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Prevention of the murine model of biliary atresia following live rotavirus vaccination of dams

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Abstract

Purpose—Biliary atresia (BA) is a neonatal disease that results in the obliteration of the biliary tree. The murine model of biliary atresia (BA) has been established where rhesus rotavirus (RRV) infection of newborn mice leads to an obstructive cholangiopathy. We determined whether maternal, post-conception rotavirus vaccination could prevent the murine model of biliary atresia.

Materials and Methods—Female mice were mated and injected intraperitoneally with one of the following materials: purified rotavirus strains RRV or Wa, high or low dose Rotateq® (a pentavalent rotavirus vaccine (PRV)), purified recombinant viral antigens of rotavirus (VP6) or influenza (NP), or saline. B-cell-deficient females also underwent post-conception PRV injection. Maternal vaccination with PRV improves survival of pups infected with RRV.

Results—Maternal vaccination with PRV improves survival of pups infected with RRV. Serum rotavirus IgG, but not IgA, levels were increased in pups delivered from dams who received RRV, Wa, PRV, or VP6, but in the case of the Wa, PRV, and VP6 groups, these antibodies were not neutralizing. Post-conception injection of high dose PRV did not improve survival of pups born to B-cell deficient dams.

Conclusion—Maternal vaccination against RRV can prevent the rotavirus-induced murine model of biliary atresia in newborn mouse pups.

Keywords

Biliary atresia; rotavirus; Rotateq®; maternal immunization

Introduction

Biliary atresia (BA) is a neonatal disease characterized by inflammation and fibrosis which results in progressive obliteration of the extrahepatic biliary tree. Worldwide, the incidence of this disease is estimated between 1 in 5,000-18,000 live births. BA is the most common

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indication for pediatric liver transplantation in the United States, accounting for 50% of these cases yearly¹.

Despite its clinical significance, the etiology of BA has not been established. In 1972, Landing proposed that cholangiopathic disease including BA may be due to an external insult that leads to a progressive inflammatory process². Although it is unclear what this external factor may be, both patient based clinical reports and basic science research suggest that viral infection may be a possible trigger. A viral “initiator” in the pathogenesis of BA has been supported by human studies that have identified a number of putative viruses³⁻⁵, but the most compelling evidence exists for cytomegalovirus (CMV)^{4, 6, 7}, reovirus⁸, and rotavirus⁹.

Murine studies involving reovirus and rotavirus, both members of the *Reoviridae* family, have supplemented our understanding of BA. The murine model of biliary atresia introduced by Riepenhoff-Talty results in clinical and histological features similar to that of the human disease and has subsequently become an important tool in BA research¹⁰. In this model, neonatal infection of BALB/c mice with rhesus rotavirus (RRV), a double-stranded RNA virus, leads to an obstructive cholangiopathy and subsequent signs of disease including jaundice, bilirubinuria, and acholic stool. Our group has previously demonstrated co-localization of RRV in biliary epithelial cells (BECs) resulting in an 81% mortality rate¹¹. Recent research has begun to bridge the pathophysiologic gap between a possible viral etiology and the subsequent T-cell-mediated destruction of BECs. Harada and colleagues demonstrated *in vitro* and *in vivo* that human BECs mount an anti-viral response and initiate apoptotic pathways in response to a synthetic ds-RNA analog¹².

Rotavirus is a ubiquitous human pathogen and the leading cause of severe diarrheal illness in children worldwide. In 2004, the World Health Organization (WHO) estimated that rotaviral gastroenteritis was responsible for approximately 527,000 deaths, mostly occurring in developing nations¹³. The Centers for Disease Control estimate that rotavirus is responsible for nearly 50,000 hospital admissions of children under age 5 in the United States per year¹⁴. As a result, significant efforts were made to develop a viable rotavirus vaccine. These efforts came to fruition with the Food and Drug Administration’s approval of two live rotavirus vaccines for use in humans, Rotateq® in 2006 and Rotarix® in 2008. Since then, phase III clinical trials have demonstrated these vaccines’ ability to significantly decrease rotavirus gastroenteritis (RVGE) of any severity as well as severe cases of RVGE¹⁵⁻¹⁸.

The aim of this study was to determine whether maternal, post-conception vaccination could prevent the murine model of biliary atresia and to begin to determine the mechanism that mediates this protection. To do so, we monitored RRV-infected pups born to vaccinated dams for symptoms related to BA and established whether these clinical findings correlated with any histological appearance of biliary tract damage. We also determined viral presence in pup biliary trees, and collected maternal and pup serum for quantitative rotavirus IgG and IgA levels as well as anti-RRV neutralizing antibody titers. We also performed post-conception vaccination studies on a B-cell deficient strain of mice to determine the role of rotavirus antibody.

Materials and Methods

Rotavirus Strains

Rotavirus strains have been characterized according to differences in structural proteins and classified into seven major groups (A-G). For these studies we used two strains of group A rotavirus — the simian strain RRV, obtained from Dr. Harry Greenberg (Stanford University, Palo Alto, CA), and the human strain Wa (kindly provided by R. Wyatt, National Institutes of Health, Bethesda, MD). We included Wa because, in previous studies, it has been shown that

Wa a human, group A rotavirus strain does not induce the murine model of BA¹¹. Of the group A rotaviruses, Wa G1P[8] and the simian strain RRV G3P[3] demonstrate the least homology of proteins VP4 and VP7¹⁹; thus, we hypothesized that Wa injection would not elicit neutralizing antibodies against RRV. The two strains of rotavirus were maintained in the monkey kidney epithelial MA104 cell line. The concentration of each strain was determined by focus forming viral titration assays. RRV and Wa obtained from cell culture lysates were used for live virus immunization.

