The rhesus rotavirus gene encoding VP4 is a major determinant in the pathogenesis of biliary atresia in newborn mice.

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GRANTS
This work was supported by the National Institute of Diabetes and Digestive and Kidney Diseases grant K08-DK-728858, R03-DK-087974 and R01-DK-091566 to G. Tiao.

Conflict of interest statement
No conflicts of interest exist

Abstract Word Count: 247
Total Word Count: 5133
Abstract

Background: Biliary atresia (BA) is a devastating disease of childhood for which increasing evidence supports a viral component in disease pathogenesis. The murine model of BA is induced by perinatal infection with rhesus rotavirus (RRV) but not by other strains of rotavirus an example of which is TUCH. To determine which RRV gene segment(s) are responsible for pathogenesis, we used RRV and TUCH strains to generate a complete set of single gene reassortants. Methods: Eleven single-gene “loss-of-function” reassortants in which a TUCH gene replaced its RRV equivalent and eleven single gene “gain-of-function” reassortants in which a RRV gene replaced its TUCH equivalent were generated. Newborn BALB/c mice were inoculated with the reassortants and monitored for biliary obstruction and mortality. In vitro, the ability to bind and replicate within cholangiocytes was analyzed. Results: Infection of mice with the “loss-of-function” reassortant RT(VP4) where gene 4 from TUCH was placed on an RRV background eliminated RRV’s ability to cause murine BA. In a reciprocal fashion, the “gain-of-function” reassortant TR(VP4) resulted in murine BA with 88% mortality. Compared with RRV, RT(VP4) binding and titer in cholangiocytes were significantly attenuated while TR(VP4) binding and titer were significantly increased. Reassortant RT(VP3) and TR(VP3) induced an intermediate phenotype. Conclusions: RRV gene segment 4 plays a significant role governing tropism for the cholangiocyte and the ability to induce the murine BA. Gene segment 3 did not affect RRV infectivity in vitro, but altered its in vivo effect.

Key word: Biliary atresia; Cholangiocyte; Reassortant; VP4, Rotavirus
Introduction

Biliary atresia (BA) is a progressive, inflammatory cholangiopathy of infancy leading to obstruction of the biliary tract. Despite current therapy, BA results in cirrhosis and end stage liver disease. Among children, it is the most common indication for liver transplantation\(^{(23,35)}\). Although the etiology of BA is unclear, evidence from both human and murine studies support the hypothesis that biliary atresia is induced by a perinatal viral infection which triggers a host inflammatory immune response\(^{(25,35)}\). The evidence includes patient-based investigations in which reovirus, cytomegalovirus, human papillomavirus, Epstein-Barr virus, and rotavirus\(^{(7,9,25,31-34,36,39)}\) were found in the livers of infants diagnosed with biliary atresia and murine studies in which newborn BALB/c pups infected with rhesus rotavirus (RRV) develop an inflammatory cholangiopathy and bile duct obstruction in a fashion that parallels the disease process that occurs in infants\(^{(33)}\). In this invaluable mouse model, the initiating event is RRV infection of the biliary epithelial cell (cholangiocyte)\(^{(1)}\).

The molecular basis for RRV tropism for cells of hepatobiliary origin has not been defined. Previously, we showed that the tropism for the biliary epithelial cell is strain specific. Among five strains studied, only RRV and SA11-FM (a simian/bovine reassortant) were found to directly infect cholangiocytes and induce extra-hepatic bile duct inflammation and obstruction\(^{(1)}\). Interestingly, the parent strain of SA11-FM, SA11-SM could be found in the hepatobiliary tissue but did not cause direct cholangiocyte injury. In vitro, only RRV and SA11-FM could infect cholangiocytes mirroring the in vivo findings. Since that study, we tested many other strains of rotavirus and identified another simian strain TUCH, that could be found in liver extracts of inoculated mice but did not cause murine BA nor could it infect cholangiocytes in vitro.

