Table 1),
TABLE 1. Protection rates against rotavirus shedding and diarrhea in gnotobiotic pigs after challenge with VirHRV and the corresponding antibody titers prevaccination (PID 0) and prechallenge (PCD 0)

<table>
<thead>
<tr>
<th>Expt</th>
<th>Vaccine regimen</th>
<th>Day of challenge (Piddle)</th>
<th>PID 0</th>
<th>PCD 0</th>
<th>Peak virus shed (103 FFU)</th>
<th>Protection rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>VN</td>
<td>IgG</td>
<td>VN</td>
<td>95% CI</td>
</tr>
<tr>
<td>I</td>
<td>MatAb-AttHRV/VLP</td>
<td>28</td>
<td>2,000A</td>
<td>65,536A</td>
<td>960B</td>
<td>20,643A</td>
</tr>
<tr>
<td>I</td>
<td>AttHRV/VLP</td>
<td>28</td>
<td>2B</td>
<td>2B</td>
<td>635B</td>
<td>11,585A</td>
</tr>
<tr>
<td>I</td>
<td>MatAb-VLP</td>
<td>28</td>
<td>2,251A</td>
<td>114,105A</td>
<td>1,621A</td>
<td>32,768A</td>
</tr>
<tr>
<td>I</td>
<td>VLP</td>
<td>42</td>
<td>2B</td>
<td>2B</td>
<td>2D</td>
<td>4,096B</td>
</tr>
<tr>
<td>I</td>
<td>MatAb-ISCOM</td>
<td>42</td>
<td>2,317A</td>
<td>65,536A</td>
<td>1,015B</td>
<td>16,384A</td>
</tr>
<tr>
<td>I</td>
<td>ISCOM</td>
<td>42</td>
<td>2B</td>
<td>2B</td>
<td>2D</td>
<td>3C</td>
</tr>
<tr>
<td>II</td>
<td>LTMatAb-AttHRV/VLP</td>
<td>42</td>
<td>1,600A</td>
<td>104,008A</td>
<td>262C</td>
<td>4,598B</td>
</tr>
<tr>
<td>II</td>
<td>LT-AttHRV/VLP</td>
<td>28</td>
<td>2B</td>
<td>2B</td>
<td>2D</td>
<td>257C</td>
</tr>
<tr>
<td>II</td>
<td>LTMatAb-ISCOM</td>
<td>42</td>
<td>1,203A</td>
<td>92,682A</td>
<td>126C</td>
<td>7,585AB</td>
</tr>
<tr>
<td>II</td>
<td>LT-ISCOM</td>
<td>42</td>
<td>2B</td>
<td>2B</td>
<td>2D</td>
<td>3C</td>
</tr>
</tbody>
</table>

a Geometric mean serum antibody titers in the same column with different superscript letters differ significantly (one-way ANOVA followed by Duncan’s grouping; P < 0.05).

b Geometric means and 95% confidence intervals of the peak virus shedding titers measured by CCIF. The HRV detection limit for the CCIF is 250 FFU/ml. Samples negative for HRV by CCIF were arbitrarily given a titer of 130 for calculation of the geometric means.

c Protection rate, [1 – (percentage of vaccinated pigs in each group with diarrhea or shedding/percentage of ISCOM control pigs with diarrhea or shedding)] × 100.

Proportions in the same column with different superscript letters differ significantly (Fisher’s exact test; P < 0.05). Five to seven pigs in each group were challenged with VirHRV. Five to six pigs in the same group were not challenged and were euthanized at PID 28 (Exp I) or PID 42 (Exp II).

confirming the neutralizing effects of MatAbs on the challenge with VirHRV.

In Exp II, although the titer of the remaining MatAbs in the circulation decreased by PID 42/PCD 0 (VN titer of 126 and IgG antibody titer of 7,585), a low rate of protection against diarrhea (20%) still occurred in the LTMatAb-ISCOM group (Table 1). Again, we noted a trend for reduced protection rates against both viral shedding and diarrhea in the LTMatAb-AttHRV/VLP group (67% and 33%, respectively), although not significantly different compared to the higher (80%) protection rates (shedding and diarrhea) in the LT-AttHRV/VLP group.