Rotavirus Vaccine

Rotateq® (Merck & Co., Inc., Whitehouse Station, NJ) is a live, pentavalent rotavirus vaccine (PRV) containing five human-bovine rotavirus reassortants approved for oral use in human infants for the prevention of rotavirus gastroenteritis. The murine maternal dose was estimated by first calculating the average cumulative dose received by a human infant normalized by weight as the vaccine is intended to be given as a series of doses. This weight-based dose was then adjusted to average adult female mouse weight and yielded a dose of 60 µl. Prenatal, post-conception immunization was then carried out as described below. A lower dose (30 µl) of vaccine was also injected to assess dose response.

Recombinant Rotavirus Protein

VP6 is a protein that comprises the intermediate layer of the rotavirus particle and possesses a highly conserved genomic sequence across group A rotavirus species²⁰. Previous studies have demonstrated that mucosal administration of recombinant VP6 protein with an adjuvant to adult mice elicited a protective immune response against subsequent oral rotavirus challenge²¹. Given its antigenic properties and cross-strain homology, a recombinant form of this protein has been developed as a non-living, rotavirus vaccine candidate^{21, 22}. Synthesis of the plasmid containing the VP6 of EDIM, a murine strain of rotavirus, expression of VP6 as a chimera with maltose binding protein (VP6::MBP), and purification of the protein have been previously described²³. A total dose of 9 µg of protein was administered to pregnant dams.

Recombinant Influenza Protein

The nucleocapsid protein (NP) of the influenza virus is a major structural protein that in order to stabilize the viral genome binds to the virus' RNA segments. To ascertain whether protection of pups could be conferred non-specifically by a generalized immune response to a prenatal antigenic injection, we also administered recombinant influenza NP protein to pregnant dams. As with the recombinant VP6 described above, NP was expressed via a bacterial plasmid system and purified using a hexahistidine system²³. A total dose of 9 µg of protein was administered to pregnant dams.

Experimental Model of Biliary Atresia

All animal procedures were conducted in accordance with the Cincinnati Children's Hospital Research Foundation Institutional Animal Care and Use Committee. Breeding pairs of BALB/c mice (Harlan Labs, Indianapolis, IN) were kept in microisolator cages in a virus-free environment. The mice were bred and pregnant females separated when found to have a vaginal plug, an indicator of conception. One week after plug appearance, pregnant dams were administered intraperitoneal (i.p.) injections of one of the following: RRV, Wa, 60 µl PRV, 30 µl PRV, VP6, NP, or saline control. Purified rotavirus strains were injected at a dose of 1.25×10^6 focus forming units (ffu)/gram of dam weight. Dams had free access to sterilized chow and water. Upon delivery, only litters of greater than 4 pups were used. An overview of the experimental design is summarized in Figure 1.

To induce the murine model of biliary atresia, newborn pups were injected i.p. with RRV at a dose of 1.5×10^6 ffu per mouse or with saline within 24 hours of birth. After injection, pups were monitored for 21 days. Weight gain, clinical signs of hepatobiliary injury - jaundice in non-fur covered skin, acholic stools, and bilirubinuria - and survival were recorded. The presence of bilirubin in the urine was detected quantitatively using commercially available urine dipsticks (Bayer, Elkhart, IN). Of note, the presence of symptoms is reported as a percentage calculated as (# of symptomatic animals/# of surviving animals).

In a separate series of experiments, J_HD mice, a genetically engineered strain of BALB/c mouse underwent study. J_HD mice (Taconic, Hudson, NY) cannot produce immunoglobulin heavy chain due to a targeted deletion of the J_H gene segments in embryonic stem cells. These mice lack surface Ig⁺ cells which inhibits B cell differentiation at the large CD43⁺ precursor stage. After mating and the appearance of vaginal plug, dams were injected with 60 µl of PRV or saline. Pups born to these dams were then injected with RRV on day of life (DOL) 0 and monitored for 21 days.

Histologic Assessment of Biliary and Hepatic Injury

On DOL 10, the extrahepatic biliary trees and livers of mice born to dams injected with PRV, RRV, or saline were microdissected and preserved in formalin. After being embedded in paraffin, samples were sectioned at 5µm along the length of the sample serially. Sections were allowed to dry overnight, deparaffinized at 60°C for 30 minutes, and stained with hematoxylin and eosin (H&E) using standard techniques. All sections were analyzed using an Olympus BX51 microscope and photographed with an Olympus Digital Camera DP71.

Quantification of Infectious Rotavirus

On DOL 2 and 7, a subset of pups was sacrificed, and their extrahepatic biliary trees were harvested. The specimens were weighed (wet weight) and homogenized in Earle's balance salt solution (EBSS) with Ca²⁺. Samples were stored at -80°C until analyzed. Tissue samples were analyzed for the presence of infectious rotavirus by fluorescent focus assay as described previously^{11, 24}. Viral quantities were expressed as focus forming units (ffu)/ml/mg, with each ffu representing one infectious viral particle.

Rotavirus Antibody Determinations

On DOL 0, pups were sacrificed, and their sera subsequently pooled in pairs. At the same time, blood specimens of a subset of dams were collected by retro-orbital capillary puncture. Dam and pup serum samples were heat inactivated (56°C, 30 min) and analyzed for rotavirus IgG and IgA. The amount of rotavirus-specific antibody was determined by a sandwich ELISA as previously described and expressed as ng of antibody/ml^{25, 26}.