Rotaviruses are members of the \textit{Reoviridae} family. A rotaviral particle consists of three concentric protein layers surrounding a genome of 11 double-stranded RNA (dsRNA) segments encoding six structural viral proteins (VP1 to VP4, VP6, and VP7) and six nonstructural proteins (NSP1 to NSP6). Reverse genetics is difficult to perform in rotavirus; however, rotavirus can undergo
genetic reassortment after mixed infection in vivo or in cell culture \((11, 18-19, 28, 30, 37-38)\). When two strains of rotavirus are co-infected into a host cell, progeny virus, termed reassortants, are generated that contain different combinations of the parental genes. Single-gene reassortants (i.e. all but one gene derived from one parent) potentially allow the determination of the function of that gene. Previous studies utilizing reassortants have reported gene segments encoding for VP3, VP4, VP7, NSP1, NSP2 and NSP4 to be associated with virulence \((2-4, 15, 26-27)\) and NSP3 as a determinant of RRV’s extra-intestinal spread \((26)\).

The goal of this study was to determine the specific RRV gene(s) that govern the induction of murine BA. We utilized the property of reassortment to systematically generate a complete set of 22 single gene reassortants derived from the parental strains RRV and TUCH - eleven loss-of-function “knock-out” reassortants that contain 10 genes derived from RRV with one gene replaced by its TUCH equivalent and eleven reciprocal gain-of-function “knock-in” reassortants that contain one gene derived from the RRV with 10 genes derived from TUCH. Administration of these reassortants to neonatal mice and characterization of their disease phenotype indicated gene segment 4, encoding for rotavirus protein VP4 was a primary determinant for biliary injury. In vitro, gene segment 4 governed RRV attachment and infection of the cholangiocyte. In vivo, gene segment 3 was found to affect RRV’s ability to cause BA. The mechanism by which these genes and their translated proteins determine RRV tropism for bile duct epithelial cells requires further study.

**MATERIALS AND METHODS**

**Cells, viruses and animals**

MA104 cells (BioWhittaker, Walkersville, Md.) were grown in Dulbecco’s Modification of Eagles Medium (DMEM) (Cellgro) supplemented with 10% fetal bovine serum (FBS) (Gibco/BRL, Gaithersburg, Md.), 0.01% Penicillin / Streptomycin (Gibco/BRL), 0.01% L-glutamine (Gibco/BRL) and 0.005% Amphotericin B (Cellgro). The mouse cholangiocyte cell line (mCL) generously provide by the laboratory of Dr. James Boyer (Yale Liver Care Center, Hartford,
CT) was cultured as previously described (16). We used two rotavirus strains: a) RRV, a simian strain of genotype G3P[3] (kindly provided by H. Greenberg, Stanford University, and Palo Alto, CA) and b) TUCH (named after location where the strain was isolated - Tulane National Primate Research Center and Cincinnati Children’s Hospital), a simian strain of genotype G3P[24] (and subgroup 1 (24)).

Breeding pairs of BALB/c mice (Harlan Labs, Indianapolis, IN) were kept in micro-isolator cages in a virus-free environment with free access to sterilized chow and water. The mice were bred and pups in litter size of greater than 4 were used.

Generation, purification and analysis of reassortants

Reassortants were generated by co-infection of MA104 cells, the standard kidney epithelial cell line used to maintain rotavirus strains in cell culture. MA104 cells were seeded in polystyrene tubes in 2 ml DMEM+10% FBS. Monolayers were co-infected at varying multiplicities of infection (MOI) of RRV and TUCH. After 1 hr of absorption, serum free DMEM+ 4µg/ml trypsin (1:250)(Invitrogen, Carlsbad, CA) was added. At 24 and 48 hours, the cultures were evaluated for cytopathic effect (CPE) and frozen when CPE was complete. Several single-gene reassortants were derived by backcrossing double or multi-gene segment reassortants with parental or other multi-gene reassortants.

Reassortants were plaque purified in confluent cell culture plates containing MA104 cells in DMEM+10% FBS. The cells were infected with 0.2 ml of serial diluted virus supernatants for 1 hr, overlaid with 5 ml of media containing 0.2% agarose (Lonza, Rockalnd, ME) and incubated for 3-4 days at 37°C. Plaques were picked and amplified in MA104 cells. Viral RNA was extracted and the dsRNA segments were visualized by silver stained sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis as described previously (14). The migration patterns of the reassortants' dsRNA genes were compared with those of the parental strains and gene assignments were made based on how RRV gene segments migrated. Progeny with single gene substitutions were
identified and re-amplified. The parental origin of the single gene reassortants was re-confirmed by gel electrophoresis and sequencing after amplification.