Small intestinal antibody responses to the AttHRV/VLP and VLP vaccines were inhibited by circulating MatAbs regardless of the time of challenge (Exp I and II). In Exp I, the only antibody isotype observed in the SIC of vaccinated or control pigs at challenge (PID 28/PCD 0) was IgG, at a low titer, likely due to transudation of the i.p. inoculated maternal serum into the gut (Fig. 2). However, between the MatAb-VLP and VLP vaccine groups, the mean numbers of IgA ASCs postchallenge among all corresponding groups without MatAb. However, the IgA antibody titers of the MatAb-vaccine groups were significantly lower than the corresponding groups without MatAb, suggesting that MatAb continued to negatively impact IgA antibody responses in the intestine even after virus challenge. There were no significant differences between the patterns of antibody responses in the SIC between Exp I and Exp II (data not shown). Thus, the antibody responses in SIC to the AttHRV/VLP and VLP vaccines were inhibited by the circulating MatAbs regardless of the time of challenge (PID 28 or 42).

Isotype-specific ASC responses in vaccinated pigs before and after challenge in Exp I. The intestinal ASC responses induced by the AttHRV/VLP vaccine were higher or significantly higher than those induced by the VLP vaccine in the absence of MatAb, suggesting lower antigenicity of the VLP vaccine compared to that of the AttHRV/VLP vaccine (Fig. 3 to 5). In the presence of MatAb, this trend was not observed, suggesting the differential effects of MatAbs on the two vaccines.

IgM ASC responses induced by the AttHRV/VLP vaccine postchallenge (PID 35/PCD 7, Exp I) were generally suppressed by MatAbs. In Exp I, the prechallenge (PID 28/PCD 0) IgM ASC responses were low in all groups, and there was no difference in these responses induced by either vaccine in the presence or absence of MatAb (Fig. 3). However, after challenge (PID 35/PCD 7), the IgM ASC numbers increased slightly in all tissues in the AttHRV/VLP group without MatAbs but not in the MatAb-AttHRV/VLP group. The mean numbers of intestinal IgM ASCs were two- to fivefold lower in the MatAb-AttHRV/VLP group than in the AttHRV/VLP group, suggesting suppression by MatAbs. However, between the MatAb-VLP and VLP vaccine groups, the mean numbers of IgM ASCs postchallenge among all tissues did not differ significantly, indicating that MatAbs did not suppress the IgM ASC responses to the nonreplicating VLP vaccine. Thus, the IgM ASC responses induced by the AttHRV/VLP but not the VLP vaccine postchallenge were generally suppressed in the pigs with MatAbs.

Intestinal IgA ASC responses were strongly suppressed in the AttHRV/VLP vaccine group with MatAbs pre- and postchallenge but not in the VLP vaccine group (Exp I). Prechallenge, in Exp I (Fig. 4), low numbers of IgA ASCs, indicative of active immune responses, were induced by the AttHRV/VLP vaccine in the presence of MatAbs. However, the IgA ASC numbers in the duodenum and ileum of the MatAb-AttHRV/VLP group were significantly lower (three- to five-
Bars, vaccine with no MatAbs; solid bars, vaccine with MatAbs.

...was strongly suppressed in the AttHRV/VLP but not the VLP vaccine group with MatAbs both pre- and postchallenge. Postchallenge, systemic IgA ASC responses were suppressed by MatAbs for both vaccines.

**Intestinal and spleen IgG ASC responses were suppressed in the AttHRV/VLP (pre- and postchallenge) and VLP (postchallenge) vaccine groups in the presence of MatAbs (Exp I).** In Exp I, prechallenge IgG ASC responses were higher or significantly higher in all tissues of the AttHRV/VLP group than those of the MatAb-AttHRV/VLP group, indicative of MatAb suppression of active IgG responses not only in the intestinal tissues (similar to the suppression of IgM and IgA responses) but also in the systemic tissues (Fig. 5). For the VLP vaccine group, there were significantly higher numbers of IgG ASCs in spleen and slightly higher numbers in the ileum and PBL than in the MatAb-VLP group. Thus, MatAb suppression of IgG ASC responses also occurred in the nonreplicating VLP vaccine group.