Evaluation of rotavirus neutralization

Neutralizing antibody titers for serum specimens obtained from dams injected with RRV, Wa, PRV (60, 30 µl), VP6::MBP, NP, and saline as well as their corresponding pups on DOL 0 were determined against RRV as previously described²⁴. Heat inactivated maternal and pup serum was serially diluted 2-fold starting at 1:10 in a diluent consisting of 0.5% albumin from bovine serum (Gibco, Carlsbad, CA) and Dulbecco's Modified Eagle Medium with penicillin/streptomycin, amphotericin-B, and glutamine. 4200 ffu of RRV were then added to the serum dilutions and incubated for one hour at 37°C. Positive virus controls and negative diluent controls were added to each row. 96 well plates seeded with MA104 cells were prepared 4 days prior to this experiment. Once confluent, the MA104 cells were washed twice with BSA diluent. The sera-virus mixture was then further diluted 80 fold with diluent, and 100 µl were added to the cell plates. Plates were centrifuged for one hour at 2000 rpm, room temperature. The plates

were then washed and overlaid with a solution of media plus 4 µg/mg trypsin and allowed to incubate at 37°C for 14-16 hours. Plates were stained for presence of virus by methods described previously²⁷. The number of foci in each well was counted and the neutralizing antibody titer was defined as the reciprocal of the dilution producing a 60% reduction in ffu.

Statistical Analysis

Results of morbidity and mortality from rotavirus infection were based on at least 6 pups per infection. Findings were expressed as percent survival and percent symptomatic. Analysis of these variables was done using Fisher Exact testing. Each subset of pups utilized for antibody assays consisted of at least 10 animals. The subset of pups tested for bile duct ffu consisted of at least 6 pups. Results of continuous variables including serum antibody levels, concentration of live virus, concentration of viral antigen, and pup body weight were expressed as mean +/– SEM and analyzed using Student's t test and ANOVA with post-hoc testing as appropriate. Results of these analyses were also expressed as a p value. P values <0.05 were considered significant.

Results

Attenuation of clinical symptoms in the murine model of BA by prenatal immunization of dams with pentavalent rotavirus vaccine

100% of RRV—infected pups born to saline-injected dams manifested signs (jaundice in non-fur-covered skin, acholic stools, and bilirubinuria) of biliary obstruction by DOL 13 (Figure 2A). In contrast, pups born to mothers injected with RRV, Wa, or either dose of PRV displayed no symptoms on DOL 13 ($p < 0.001$). Interestingly, 16% of pups in the high dose PRV group became transiently symptomatic at a later time point. Although this was statistically significant (+, $p < 0.05$), the symptoms disappeared by day of life 21. Furthermore, Figure 2B demonstrates the mean weights of each subset of pups by DOL 21. Mean weights for RRV-infected pups born to dams injected only with saline were consistently lower than those of pups born to mothers injected with RRV, Wa, or 60 µl PRV.

The mortality rates for pups born to mothers injected prenatally with RRV, Wa, 60µl PRV, and 30 µl PRV were 0% ($n=19$), 0% ($n=11$), 8% ($n=13$), and 0% ($n=11$) at 21 days respectively (Figure 2C). In the case of the high dose PRV group, the mortality rate reflects 1 death out of 13 pups. Of note, this mouse did not demonstrate signs of biliary obstruction before its demise on DOL 8. Furthermore, a death at this time point from biliary obstruction would be early in the disease model. In contrast, 82% of pups ($n = 17$) derived from dams that received saline control died by DOL 20 ($p < 0.001$ vs. vaccination), a mortality rate consistent with previously described studies utilizing the murine model of BA¹¹.

To confirm these macroscopic clinical findings we performed histological analyses of the extra-hepatic biliary trees and livers of animals born to dams vaccinated with either RRV or PRV. 10 days after injection with RRV, these pups demonstrated none of the histological characteristics pathognomonic for murine biliary atresia as the normal morphology of the biliary and hepatic tissues was intact (Figure 3). Most significantly, tissue sections taken from RRV-infected pups born to dams who received either RRV or PRV after conception appear identical to the biliary and hepatic sections from non-diseased, control animals (i.e., pups who received saline at the time of birth born to dams who also received saline post-conception). In contrast, biliary and hepatic sections taken from newborn mice injected with RRV born to dams injected with saline demonstrate findings concurrent with murine biliary atresia. Specifically, 10 days after injection with RRV, these animals' biliary trees demonstrate robust inflammatory-cell infiltrates, destruction of the biliary epithelium, and obliteration of the common bile duct lumen. Concurrently, the portal triads from the livers of these animals

demonstrate a significant periportal inflammatory process with destruction of biliary structures as well as focal areas of hepatocellular necrosis (Figure 3).

Prevention of the murine model of biliary atresia using recombinant VP6 but not recombinant NP

The observation that post-conception immunization of dams with RRV, Wa, or PRV protected newborn pups from murine BA was of particular interest given the genetic heterogeneity of these rotavirus strains. Of all the genes in the rotavirus RNA genome, the VP6 gene is one of the most conserved across species. An immune response against the inner capsid protein product of this gene may provide protection against rotavirus infection. With regard to mean weight and symptom manifestation, mice from the VP6-immunized group demonstrated increasing mean weight and lacked symptoms related to extrahepatic biliary obstruction as compared to the pups born to dams injected with saline or influenza NP protein. Furthermore, mean weight and symptom manifestation in the NP group was similar to that of RRV-infected pups born to dams injected only with saline (Figure 4A, B). The mortality rate of RRV-infected pups born to dams who received VP6 protein after conception via i.p. inoculation was 0% at DOL 20 (n=23) ($p < 0.001$). Figure 4C shows that RRV-infected pups born to dams injected with NP protein manifested a mortality rate (73%) similar to that of the saline control mice but significantly different from the VP6 group (n=11) ($p < 0.001$).

Levels of infectious rotavirus in the extrahepatic biliary systems of neonatal mice born to vaccinated dams

After observing the clinical protection afforded by prenatal immunization with Wa, PRV, or recombinant VP6, we next determined the amount of live rotavirus, if any, present in the extrahepatic biliary system of neonatal pups after challenge with RRV. On DOL 2, elevated quantities of infectious RRV were found in the biliary trees of RRV-injected pups born to dams who received saline (n=6) or recombinant influenza NP protein (n=6). In contrast, the RRV level in the biliary trees of those pups born to dams injected with RRV, Wa, or 60 μ l PRV was significantly lower (n=10, 7, and 6, respectively). In the case of pups born to dams injected with 30 μ l of vaccine (n=6) or recombinant VP6 protein (n=6), RRV was undetectable in the extrahepatic biliary system (Table 1).