**Sequencing of the reassortant genes using RT-PCR**

To verify the findings based on gel electrophoresis, all eleven genes found in each of the twenty-two single gene reassortants were amplified and underwent partial sequence analysis. Genomic dsRNA was isolated from infected cell lysates as described before. The RNA was used as a template for the preparation of viral cDNAs using a OneStep RT-PCR kit (Invitrogen) and appropriate segment-specific primers (listed in supplemental file 1). PCRs were performed in 50 µl reaction mixtures containing 10 mM Tris buffer (pH 8.3), 3 mM MgSO₄, 0.2 mM concentration of each dNTP, 0.25 µM concentration of each primer, and 2 U of PlatnimTaq polymerase (Invitrogen). PCR conditions were 35 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min 30 s, using a thermocycler PTC-200 (MJ research). PCR products were confirmed by separating in 1.5% agarose gels stained with ethidium bromide. DNA sequencing of RT-PCR products was performed according to the dideoxynucleotide chain termination method, using an ABI Prism Big Dye Terminator cycle sequencing ready reaction kit and an ABI Prism 3730 XL DNA analyser (Applied Biosystems) according to the manufacturer’s instructions (see supplemental file 1 for sequence data).

**Viral inoculation of newborn mice: phenotypic characterization**

Intraperitoneal injection of newborn pups with single gene reassortants at a dose of 1.5 x 10⁶ ffu per mouse was performed within 24 hr of birth. Saline injected pups served as controls. Clinical signs of hepatobiliary injury (i.e., 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 Co., Elkhart, IN). A subset of injected mice were sacrificed 7 days post injection and the liver and extrahepatic biliary tract were harvested, preserved in formalin and analyzed histologically as previously described (1). In another subset of mice, the extrahepatic biliary tract was weighed (wet weight) and homogenized in Earl’s Balanced Salt Solution...
(EBSS). Tissue samples were analyzed for the presence of infectious rotavirus by a focus forming assay (FFA) and quantities of infectious virus were reported as focus forming units (ffu) per milliliter per milligram (wet weight) as previously described \(^1\).

**Measurement of viral binding using attachment assay**

The ability of the virus to attach to mCL cells was assessed by binding assays as previously described \(^{16-17}\). In brief cultured cells were grown to confluence in 24-well plates. Attachment assays were performed in triplicate. At the time of assay, the cells, media, and inoculating virus were cooled to 4°C. Cells were inoculated with varying amounts of virus and incubated for 1 hour at 4°C. The inoculum was removed and the cells were washed twice to remove any unbound virus. The washes and the residual inoculum were combined to account for all unbound viruses. The cells underwent 2 freeze/thaw cycles and any virus found within the final cell fraction reflected bound virus. The amount of bound and unbound virus was determined by FFA analysis. The amount of bound virus was expressed as a percentage of the total amount of virus used to inoculate the cells.

**In vitro infectious viral assay in cholangiocytes**

For the infectious assay, mCL cells were seeded in 24-well plates and grown to confluence. Wells were washed with EBSS and inoculated with reassortants at an MOI of 1 at 37°C for 1 hr. The cultures were washed and incubated with serum-free DMEM+ 4µg trypsin/ml at 37°C for 48 hr. Cultures were monitored for the development of CPE and viral yield was assessed by FFA using MA104 cells as described below.

**Focus Forming Assay**

The focus forming assay was performed by seeding 96-well plates with MA104 cells and grown for 4 days. Once confluent, they were exposed to serially diluted virus samples for 1.5 h. The cells were washed with DMEM and incubated at 37°C for 14 to 16 h with DMEM containing 4 ug of trypsin/ml. The media was aspirated and the cells were fixed with cold 80% acetone for 15 min at 20°C. Following washing with phosphate-buffered saline (PBS) guinea pig anti-rotavirus
immunoglobulin G (IgG) primary antibody (1:1,000) was added and incubated for 30 min. Wells were washed with PBS and fluorescein isothiocyanate (FITC)-tagged goat anti-guinea pig IgG secondary antibody (1:500) was added and incubated for 30 min at 37°C. Wells were washed twice and allowed to dry completely. Plates were scored using a UV microscope (10X objective) and quantities of infectious virus were reported as ffu per milligram (wet weight) of tissue.