After challenge, the numbers of intestinal and spleen IgG ASCs increased in both the MatAb-AttHRV/VLP and MatAb-VLP groups compared to the numbers prechallenge (7- to 30-fold) (Fig. 5). The substantial increase in splenic IgG ASC numbers in the MatAb-AttHRV/VLP group coincided with the presence of moderate numbers of splenic IgG memory B cells in this group, suggesting that active immune responses occurred in the presence of MatAbs (see below). Nevertheless, the numbers of IgG ASCs in all tissues remained significantly lower in both vaccine groups with MatAbs, confirming MatAb suppression of the IgG ASC responses to both vaccines postchallenge. These findings are in contrast to the lack of MatAb suppression observed for intestinal IgM and IgA ASC responses in the VLP vaccine group. Similarly, significantly lower numbers of intestinal IgG ASCs were detected in the MatAb-ISCOM group than in the ISCOM group after VirHRV challenge, suggesting that the MatAbs also significantly suppressed the intestinal IgG ASC responses to the VirHRV at PID 35/PID 7. Thus, the intestinal and spleen IgG ASC responses were suppressed in the presence of MatAbs in the AttHRV/VLP (pre- and postchallenge) and VLP (postchallenge) vaccine groups. However, the increased intestinal and spleen IgG ASC responses postchallenge in the MatAb-AttHRV/VLP vaccine group were indicative of the induction of memory B-cell responses in the presence of MatAbs.

**IgM and IgA ASC responses were suppressed to a greater extent in the LT vaccination study (Exp II) than in the short-term study (Exp I).** Based on the observation that MatAb suppression of the ASC responses induced by the AttHRV/VLP vaccine remained at PID 28, we challenged other AttHRV/VLP-vaccinated pigs at a later time (PID 42), when the MatAb titer had further declined. The VLP vaccine group was not included in this experiment because of the lesser impact of MatAbs on this vaccine in Exp I, and the VLP vaccine did not induce protection in pigs.

In Exp II, MatAb suppression was still evident in the LTMatAb-
AttHRV/VLP group both prechallenge (PID 42/PCD 0) and postchallenge (PID 49/PCD 7) with significantly lower IgM, IgA, and IgG ASC responses in the intestine than those in the LT-AttHRV/VLP group (Fig. 6). Surprisingly, MatAb suppression of the IgM and IgA ASC responses was greater in Exp II than in Exp I. IgM ASC numbers were decreased 6- to 32-fold and IgA ASC numbers were decreased 8- to 87-fold in the LTMatAb-AttHRV/VLP group compared to the LTAttHRV/VLP group pre- and postchallenge. In comparison, in the short-term study (Exp I), the IgM and IgA ASC numbers were decreased two- to fivefold in the MatAbAttHRV/VLP group compared to the AttHRV/VLP group pre- or postchallenge. The AttHRV/VLP vaccine without MatAb induced significantly higher numbers of intestinal IgM and IgA ASCs in the longer-term study compared to those in the short-term study; in the presence of MatAb, the intestinal ASC numbers were similar. Thus, the extended vaccination scheme increased the magnitude of ASC responses in the absence of MatAbs, but it did not alleviate the suppressive effect of MatAbs.

In Exp II, in systemic tissues, the IgA ASC numbers of the AttHRV/VLP vaccine group with or without MatAbs were low and did not differ between the two groups pre- and postchallenge (data not shown).