We also evaluated the biliary tract of RRV-infected pups on DOL 7 for infectious virus by focus forming assay. Allen, et al demonstrated RRV's tropism for BECs as compared to other rotavirus strains and found that the highest titers of RRV in the extrahepatic biliary system of RRV-infected newborn mice was highest on DOL 7¹¹. As with the DOL 2 data, pups born to dams injected with saline or recombinant NP protein had an elevated quantity of RRV in their biliary trees (n=9, 6 respectively). Interestingly at this point, RRV was detected in the biliary trees of pups born to dams injected with 30 μ l of vaccine (n=6). RRV was not found in the extrahepatic biliary systems of pups born to dams injected with RRV, Wa, 60 μ l PRV, or VP6 (n=7, 7, 7, and 6, respectively) (Table 1).

Post-conception immunization of dams elicits rotavirus antibody production

In order to determine the immunologic mechanism that prevented the murine model of BA, antibody studies were performed. Previous studies have demonstrated that RRV infection of adult mice can result in the passive transfer of immunologic factors in milk to suckling pups²⁸. Furthermore, recent data confirmed the vertical transmission of IgG antibodies from dam to pup via the harvest of amniotic fluid and fetal sera at term pregnancy²⁹. Rotavirus IgG levels on the day of delivery in serum from dams injected with RRV, Wa, 60 μ l PRV, 30 μ l PRV, or recombinant VP6 protein were significantly higher (n=3 for all groups) than those levels found in dams injected with saline or NP (n=3, $p < 0.05$) (Figure 5A). Pups born to dams injected with saline or NP had nearly undetectable levels of rotavirus IgG (n=8 for both groups,

$p < 0.05$) in serum as compared to pups born to dams injected with RRV, Wa, 60 μ l PRV, 30 μ l PRV, or recombinant VP6 protein ($n=8, 9, 9, 6$, and 10 , respectively, Figure 5B). Serum levels of rotavirus IgA from both dams and their pups were below the limit of detection (data not shown).

Once it was determined that elevated levels of serum IgG were present in the experimental groups that demonstrated protection against the murine model of BA, we sought to elucidate how these rotavirus antibodies might be conferring protection. Furthermore, it was evident that an interaction between these rotavirus antibodies and RRV was preventing post-natal infection of the BECs by rotavirus. Previous studies have demonstrated an association between levels of rotavirus antibodies and subsequent immunity after RRV immunization^{25, 30}. One commonly accepted mechanism of rotavirus immunity involves generation of neutralizing antibodies against the virus' outer capsid proteins VP4 and/or VP7³¹. As a result, it was of interest to determine whether these antibodies demonstrated neutralizing antibody (NA) against the RRV challenge strain. The neutralizing titer of antibodies obtained from dams injected with saline as well as their pups was expectedly low and thus, represent baseline neutralization (Table 2). Serum antibodies obtained from dams injected with RRV and their pups demonstrated significant NA. However the NA of dams injected with Wa, 60 μ l PRV, 30 μ l PRV, or VP6 and their pups were not significantly higher than baseline.

Maternal vaccination does not prevent murine biliary atresia in B-cell deficient mice

As previously mentioned, the correlation between survival of pups born to dams vaccinated with PRV and level of serum rotavirus antibody highlighted the role of passively acquired antibody as the mechanism protecting BECs from RRV infection. To evaluate this hypothesis further, B-cell-deficient BALB/c females (J_HD) received i.p. injections of either 60 μ l PRV or saline after conception. Only the higher dose of PRV was compared to saline injection given our hypothesis that post-conception vaccination of this mouse strain would not confer protection due to the lack of antibody production by dams. At the time of birth, pups were injected with RRV to induce the murine model of biliary atresia. 100% of pups born to J_HD dams manifested signs of biliary obstruction by DOL 10, and by DOL 15 (Figure 6A), and the overall mortality rate for these animals was 100% (Figure 6B). Similarly, those pups injected RRV at birth born to dams that received post-conception saline also demonstrated a high rate of mortality (78% by DOL 21), and 100% of these pups were symptomatic by DOL 12. This set of experiments further confirms that maternal antibody is involved in the protection against BA that was induced in dams immunized with RRV, Wa, PRV and VP6 protein and that antibody does not have to be neutralizing.

Discussion

Although the pathogenesis of biliary atresia in humans is still unknown, recent research in both humans and animals support the position that a primary perinatal viral infection of the fetus may initiate a host-driven, auto-immune destruction of biliary epithelial cells³². In the current study, our goal was to determine whether maternal, post-conception rotavirus vaccination could mitigate or prevent the development of BA in this murine model of BA. A previous review identified the primary effectors in human and animal studies of rotavirus immunity against oral infection generated by both live rotavirus and non-living rotavirus vaccines³³. Orally-administered, live rotavirus has been demonstrated to generate a neutralizing, IgA rotavirus antibody-mediated protection. In contrast, non-living rotavirus vaccines rely upon a CD4⁺ T cell mediated response. Our study is the first to demonstrate that maternal injection with live rotavirus, including strains that are contained in an approved vaccine for infants, or a recombinant viral protein, namely rotavirus VP6, elicits a non-neutralizing, rotavirus IgG response in both dams and their pups that subsequently prevents BA.