**Statistical analysis**

Development of symptoms and mortality rates following rotavirus inoculation were based on at least 12 pups per experimental group. Findings were presented as percentages of pups expressing at least two symptoms and percent survival. Analysis of these non-continuous variables was done using an arc sin square root transformation to make comparisons between control and treatment groups. A multiple testing adjustment was made by calculating the Bonferroni adjusted p-values due to the control being compared to the eleven treatment groups (10). Each subset utilized for the FFA assays consisted of at least 5 pups. Results of these continuous variables were expressed as arithmetic means ±S.E. and were analysed by using ANOVA with post hoc testing where appropriate. A p< 0.05 was considered significant.

**Results**

**Perinatal infection with TUCH does not cause the murine model of BA**

Compared to RRV which induces the murine model of BA, the i.p. inoculation of newborn BALB/c pups with the TUCH virus caused no signs of hepatobiliary injury even though both RRV and TUCH was detected in liver and common bile duct extracts within 2 days of inoculation (Table 1). None of the pups displayed jaundice during the 21 days after inoculation and the mortality rate was 0% (Figure 1A-B). In vitro, TUCH had poor binding capacity and virtually no ability to replicate within cholangiocytes correlating with its in vivo effect. In contrast TUCH bound to and replicated similarly to RRV in MA104 cells.
(Figure 1C). These qualities made TUCH an ideal virus to generate single gene reassortants when co-infected with RRV.

**Generation of viral reassortants**

To generate single gene reassortants, MA104 cells were co-infected with the parental strains RRV and TUCH at varying MOI. The resulting progeny underwent plaque purification followed by polyacrylamide gel electrophoresis and sequencing to determine parental origin of the genetic content. Reassortants were classified according to the source of their individual genes (Figure 2). A total of 1226 plaques were picked: 703 plaques were generated by co-infections with different MOI combinations of RRV and TUCH. 225 of the 703 plaques contained copies of both parental genes indicating the plaque was not pure; these plaques were not used further. Of the 478 pure plaques, 337 clones were of a parental phenotype and 141 were reassortants. Among the reassortants, 71 were of a RRV background and 70 were on a TUCH background. Background was determined by which parent contributed 6 or more genes to the reassortant strain. 59 plaques contained single-segment reassortants, 39 were double, 18 were triple, and 25 had 4 or 5 gene segment substitutions. Because we could not isolate all single gene reassortants from co-infection with the parental strains, backcrossing first generation double or multiple-gene segment reassortants with a parental strain was performed; 523 plaques were picked under those conditions. From these back crosses, six single-gene reassortants were generated - $T_{R(VP2)}^R$, $T_{R(VP3)}^R$, $T_{R(VP4)}^R$, $T_{R(NSP2)}^R$, $T_{R(NSP5)}^R$ and $R_{T(VP6)}^T$.

Initially, reassortants were identified by gel electrophoresis. In addition, upon identification of the complete set of 22 gene reassortants, all eleven genes within each of the 22 reassortants underwent partial sequence analysis using RT-PCR. The sequence data (supplemental file 1) verified the single gene change within the background of ten genes derived from the parent strain of RRV and TUCH and validate the findings of gel electrophoresis.

The 22 single-gene reassortants generated were labeled according to the following nomenclature - $R_{T(oooo)}^T$ (reassortant containing 10 genes derived from RRV with the xxxx gene segment derived from TUCH) or the reciprocal $T_{R(oooo)}^R$. 


(reassortant containing 10 genes derived from TUCH with the xxxx gene derived from RRV). For example, the single gene reassortant in which the TUCH VP1 gene was placed on an RRV background is labeled as the reassortant $R^T(VP1)$. The reciprocal reassortant in which the RRV VP1 was placed on the TUCH background is described as $T^R(VP1)$.