Prechallenge memory B-cell responses induced by the AttHRV/VLP and VLP vaccines were also inhibited by circulating MatAbs with greater inhibition in the longer-term (PID 42) than in the short-term (PID 28) pigs. In Exp I, the IgA and IgG memory B-cell numbers were higher or significantly higher in the systemic lymphoid population (spleen and blood) of the AttHRV/VLP and VLP vaccine groups (without MatAbs) than in the MatAb-AttHRV/VLP and MatAb-VLP groups, respectively. A striking difference regardless of the presence or absence of MatAbs was the significantly lower numbers of IgA and IgG memory B cells in spleen and PBL of the VLP vaccine groups compared to the AttHRV/VLP groups, suggesting a need for live virus priming to induce memory B-cell responses. Insufficient numbers of memory B cells likely had a major impact on the lack of protection seen in the VLP vaccine groups.

MatAb suppression of the memory B-cell responses induced by the AttHRV/VLP vaccine was greater at PID 42 than at PID 28. In all tissues (intestines, spleen, and blood), the numbers of IgA and IgG memory B cells in the LTMatAb-AttHRV/VLP group were lower or significantly lower than that in the LT-AttHRV/VLP group before challenge (Table 2). Interestingly, without MatAb, the numbers of IgA and IgG memory B cells in the LT-AttHRV/VLP group were significantly higher in ileum (higher in duodenum) but significantly lower in blood than those in the short-term AttHRV/VLP group (Table 2), suggesting a different distribution/dynamic of memory B cells in the long-term experiments compared to the short-term experiments. One possible scenario is that a major portion of the IgG and the majority of the IgA memory B cells were still in the circulation at PID 28, whereas by PID 42, these memory B cells had reached their resident sites (intestine and spleen). To summarize, the prechallenge memory B-cell responses induced by the AttHRV/VLP and VLP vaccines were also inhibited by circulating MatAbs. Greater inhibition of the memory B-cell responses occurred in the AttHRV/VLP vaccine group with MatAbs at PID 42 than at PID 28 and was associated with a different distribution of memory B cells in the lymphoid tissues.
DISCUSSION

We studied the effects of high titers of MatAbs on the protective efficacy and B-cell responses induced by two candidate rotavirus vaccines that were previously evaluated in colostrum-deprived seronegative gnotobiotic pigs (8). Although protection rates induced by the AttHRV/VLP vaccine in the presence of high titers of MatAbs were reduced, they did not differ statistically from those induced by this vaccine without MatAbs. However, the mechanism of protection differed based on the magnitude of the immune responses we observed. The

FIG. 4. IgA ASC responses to Wa HRV in pigs vaccinated in the presence or absence of MatAbs. The MNCs from duodenum, ileum, spleen, and PBLs of pigs were collected and assayed on PID 28/PCD 0 and PID 35/PCD 7 in Exp I. For an explanation of symbols, see the legend of Fig. 2. Note the change of vertical scale from Fig. 3.

FIG. 5. IgG ASC responses to Wa HRV in pigs vaccinated in the presence or absence of MatAbs. The MNCs from duodenum, ileum, spleen, and PBLs of pigs were collected and assayed on PID 28/PCD 0 and PID 35/PCD 7 in Exp I. For an explanation of symbols, see the legend of Fig. 2. Note the change of vertical scale from Fig. 3.
FIG. 6. The intestinal ASC responses to Wa HRV in pigs vaccinated in the presence or absence of MatAbs and challenged at PID 42 (Exp II). For an explanation of symbols, see the legend of Fig. 2. The "Δ" denotes a significant difference in the ASC responses between LT-AttHRV/VLP (Exp II) and AttHRV/VLP (Exp I) at the same time point in the same tissue for the same antibody isotype. The numbers in or above the columns are the ratios of intestinal ASC numbers between LTNoMatAb and LTMatAb groups receiving the AttHRV/VLP vaccine at the same time point.
higher protection rates observed in the MatAb-AttHRV/VLP group were likely due to the combination of active and passive immunity, because the MatAb-ISCOM and LTMatAb-ISCOM control groups were also partially protected (but with much lower protection rates) from diarrhea upon challenge due to the remaining MatAbs in the serum. The remaining MatAbs in serum, however, did not completely protect the pigs from virus shedding, which is consistent with previous findings by Hodgins et al. (9). Although the window of susceptibility to HRV in gnotobiotic piglets is at least 8 weeks (37), due to the expense and difficulties in maintaining the pigs in the isolator units for more than this period, we were unable to access the immune responses in pigs at still later times, when MatAb is no longer protective. However, it is clear that memory responses induced by the AttHRV/VLP vaccine regimen were strongly suppressed, predicting suboptimal long-term protection against rotavirus when MatAb declines to unprotective levels.