The murine model of biliary atresia, first described by Riepenhoff-Talty, recapitulates clinical symptoms common to the human disease including jaundice, bilirubinuria, and acholic stools¹⁰. However, mortality rates of BALB/c mice born to dams injected with rotavirus, rotavirus vaccine, or recombinant rotaviral capsid protein were significantly lower than those of mice born to dams injected only with saline or the recombinant influenza protein, NP. Furthermore, mice born to vaccinated mothers rarely manifested the sequelae of biliary obstruction and grew at rates similar to those of control mice. It should be noted however, that in **Figures 2B and 4B**, mean weights are representative of animals that survived and cleared RRV infection. Functionally, this aspect of the data may bias our findings thereby minimizing the actual clinical effect of BA. However, when analyzed in the context of the symptom and survival data, this bias is mitigated because RRV-infected pups born to vaccinated dams almost uniformly develop normally and without any sequelae of RRV infection. Therefore, this novel finding indicates that prenatal, maternal vaccination confers a protective immunity to BA in pups subsequently infected with RRV.

The protective effect of prenatal vaccination with PRV or RRV can be explained by the histological appearance of bile ducts and livers taken from pups injected with RRV. The bile ducts of these animals are patent with intact epithelium and are without evidence of inflammation 10 days after RRV infection (Figure 3). Similarly, portal triad and hepatocellular architecture are preserved. In contrast, animals infected with RRV born to dams injected only with saline in the prenatal period demonstrate inflammation and obliteration of both their extra- and intra-hepatic biliary systems. These findings are consistent with macroscopic manifestations of biliary atresia including jaundice and bilirubinuria.

The clinical protection of newborn pups also correlates with the observation that very low amounts of infectious RRV were present in the extrahepatic biliary trees of mice born to vaccinated dams on DOL 2. Furthermore, similar findings were observed on DOL 7, a time point at which previous research has demonstrated the highest titer of infectious RRV in the biliary tree for this model of biliary atresia¹¹. This prevention of RRV infection correlated to an antibody mediated protective mechanism as demonstrated by the results of experiments performed in B-cell deficient mice. Post-conception vaccination with PRV did not prevent obstructive cholangiopathy in B-cell-deficient mice. Given the J_HD dams' inability to generate rotavirus antibodies against PRV, these animals were unable to pass the crucial protective immunologic factor onto their offspring. Although this study cannot say if any subsequent immunologic processes such as antibody-dependent cell cytotoxicity or phagocytosis are involved in viral clearance, these data demonstrate that antibodies are absolutely necessary for rotaviral immunity and protection against murine BA.

Furthermore, this study demonstrates that passive transfer of IgG antibodies from immunized dams to pups is necessary for protection against rotavirus infection. One previous study indicates that anti-rotaviral maternal antibodies can be passively acquired by their offspring in humans³⁴. Two other studies, one in mice and the other in non-human primates, demonstrated that an anti-rotaviral vaccine administered to pregnant females led to elevated antibody levels in both mothers and their offspring^{35, 36}. Our results are consistent with these findings as serum levels of rotavirus IgG were elevated in dams injected with virus, vaccine or VP6 protein and their corresponding pups. In contrast, rotavirus IgG levels were undetectable in saline-injected dams and their pups. Given the different responses to the high and low doses of vaccine, further study is warranted to determine what the smallest dose of vaccine, as well as the subsequent serum rotavirus IgG level necessary for clinical protection against the murine model of biliary atresia.

Rotavirus IgA in all groups was undetectable on DOL 0. This finding is consistent with the data of Connor et al which determined that parenteral rotavirus vaccination elicited an intestinal

IgG response but not an IgA response in rabbits³⁷. It was not until these investigators challenged animals with an oral vaccine did they see a corresponding IgA response in the intestine. Yet our study is novel insofar as we have demonstrated the protective effect of serum rotavirus IgG on a parenterally administered RRV challenge. A recent study demonstrated that passively transferred, parenterally administered IgG prevented enteric rotavirus infection in primates with elevated levels of fecal IgG after antibody infusion³⁸. This finding led the authors to conclude that local IgA in the gut was not essential for protection, and they hypothesized that serum IgG antibody, whether passively acquired or actively induced, may be a surrogate measure for determining protection against viral infection. Given this information, we could have assayed newborn pups for intestinal levels of IgG. However, in contrast to the previously cited study which subsequently challenged animals orally with rotavirus, the transit of parenteral RRV to target BECs in the murine model of BA is not well described. Nevertheless, these results suggest that parenteral administration of both live virus and non-living rotavirus proteins have the capacity to elicit a protective rotavirus antibody response.

Despite the importance of elevated levels of serum rotavirus IgG in dams injected with PRV and their pups, RRV-specific neutralizing antibodies are not necessary for protection in this vaccination model. The NA of both dams and pups injected with either high or low dose vaccine were not significantly different from those of animals injected only with saline. In contrast, the serum antibody of dams injected with live RRV and their pups demonstrated significant RRV neutralizing capability. The human rotavirus strain, Wa, did not elicit a neutralizing antibody response either. Additionally, clinical protection is maintained in pups born to dams injected with recombinant VP6 protein. By definition, rotavirus antibodies elicited by VP6 are non-neutralizing. Our finding is supported by Franco and Greenberg's assertion that rotavirus-specific antibodies, both in serum and the intestine, are most often directed against VP6, a non-neutralizing epitope³⁹. Recent studies have demonstrated that non-neutralizing, anti-VP6 antibodies transcytosed into cells are able to bind uncoated RRV and subvert the virus' life cycle by blocking RNA secretion pores^{40, 41}. However, these studies differ from this one in that they identified IgA, but not IgG, antibodies as the primary mediator of this mechanism. Yet, given the data presented here, it is a possibility that IgG antibodies generated by a parenteral vaccination could block rotavirus intracellular replication in the murine model of BA in light of published data that provide a mechanism of intracellular IgG transcytosis. This process is mediated by the FcRn receptor found on the cell surfaces of polarized epithelial cells of diverse phenotypes including the respiratory, gastrointestinal, and genitourinary systems⁴²⁻⁴⁴. These studies have demonstrated this receptor's ability to take IgG from the apical to the basolateral cell surface and vice versa through the cell. Further studies to determine whether this mechanism is plausible in our disease model are warranted.