**Gene segment 4 governs the RRV induced murine model of BA**

Groups of newborn pups were inoculated with the 22 single-gene reassortants. The ability to cause clinical manifestations of biliary obstruction and mortality rates were determined (Table 2). Dramatic changes were found with reassortants $R^T(VP4)$ and $T^R(VP4)$. The reassortant $R^T(VP4)$ (containing gene segment 4 from TUCH placed on a RRV background) did not elicit any manifestation of biliary injury and 100% of injected pups survived completely eliminating the RRV parental effect (Table 2). In contrast, reassortant $T^R(VP4)$ (containing gene segment 4 from RRV on a TUCH background) induced signs of biliary obstruction in 100% of pups and produced a mortality rate of 88.2%, reversing the TUCH parental effect (Table 2). An intermediate phenotype was seen in $R^T(VP3)$ infected mice. Both the symptom and mortality rates were significantly lower than that of RRV injected mice ($p<0.05$, Table 2). In mice infected with $T^R(VP3)$, significantly more pups developed signs of obstructive jaundice than TUCH but the mortality rate remained low.

Of the remaining nine “knock-out” strains, all behaved similarly to RRV causing biliary obstruction in 76.9-100% with mortality rates ranging from 53.9-89.5%. There were only subtle differences in onset and duration of clinical symptom (data not shown). Among the remaining nine “gain-of-function” reassortants, $T^R(NSP1)$, $T^R(VP6)$, $T^R(NSP2)$, $T^R(NSP4)$ and $T^R(NSP5)$ caused some symptoms of hepatobiliary injury; but, the rate was significantly lower than that of RRV. All pups injected with $T^R(NSP1)$, $T^R(VP6)$, $T^R(NSP2)$, and $T^R(NSP5)$ survived ($p<0.05$) while there was a slight increase in mortality in pups infected with $T^R(NSP3)$. Reassortants $T^R(VP1)$, $T^R(VP2)$ or $T^R(VP7)$ did not elicit any sign of hepatobiliary injury or cause mortality.
The yield of virus in extrahepatic biliary samples mirrored the development of clinical symptoms of BA. Although “knock-out” of gene segments 1 through 7, 10 and 11 all reduced the yield of virus in the bile ducts compared to RRV (p<0.05, Table 2), the most dramatic reduction was seen with reassortant RT(VP4) with a viral yield 27 fold less than RRV (1.1 ± 0.1 x 10^4 ffu/ml/mg vs 29.5 ± 4.5 x 10^4 ffu/ml/mg, p<0.05, respectively). In contrast, the yield of virus following infection with TR(VP4) was 9 times higher than that of its parent strain TUCH (25.9 ± 4.0 x 10^4 ffu/ml/mg vs 2.9 ± 0.6 x 10^4 ffu/ml/mg, respectively, p<0.05, Table 2). Among the “knock-in” strains that induced signs of obstructive jaundice (TR(VP3), TR(NSP1), TR(VP6), TR(NSP3), TR(NSP2), TR(NSP4), TR(NSP5)), the viral titer was slightly higher than that of parental strain TUCH, but only the titer after infection with TR(VP6) was statistically significant (p<0.05). Consistent with the observation that mice injected with TR(VP1), TR(VP2) and TR(VP7) did not show any signs of disease, the viral titers in the bile ducts were low.

**Histological assessment of the liver and extra-hepatic biliary tract**

Histologic analysis of the liver and extrahepatic bile duct harvested from mice at 7 days after inoculation with reassortants was performed and compared to specimens obtained from mice inoculated with the parental strains (Figure 3). The histologic appearances of the portal area as well as the extra-hepatic bile duct after infection of mice with different mono-reassortant strains were consistent with the symptoms. The histology of livers from mice injected with the reassortants on an RRV background revealed marked infiltration of inflammatory cells within the area of the portal tract except for those inoculated with RT(VP4) (Figure 4). Peri-ductal inflammatory infiltration, epithelial sloughing, stromal proliferation and lumen obstruction were universally seen in extrahepatic bile duct of these groups. In contrast, histology of extrahepatic bile ducts as well as livers of pups injected with TR(VP4) was indistinguishable from TUCH or saline control injected mice (Figures 3 and 4).

Minimal (TR(VP1), TR(VP2), TR(VP7)) to medium (TR(VP3), TR(NSP1), TR(VP6), TR(NSP3), TR(NSP2), TR(NSP4), and TR(NSP5)) inflammatory infiltration in the portal area of liver was observed in all the TUCH background reassortants infected mice.
excluding those inoculated with T^R(VP4) in which the portal tract infiltration was rather extensive. The histological changes of extrahepatic bile duct of T^R(VP4) injected mice were indistinguishable with that of RRV injected mice (Figures 3 and 5). Epithelial edema and submucosal inflammation were commonly seen in extrahepatic bile ducts of mice infected with other TUCH based clones. In addition epithelial cell sloughing was also found in the T^R(VP3), T^R(NSP3) and T^R(NSP5) group, but intra-luminal infiltration and stromal proliferation were rarely seen and the lumens of bile ducts were patent as demonstrated by T^R(VP2).