Because the B-cell responses were suppressed (but not completely) in the MatAb-AttHRV/VLP group, as indicated by the lower or significantly lower antibody titers and ASC and memory B-cell responses, the T-cell responses might also contribute to the protection induced by the AttHRV/VLP vaccine in the presence of MatAbs. It has been shown that in the presence of MatAbs, B-cell responses were depressed, but the T-cell responses were not affected (2). In infants given 2 doses of measles vaccine, booster vaccinations led to higher seroconversion rates and enhanced T-cell responses even in the presence of MatAb, suggesting that the infants would benefit from the additive protective effects mediated by both MatAbs and active T-cell responses (7). On the other hand, cellular immune responses induced by a DNA vaccine for human immunodeficiency virus did not improve the protection conferred by circulating antibodies (13). Thus, the role of T-cell responses as a determining factor for the success of a vaccine in the presence of MatAbs is still controversial.

In this study, the suppressive effects of MatAbs on the numbers of ASCs, antibody production, and memory B-cell responses were observed in both AttHRV/VLP and VLP vaccine regimens, especially for the AttHRV/VLP vaccine regimen at PID 28. This suppression remained and was more pronounced at PID 42. The long-term suppressive effect of MatAbs observed in this study agrees with previous findings. Feeding or i.p. injection of rats as neonates with monoclonal IgG2a or IgG1 antibodies led to the suppression of humoral immune responses in these rats as adults; thus, the suppression by MatAbs lasted for up to 5 months (20). Furthermore, we observed that the suppression was higher with the longer vaccination/challenge interval (42 days) than with the shorter interval (28 days) in both the magnitude of the ASC responses and the extent of lymphoid tissues affected. The stronger inhibition of the effector/memory B-cell response in the intestine of the LTMatAb-AttHRV/VLP group may be explained by the differences in the distribution of memory B cells in the short-term (Exp I) experiment compared to the long-term (Exp II) experiment. In Exp I, where the memory B-cell responses without MatAbs were mostly IgG in spleen and blood, with few memory B cells in the duodenum and ileum, MatAb suppression of the memory responses occurred only in spleen and blood. In Exp II, where the IgA and IgG memory B-cell responses were found in all tissues in the absence of MatAbs, MatAb suppression of the memory responses occurred in all tissues, including the intestine. Based on these observations, we postulate that in the presence of high titers of maternal serum, the homing of the IgA and IgG memory cells to the resident sites (intestine and spleen) in the long-term pigs (Exp II) was strongly inhibited. The suppression of the ASC responses by MatAbs in the MatAb-AttHRV/VLP group continued even after challenge with VirHRV. The effects on the memory B-cell responses were also observed in the presence of low titers of MatAbs (17). In the AttHRV/VLP vaccine group with low titers of MatAbs, there was a high level of suppression of the IgG memory B cells in blood and a low level of suppression of the IgG memory B cells in spleen and blood. Thus, the MatAb effects on the memory B-cell responses extend to a wide range of MatAb titers. These observations may have implications for