With regard to human BA, no viral etiology has been definitely identified as the inciting agent for biliary atresia in humans nor has any mechanism been elucidated as to how such a virus would transit to the fetal or neonatal biliary tree. Even though RRV induces the disease in the murine model, the correlation between it and the human disease are not exact. Human BA has been difficult to characterize fully due to its low incidence and frequently delayed diagnosis⁴⁵. And although a recent high-profile study prospectively demonstrated the benefit of maternal immunization against influenza in the prenatal period for infants⁴⁶, the application of our data to human BA would be highly speculative, and the need for further basic science and clinical insight into the initiation of this disease process is critical.

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References

1. Mack CL, Sokol RJ. Unraveling the pathogenesis and etiology of biliary atresia. *Pediatr Res* 2005;57:87R–94R.
2. Landing BH. Considerations of the pathogenesis of neonatal hepatitis, biliary atresia and choledochal cyst--the concept of infantile obstructive cholangiopathy. *Prog Pediatr Surg* 1974;6:113–39. [PubMed: 4856850]
3. Drut R, Drut RM, Gomez MA, Rua E Cueto, Lojo MM. Presence of human papillomavirus in extrahepatic biliary atresia. *J Pediatr Gastroenterol Nutr* 1998;27:530–5. [PubMed: 9822318]
4. Fjaer RB, Bruu AL, Nordbo SA. Extrahepatic bile duct atresia and viral involvement. *Pediatr Transplant* 2005;9:68–73. [PubMed: 15667615]
5. Mahjoub F, Shahsiah R, Ardalan FA, et al. Detection of Epstein Barr Virus by Chromogenic In Situ Hybridization in cases of extra-hepatic biliary atresia. *Diagn Pathol* 2008;3:19. [PubMed: 18442403]
6. Fischler B, Ehrnst A, Forsgren M, Orvell C, Nemeth A. The viral association of neonatal cholestasis in Sweden: a possible link between cytomegalovirus infection and extrahepatic biliary atresia. *J Pediatr Gastroenterol Nutr* 1998;27:57–64. [PubMed: 9669727]
7. De Tommaso AM, Andrade PD, Costa SC, Escanhoela CA, Hessel G. High frequency of human cytomegalovirus DNA in the liver of infants with extrahepatic neonatal cholestasis. *BMC Infect Dis* 2005;5:108. [PubMed: 16321152]
8. Tyler KL, Sokol RJ, Oberhaus SM, et al. Detection of reovirus RNA in hepatobiliary tissues from patients with extrahepatic biliary atresia and choledochal cysts. *Hepatology* 1998;27:1475–82. [PubMed: 9620316]
9. Riepenhoff-Talty M, Gouvea V, Evans MJ, et al. Detection of group C rotavirus in infants with extrahepatic biliary atresia. *J Infect Dis* 1996;174:8–15. [PubMed: 8656017]
10. Riepenhoff-Talty M, Schaekel K, Clark HF, et al. Group A rotaviruses produce extrahepatic biliary obstruction in orally inoculated newborn mice. *Pediatr Res* 1993;33:394–9. [PubMed: 8386833]
11. Allen SR, Jafri M, Donnelly B, et al. Effect of rotavirus strain on the murine model of biliary atresia. *J Virol* 2007;81:1671–9. [PubMed: 17121809]
12. Harada K, Sato Y, Itatsu K, et al. Innate immune response to double-stranded RNA in biliary epithelial cells is associated with the pathogenesis of biliary atresia. *Hepatology* 2007;46:1146–54. [PubMed: 17661372]
13. Rotavirus vaccines. *Wkly Epidemiol Rec* 2007;82:285–95. [PubMed: 17691162]
14. Parashar UD, Alexander JP, Glass RI. Prevention of rotavirus gastroenteritis among infants and children. Recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2006;55:1–13. [PubMed: 16902398]
15. Rodriguez ZM, Goveia MG, Stek JE, et al. Concomitant use of an oral live pentavalent human-bovine reassortant rotavirus vaccine with licensed parenteral pediatric vaccines in the United States. *Pediatr Infect Dis J* 2007;26:221–7. [PubMed: 17484218]
16. Vesikari T, Matson DO, Dennehy P, et al. Safety and efficacy of a pentavalent human-bovine (WC3) reassortant rotavirus vaccine. *N Engl J Med* 2006;354:23–33. [PubMed: 16394299]
17. Vesikari T, Karvonen A, Prymula R, et al. Efficacy of human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in European infants: randomised, double-blind controlled study. *Lancet* 2007;370:1757–63. [PubMed: 18037080]
18. Linhares AC, Velazquez FR, Perez-Schael I, et al. Efficacy and safety of an oral live attenuated human rotavirus vaccine against rotavirus gastroenteritis during the first 2 years of life in Latin American infants: a randomised, double-blind, placebo-controlled phase III study. *Lancet* 2008;371:1181–9. [PubMed: 18395579]
19. Flores J, Hoshino Y, Boeggeman E, Purcell R, Chanock RM, Kapikian AZ. Genetic relatedness among animal rotaviruses. *Arch Virol* 1986;87:273–85. [PubMed: 3004389]
20. Fields virology. Vol. fourth edition. Lippincott Williams & Wilkins; 2002. (Accessed at
21. Choi AH, McNeal MM, Flint JA, et al. The level of protection against rotavirus shedding in mice following immunization with a chimeric VP6 protein is dependent on the route and the coadministered adjuvant. *Vaccine* 2002;20:1733–40. [PubMed: 11906760]