RRV and TUCH binding to cholangiocytes was significantly altered by manipulating gene segment 4

To establish a mechanistic basis for the in vivo findings, we tested the reassortants in our in vitro model of BA in which RRV but not TUCH binds to and replicates within cholangiocytes. We found that gene segment 4 of TUCH when inserted into a RRV background reduced viral binding when compared to the parental RRV strain (4.0%±0.5 vs 11.9%±0.1, p<0.05, Table 3). While binding ratios of reassortants R^T(VP1), R^T(VP2), R^T(VP3) and R^T(NSP1) were similar to that of RRV, reassortants R^T(VP6), R^T(VP7), R^T(NSP3), R^T(NSP2), R^T(NSP4), and R^T(NSP5) were all slightly higher (Table 3).

Placement of RRV gene segment 4 onto a TUCH background enhanced viral attachment. Reassortant T^R(VP4) exhibited a significantly higher binding ratio as compared with that of parental strain TUCH (12.4%±0.5 vs 5.3%±0.6, p<0.01, Table 3). The binding abilities of none of the other reassortants were different from TUCH except for strain T^R(VP2) and T^R(NSP3), whose binding ratio (1.37%±0.2, 2.87%±0.2 respectively) were significantly lower (p<0.05).

Reassortant replication in cholangiocytes in vitro

Infection of cholangiocytes in vitro with “knock-out” reassortants resulted in viral replication rates that mirrored the in vivo findings. R^T(VP4) dramatically lowered the yield of virus when compared to RRV(0.8 ± 0.1 x 10^4 ffu/ml vs 3850.0 ± 449.2 x 10^4 ffu/ml respectively) (p<0.05, Table 3). The remaining “knock-out” reassortants had viral yields that were similar to that of the parental strain RRV.
The “knock–in” clones $T^{R(VP3)}$, $T^{R(VP4)}$, $T^{R(NSP1)}$, $T^{R(VP6)}$, $T^{R(NSP2)}$, $T^{R(NSP4)}$, and $T^{R(NSP5)}$ titers were significantly higher than that of TUCH ($p<0.05$, Table 3). Consistent with the in vivo findings, the reassortant $T^{R(VP4)}$ replicated to the highest titer, 174 times greater than that of TUCH ($226.3 \pm 50.4 \times 10^4$ ffu/ml vs $1.3 \pm 0.1 \times 10^4$ ffu/ml) and 3 times greater than the second highest reassortant, $T^{R(VP3)}$. Reassortants $T^{R(VP1)}$, $T^{R(VP2)}$, and $T^{R(VP7)}$ had significantly reduced titers ($p<0.05$, Table 3).