<table>
<thead>
<tr>
<th>Expt and vaccine regimen</th>
<th>Duodenum (Mean cell no. (SEM)*</th>
<th>Ileum (Mean cell no. (SEM)*</th>
<th>Spleen (Mean cell no. (SEM)*</th>
<th>PBL (Mean cell no. (SEM)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>I (PID 28/PCD 0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MatAb-AttHRV/VLP</td>
<td>5 (3)</td>
<td>2 (2)</td>
<td>3 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>AttHRV/VLP</td>
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<td>1 (0)</td>
<td>2 (1)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>MatAbs-VLP</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>3 (1)</td>
<td>2* (1)</td>
</tr>
<tr>
<td>VLP</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>6 (6)</td>
<td>10 (3)</td>
</tr>
<tr>
<td>II (PID 42/PCD 0)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LTMatAb-AttHRV/VLP</td>
<td>1 (1)</td>
<td>0 (0)</td>
<td>2* (1)</td>
<td>8* (5)</td>
</tr>
<tr>
<td>LT-AttHRV/VLP</td>
<td>8* (5)</td>
<td>420* (29)</td>
<td>129* (58)</td>
<td>2,276* (914)</td>
</tr>
</tbody>
</table>

* Mean number of HRV-specific memory B cells (n = 4 to 5). Statistical analyses were performed using Kruskal-Wallis rank sum test (P < 0.05). Single asterisks denote significant differences between MatAb and no-MatAb vaccine groups in the same experiment. The double asterisks denote significant differences between AttHRV/VLP and VLP vaccine groups with or without MatAbs in Exp I prechallenge. " denotes significant differences between Exp II and Exp I within the AttHRV/VLP vaccine group in the presence or absence of MatAbs. SEM, standard error of the mean. Because there were no significant IgA and IgG memory B-cell responses in ISCOM and MatAb-ISCOM control groups prechallenge, these responses were not included (for both experiments).

Results from duodenum of two pigs were presented for the LT-AttHRV/VLP group (due to insufficient cell numbers collected from duodenum for the memory B-cell assay); no statistical analysis was done between the LT-AttHRV/VLP group and other groups for duodenum.
live oral rotavirus vaccines in human infants used in the presence of variable levels of MatAbs.

In addition, the presence of MatAbs in the intestine may provide feedback inhibition to the secretion of antibody by plasma cells in the intestine, which explains the failure to detect IgA antibodies in the intestinal contents of pigs in the MatAb-AttHRV/VLP and MatAb-VLP groups, although low numbers of intestinal IgA ASCs were detected at the same time point. In contrast, moderate levels of intestinal IgA antibodies were detected in the VLP group, although the numbers of intestinal IgA ASCs in the VLP group did not differ significantly from the MatAb-VLP or MatAb-AttHRV/VLP group pre- and postchallenge. Inhibition of intestinal IgA antibody responses also occurred in the vaccine groups that received low titers of MatAbs (17). The feedback inhibition of antibody secretion has been observed in hybridoma cells, which generally display high initial rates of monoclonal antibody production; however, after a few hours, the monoclonal antibody secretion was reduced to low concentrations (14). Furthermore, the ELISPOT assay allows the enumeration of activated B cells capable of antibody production, whereas the ELISA assay measures both the amount of antibodies actively produced by these cells and the remaining passive MatAbs. Of interest, the sizes of the spots generated by the ASCs in the ELISPOT assay from pigs with MatAbs appeared smaller than those from pigs without MatAbs. A similar observation was reported in a previous study using 1 dose of VirHRV in the presence of low titers of MatAbs (19). In a recent study of calves, the calves that received control colostrum (with low titers of antibodies to bovine rotavirus) exhibited lower IgA and IgG1 antibody titers in intestinal contents than the colostrum-deprived calves after challenge with bovine rotavirus, yet the intestinal IgA ASC numbers did not differ between these groups (18). Thus, in the presence of MatAbs, although the induction of antibody-containing plasma cells in terms of their numbers may not be affected, the generation of functional plasma cells or their levels of antibody secretion may be affected.