22. Choi AH, Basu M, McNeal MM, Clements JD, Ward RL. Antibody-independent protection against rotavirus infection of mice stimulated by intranasal immunization with chimeric VP4 or VP6 protein. *J Virol* 1999;73:7574–81. [PubMed: 10438847]
23. Choi AH, Basu M, McNeal MM, Bean JA, Clements JD, Ward RL. Intranasal administration of an Escherichia coli-expressed codon-optimized rotavirus VP6 protein induces protection in mice. *Protein Expr Purif* 2004;38:205–16. [PubMed: 15555936]
24. Knowlton DR, Spector DM, Ward RL. Development of an improved method for measuring neutralizing antibody to rotavirus. *J Virol Methods* 1991;33:127–34. [PubMed: 1658027]
25. McNeal MM, Broome RL, Ward RL. Active immunity against rotavirus infection in mice is correlated with viral replication and titers of serum rotavirus IgA following vaccination. *Virology* 1994;204:642–50. [PubMed: 7941332]
26. McNeal MM, Rae MN, Bean JA, Ward RL. Antibody-dependent and-independent protection following intranasal immunization of mice with rotavirus particles. *J Virol* 1999;73:7565–73. [PubMed: 10438846]
27. McNeal MM, Sheridan JF, Ward RL. Active protection against rotavirus infection of mice following intraperitoneal immunization. *Virology* 1992;191:150–7. [PubMed: 1329313]
28. Offit PA, Clark HF. Protection against rotavirus-induced gastroenteritis in a murine model by passively acquired gastrointestinal but not circulating antibodies. *J Virol* 1985;54:58–64. [PubMed: 2983120]
29. Fusaro AE, Brito CA, Victor JR, et al. Maternal-fetal interaction: preconception immunization in mice prevents neonatal sensitization induced by allergen exposure during pregnancy and breastfeeding. *Immunology* 2007;122:107–15. [PubMed: 17608811]
30. VanCott JL, Prada AE, McNeal MM, et al. Mice develop effective but delayed protective immune responses when immunized as neonates either intranasally with nonliving VP6/LT(R192G) or orally with live rhesus rotavirus vaccine candidates. *J Virol* 2006;80:4949–61. [PubMed: 16641286]
31. Angel J, Franco MA, Greenberg HB. Rotavirus vaccines: recent developments and future considerations. *Nat Rev Microbiol* 2007;5:529–39. [PubMed: 17571094]
32. Mack CL. The pathogenesis of biliary atresia: evidence for a virus-induced autoimmune disease. *Semin Liver Dis* 2007;27:233–42. [PubMed: 17682970]
33. Ward RL. Rotavirus vaccines: how they work or don't work. *Expert Rev Mol Med* 2008;10:e5. [PubMed: 18267049]
34. McLean B, Holmes IH. Transfer of antirotaviral antibodies from mothers to their infants. *J Clin Microbiol* 1980;12:320–5. [PubMed: 6260832]
35. Sheridan JF, Smith CC, Manak MM, Aurelian L. Prevention of rotavirus-induced diarrhea in neonatal mice born to dams immunized with empty capsids of simian rotavirus SA-11. *J Infect Dis* 1984;149:434–8. [PubMed: 6325551]
36. Snodgrass DR, Campbell I, Mwenda JM, et al. Stimulation of rotavirus IgA, IgG and neutralising antibodies in baboon milk by parenteral vaccination. *Vaccine* 1995;13:408–13. [PubMed: 7793139]
37. Conner ME, Crawford SE, Barone C, Estes MK. Rotavirus vaccine administered parenterally induces protective immunity. *J Virol* 1993;67:6633–41. [PubMed: 8411366]
38. Westerman LE, Xu J, Jiang B, McClure HM, Glass RI. Experimental infection of pigtailed macaques with a simian rotavirus, YK-1. *J Med Virol* 2005;75:616–25. [PubMed: 15714478]
39. Franco MA, Greenberg HB. Immunity to rotavirus infection in mice. *J Infect Dis* 1999;179(Suppl 3):S466–9. [PubMed: 10099121]
40. Feng N, Lawton JA, Gilbert J, et al. Inhibition of rotavirus replication by a non-neutralizing, rotavirus VP6-specific IgA mAb. *J Clin Invest* 2002;109:1203–13. [PubMed: 11994409]
41. Kallewaard NL, McKinney BA, Gu Y, Chen A, Prasad BV, Crowe JE Jr. Functional maturation of the human antibody response to rotavirus. *J Immunol* 2008;180:3980–9. [PubMed: 18322207]
42. Kim KJ, Fandy TE, Lee VH, Ann DK, Borok Z, Crandall ED. Net absorption of IgG via FcRn-mediated transcytosis across rat alveolar epithelial cell monolayers. *Am J Physiol Lung Cell Mol Physiol* 2004;287:L616–22. [PubMed: 15169676]
43. Rojas R, Apodaca G. Immunoglobulin transport across polarized epithelial cells. *Nat Rev Mol Cell Biol* 2002;3:944–55. [PubMed: 12461560]

44. Yoshida M, Kobayashi K, Kuo TT, et al. Neonatal Fc receptor for IgG regulates mucosal immune responses to luminal bacteria. *J Clin Invest* 2006;116:2142–51. [PubMed: 16841095]
45. Sokol RJ, Shepherd RW, Superina R, Bezerra JA, Robuck P, Hoofnagle JH. Screening and outcomes in biliary atresia: summary of a National Institutes of Health workshop. *Hepatology* 2007;46:566–81. [PubMed: 17661405]
46. Zaman K, Roy E, Arifeen SE, et al. Effectiveness of maternal influenza immunization in mothers and infants. *N Engl J Med* 2008;359:1555–64. [PubMed: 18799552]

Figure 1. Overview of experimental design. This schematic flowchart summarizes the experiments performed on the wild type (WT) BALB/c animals as described in the **Materials and Methods**. The number of animals in each experimental group is also included. For antibody studies, the sera of two pups was pooled for each sample.