Discussion

Previously, we found that among five rotavirus strains studied, RRV and SA11-FM had tropism for the biliary epithelial cell and could induce the murine model of biliary atresia; SA11-SM could be found in the hepatobiliary system but caused hepatitis instead of biliary obstruction while EDIM and Wa caused no hepatobiliary disease (1). To determine the molecular basis of RRV tropism for the biliary epithelial cell, we utilized the rotavirus property of reassortment to determine which RRV gene(s) govern its ability to induce the murine model of biliary atresia. Initially, we attempted to generate reassortants with the parental strains of RRV and EDIM; but, because EDIM does not replicate well within MA104 cells, the generation of new reassortants was challenging. To overcome this challenge, we tested other strains of rotavirus in the murine model of BA and identified the simian strain TUCH which after injection into newborn BALB/c pups could be found within hepatobiliary tissue but did not cause BA. Because it replicated well in MA104 cells we used it for generation of reassortants. By using RRV and TUCH, we generated a complete set of 11 loss-of-function “knock-out” single gene reassortants and 11 reciprocal gain-of-function “knock-in” single-gene reassortants. The gain-of-function “knock-in” reassortants were important confirming that the presence of a specific RRV gene was by itself capable of causing the murine model of biliary atresia. These rotavirus mono-reassortants permit the identification of RRV gene(s) which govern the ability to induce BA. Using these single-gene reassortants, we identified RRV gene segments 3 and 4 as important determinants in the pathogenesis of murine BA. We found that when gene segment 4 of RRV was replaced by the corresponding gene
derived from TUCH, the manifestations of biliary obstruction were totally abolished. In a reciprocal fashion, when newborn mice were infected with TR(VP4) (the TUCH background clone with the VP4 gene derived from RRV), BA was induced. Supportive results were recently found using the rotavirus strains RRV and UK (8). Experiments performed by Feng et al demonstrated the requirement of both genes VP4 and NSP1 from RRV for replication in mouse biliary tract. RRV and TUCH share a 91% amino acid homology in NSP1 which could explain why we did not observe a role for NSP1 in our experiments. The in vitro studies performed in our study, revealed that RRV gene segment 4 governed cholangiocyte binding and infectivity establishing a mechanistic basis for the in vivo results. The protein product of gene segment 4, VP4, has been found to be a major determinant of pathogenicity of rotavirus in several systems. Using a heterologous bovine × simian viral reassortants, Offit et al linked gene segment 4 to tropism for the intestine in mice (27). Two porcine rotavirus variants (4f and 4s) having different VP4 genes showed distinct pathogenicity during serial passage in gnotobiotic piglets. Inserting the pathogenic parental (4f) gene segment 4 into the non-pathogenic virus (4s) genome by reassortment caused the latter to develop tropism for the intestine and caused diarrhea in piglets (2). VP4 is essential for early virus-cell interactions as it participates in receptor binding and cell penetration (12-13).

The early rotavirus-cell interactions constitute a multistep process (21). In the multistep model, the initial contact of a neuraminidase sensitive virus strain with the cell surface occurs through a sialic acid (SA) containing cell receptor, using the VP8* subunit of VP4 which is positioned at the surface of rotavirus particles. The initial interaction of the virus with SA induces a subtle conformational change in VP4, which allows the virus to interact with a second cell receptor (currently proposed to be the α2β1 integrin) through a DGE-binding motif. After the second interaction, the virus interacts with integrins αXβ2, αVβ3 and hsc70. These interactions increase the permeability of the cell membrane facilitating penetration of the virus into the cell. Previously, we found that cell-surface expression of the α2β1-integrin confers cholangiocyte susceptibility to
RRV infection (16). The basis by which TUCH versus RRV VP4 interacts with the
433  cell surface requires further study. The VP4 proteins from these simian
434  rotaviruses share 87% homology, so it is probable that within the non-
435  homologous regions lies the mechanistic basis for their differing ability to infect
436  the cholangiocyte.
437
438  Interestingly, VP7 the other major constituent of the outer protein layer did
439  not, under these conditions, govern hepatobiliary tropism. As an outer capsid
440  protein, VP7 plays a role in viral entry; however, reassortants R_T(VP7) and T_R(VP7)
441  behaved similar to their parents. The function of VP7 is to facilitate cell entry and
442  infection by interacting with αXβ2 and αVβ3 (12) in a post-binding step. Because
443  the VP7 of RRV and TUCH were both G3 types and their protein homology was
444  90.5%, it is likely that manipulating this gene segment did not change the protein
445  structure of VP7, thus not affecting cell entry/replication.
446
447  The basis by which gene segment 3 and its translated protein VP3
governs the induction of murine BA is unknown. At least one function of VP3 is
448  that it acts as a guanylyltransferase and methyltransferase enabling capping of
449  the 5' end of the mRNA synthesized in DLPs by the RNA dependent RNA
450  polymerase, VP1 (5, 20, 29). The cap stabilizes viral mRNA potentially protecting it
451  from degradation by nucleases (6) and enables its translation by interaction with
452  cellular translation protein complexes. Association of VP3 with three other genes
453  (VP4, VP7, NSP4) in rotavirus virulence and host range restriction and
454  attenuation has been suggested in reassortant studies (27). In these studies
455  piglets failed to develop diarrhea when challenged with a single gene reassortant
456  that derived its 3rd gene from an avirulent background, while diarrhea was
457  induced only when virulence-associated genes encoding VP3, VP4, VP7, NSP4
458  were present together on an avirulent rotavirus background. Our results are
459  somewhat different from these observations in that reassortant R_T(VP3) did not
460  abolish the biliary injury, rather it elicited an attenuated phenotype as compared
461  with RRV. Reassortant T_R(VP3) caused a significant increase in biliary injury
462  symptoms similar to RRV but the mortality was minimal. We speculate that gene
463  segment 3 might affect viral replication rates thereby decreasing mortality but this
was not observed in infectivity assays. The basis for the intermediate phenotype
induced by gene segment 3 is currently unclear but it might be the result of the
interactions of multiple factors including the host immune response.