The maternal, mainly IgG antibody clearly exerted a strong inhibition of IgG ASC responses in both intestinal and systemic tissues of the MatAb-AttHRV/VLP and LTMatAb-AttHRV/VLP groups. Similarly, the IgG ASC response was suppressed in intestinal tissues before and after challenge in pigs inoculated with 3 doses of VLP vaccine, whereas the intestinal IgM and IgA ASC responses were low, but they appeared to be less affected by MatAbs. This finding coincides with the observations reported previously by Hodgins et al. (9), whereby postchallenge IgG ASCs but not IgA ASC numbers in pigs inoculated with VirHRV were affected by low titers of MatAbs. MatAb inhibition of IgG1 antibody responses that was stronger than MatAb inhibition of IgG2a antibody responses was also observed in mice immunized with HBs DNA or HBs antigen vaccine in the presence of MatAb, suggesting the preferential binding of HBs antigen epitopes by maternal IgG1 rather than IgG2a antibodies (34). Taken together, the observation that MatAb suppression in the AttHRV/VLP and VLP groups was more pronounced for IgG than for IgM or IgA antibody or for ASC induction suggests that MatAb suppression (including negative feedback) may be isotype and titer specific, because IgG antibody titers were the highest in the maternal serum. However, in the presence of the lower titers of IgG MatAbs, no IgG isotype-specific inhibition was observed in the AttHRV/VLP vaccine group (17). Moreover, the low titers of MatAbs actually enhanced intestinal IgM and IgA ASC responses induced by this vaccine and the VLP vaccine postchallenge, possibly by antibody-dependent immune enhancement (17).

The use of the nonreplicating 2/6-VLP vaccine associated with ISCOM may have partially overcome the intestinal suppression by MatAbs, as evidenced by the comparable IgM and IgA ASC responses in the MatAb-VLP and VLP groups. On the other hand, the significantly reduced IgG ASC responses and the reduced intestinal IgA antibody titers in the MatAb-VLP vaccine group suggest that (i) the challenge virus dose may have been partially neutralized by MatAbs, resulting in lower booster responses after challenge, or (ii) the lower numbers of prechallenge virus-specific IgA and IgG memory B cells induced by the nonreplicating VLP vaccine in the presence of MatAbs led to a reduced magnitude of anamnestic responses postchallenge. Alternatively, oral delivery and replication of AttHRV may be differentially affected by circulating MatAbs compared to the i.n. delivery of VLP/ISCOM. Circulating MatAbs may have less impact on intestinal IgA and IgM ASCs but a greater impact on IgG ASCs following delivery of VLPs by the i.n. route.

Because inhibition by high titers of MatAbs also extended to the nonreplicating VLP vaccine, this finding further supports the hypothesis that mechanisms other than neutralization of live virus are involved in MatAb suppression (27). In comparison, low titers of MatAbs had only temporal inhibitory effects on the IgG but not on the IgM and IgA ASC responses induced by the VLP vaccine (17). Although the 2/6-VLP vaccine alone was not sufficiently immunogenic and did not induce any protection in pigs (36), perhaps higher doses of VLPs with VP4 or and VP7 associated with ISCOM or other effective adjuvants may improve immunogenicity, overcome MatAb interference (by a wide range of titers), and induce protective neutralizing antibody responses.

In summary, this study confirmed that the presence of high titers of MatAbs has substantial effects not only on effector but also on memory B-cell responses to rotavirus vaccines in neonates. Our current and previous studies (17) have provided a more comprehensive understanding of the myriad of effects of low MatAb titers compared to high MatAb titers on antibody responses to rotavirus vaccines. The impacts of MatAbs are varied and complex, depending on the type of vaccine, the route of vaccination, the tissue examined, the antibody isotype, and the time of challenge. The effects of high titers of MatAbs were mainly inhibitory, whereas the effects of lower titers of MatAbs were either suppressing or enhancing. Our studies mimic rotavirus immunity in infants influenced by the universal presence of variable titers of MatAbs. Our findings concerning the effects of MatAbs on replicating vaccines compared to those on nonreplicating vaccines should facilitate the design of vaccines to overcome MatAb suppression. An understanding of the immune mechanisms for protection conferred by AttHRV/VLP and other vaccine regimens will enable us to develop alternative strategies to further improve the efficacy of rotavirus vaccines given to infants in the presence of MatAb.

ACKNOWLEDGMENTS

We thank Juliette Hanson, Rich McCormick, Severin Pouly, and Marcela Azevedo for technical assistance. We thank Viviana Parreño...
REFERENCES