Figure 2.

Clinical results of BALB/c pups injected with RRV born to dams injected with live rotavirus, PRV, or saline. (A) Percentage of mice that developed clinical symptoms of hepatobiliary injury. This percentage is calculated as (# of symptomatic animals/# of living animals). The percentage of symptomatic pups born to dams injected with saline was significantly higher than the other experimental groups on day of life 20 (*, $p < 0.05$); (B) average daily weight of **surviving** pups injected with RRV born to dams injected with live rotavirus, PRV, or saline. The mean weight of pups injected with RRV born to dams that received saline was 7.42 g on day of life 20; (C) Kaplan-Meier survival curves of pups injected with RRV. The mortality rate of mice born to dams injected with saline was 82%.

Figure 3.

Extra-hepatic bile duct and liver histology of BALB/c pups on day of life 10 at 40X magnification. Panels (A) and (B) depict bile ducts and livers from RRV-infected pups born to dams vaccinated with PRV and RRV, respectively. In both cases, the biliary epithelium is intact, and luminal integrity is preserved. Hepatic architecture and morphology are normal. In both cases, the number of immune cells present in these structures is limited. Furthermore, these sections appear identical to those in (C) which come from non-diseased pups, or those animals injected with saline born to dams who received post-conception saline injection. In contrast, (D) demonstrates these tissues from RRV-infected pups born to dams who received only saline. In this case, cholangiocytes are effaced and surrounded by significant numbers of inflammatory cells, both intra- and extra-luminally. Similarly, the portal structures of the liver are overrun with immune cells which are causing focal hepatocellular necrosis (*) and causing damage to the intrahepatic bile duct.

Figure 4.

Clinical results of BALB/c pups injected with RRV born to dams injected with recombinant viral proteins or saline. (A) Percentage of mice that developed clinical symptoms of hepatobiliary injury. The percentage of symptomatic pups born to dams injected with saline was significantly higher than the other experimental groups on day of life 20 (*, $p < 0.05$). On day of life 17, the number of symptomatic pups born to dams that received NP protein was statistically significant (+, $p < 0.05$); (B) average daily weight of **surviving** pups injected with RRV born to dams injected with recombinant viral proteins or saline. The mean weight of pups injected with RRV born to dams that received VP6 was 9.02 g on day of life 20; (C) Kaplan-Meier survival curves of pups injected with RRV born to dams injected with recombinant viral proteins or saline. The mortality rate of pups injected with RRV born to dams that were injected with VP6 was 0%.

Figure 5.

Geometric mean titer of serum rotavirus IgG. The lower limit of detection for this assay is 100 ng/ml. (A) Serum rotavirus IgG in dams. Dams injected with RRV, VP6, PRV (60 and 30 μ l), and VP6 had significantly higher levels of circulating rotavirus IgG (*, $p < 0.05$); (B) Serum rotavirus IgG in pups. Pups born to dams injected with RRV, VP6, PRV (60 and 30 μ l), and VP6 had significantly higher levels of circulating rotavirus IgG (*, $p < 0.05$).

Figure 6.

Clinical results of J_{HD} pups injected with RRV born to dams injected with high dose PRV or saline. (A) Percentage of J_{HD} pups injected with RRV born to dams injected with high dose PRV that developed clinical symptoms of hepatobiliary injury. By day of life 15, all of the pups injected with RRV born to dams that received vaccine were deceased; (B) Percent survival of J_{HD} pups injected with RRV born to dams injected with high dose PRV. The mortality rate of pups injected with RRV born to dams that were injected with either high dose PRV or saline was 100% and 78%, respectively.

Table 1Infectious rotavirus in homogenized extrahepatic biliary trees from pups inoculated with RRV^a

Dam treatment	Titer of infectious rotavirus ^b	
	Day of life 2	Day of life 7
RRV	11.6 ± 11.6 [*]	-- ⁺
Wa	15.0 ± 7.1 [*]	-- ⁺
60 µl PRV	23.3 ± 23.3 [*]	-- ⁺
30 µl PRV	-- ^{c, *}	18991.5 ± 5851.8 ⁺
VP6	-- [*]	-- ⁺
NP	1218.8 ± 328.0	71562.3 ± 15278.4 ⁺
Saline	2260.4 ± 459.7	585997.7 ± 122689.5

^a Groups of ≥6 pups per group were injected with 1.5×10^6 FFU of RRV after dams were treated with live rotavirus, PRV, recombinant viral protein, or saline. Extrahepatic biliary tissue was harvested on the day postinfection indicated and homogenized, and the presence of infectious rotavirus was determined by focus forming assay.

^b Values are expressed as mean ffu/ml/mg wet tissue weight ± standard errors.

^c --, value below the limit of detection.

^{*, +} P < 0.05, as compared to the saline injection group.

Table 2Neutralizing antibody titers against RRV^a

Dam treatment	Antibody titer ^b	
	Maternal ^c	Pup ^d
Saline ^e	16±10.0	10±0.0
RRV	640±282.2	113±150.6
Wa	32±6.7	12±1.5
60 µl PRV	32±20.3	19±7.2
30 µl PRV	50±20.0	13±6.0
VP6	10±0.0	10±0.0
NP	10±0.0	10±0.0

^aSera from dams and their pups were harvested within 1 to 6 hours of delivery. A sera-virus mixture was overlaid on a monkey kidney cell line. Samples were assayed for the presence of virus, and the number of foci were then counted.

^bNeutralizing antibody titer is defined as the reciprocal of the dilution producing a 60% reduction in ffu and is represented as a geometric mean of samples ± standard error.

^cn=3 dams per experimental group.

^dn≥5 samples per experimental group. For each sample, sera from 2 pups was pooled.

^eThe saline control group represents baseline neutralization.