It was interesting to note that TUCH reassortants containing RRV gene
VP6, NSP2, NSP3, NSP4 or NSP5 increased symptoms of hepatobiliary injury
without eliciting bile duct obstruction or mortality. The reverse effect was not
seen in the reciprocal RRV reassortants containing the same TUCH genes. The
TUCH reassortants produced higher amounts of replication competent virus
versus the TUCH parent both in vivo and in vitro. The increase in symptoms
encountered following infection with these reassortants could be a consequence
of viral load causing hepatobiliary inflammation rather than obstruction. Further
study will be required to determine how these genes contribute to disease
pathogenesis.

The basis by which these gene segments contribute to disease
pathogenesis may extend beyond the mechanisms discussed. Several recent
investigations have established both a cell and antibody-mediated immunological
role in biliary obstruction. A recent study by Mack et al showed that a component
of VP4 results in the generation of an antibody that recognizes alpha enolase
expressed on cholangiocytes. This mimicry may contribute to BA disease
pathogenesis. Interestingly, within the segment of interest identified in that study
lie several amino acid differences between RRV and TUCH that may cause
conformational changes in the peptide structure which could contribute to
antibody recognition. Site directed mutagenesis will be necessary to confirm
these findings.

In summary, a set of 22 single gene reassortants was generated that
allowed the identification of RRV gene segments 3 and 4 as important
determinants of murine BA. RRV gene segment 4 through its translated protein
VP4 determined RRV tropism for the biliary system. The mechanism of binding to
receptors on the surface of cholangiocytes remains to be characterized.
Substitution of RRV’s 3rd gene segment did not affect the viral infectivity in vitro;
however, it caused an intermediate phenotype in vivo. Although rotavirus has
been refractory to direct genetic manipulation, a reverse genetics system has been recently developed which permits the rescue of a viral RNA segment produced from cDNA in vitro into replication-competent progeny virus (18-19). In the future, this approach will be utilized to determine the precise molecular basis by which RRV gene segments 3 and 4 contribute to pathogenesis of BA.

Acknowledgements

We thank Dr Harry Greenberg for his editorial review of this manuscript, Dr Marepalli Rao for statistical support, and Dr Jelle Matthijnssens for help with primer design.

Reference


Table 1 – Live virus in organs extracts per strain following inoculation

<table>
<thead>
<tr>
<th>Organ</th>
<th>Day Post Injection</th>
<th>RRV</th>
<th>TUCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestines</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>28.5 ± 7.7 *c</td>
<td>1.7 ± 0.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>409.6 ± 44.9 *</td>
<td>33.5 ± 24.5</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>1199.7 ± 298.1 *</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>25.5 ± 16.0 *</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>47.7 ± 28.9</td>
<td>15.6 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>26369.6 ± 12112.0 *</td>
<td>37.8 ± 19.0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>24566.6 ± 13445.0 *</td>
<td>512.1 ± 418.6</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>33.4 ± 24.0</td>
<td>23.1 ± 13.5</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Bile Duct</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>750.6 ± 395.2 *</td>
<td>94.4 ± 31.9</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>97943.8 ± 36176.8</td>
<td>26622.9 ± 11252.7</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>234949.2 ± 76252.7 *</td>
<td>29011.6 ± 6053.2</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>4890.8 ± 3515.5 *</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>—</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

a Groups of ≥10 pups per time point were injected within 24 hours of birth with 1.5 x 10^6 ffu of rotavirus. Organs were harvested on the day post-infection indicated, homogenized, and the amount of virus was determined by focus-forming assay in MA104 cells.

b Values are expressed as arithmetic mean titer (amt): ffu/ml/mg tissue weight ± standard errors harvested from mice.

*c = p≤0.05 RRV vs. TUCH

d, value below the limit of detection
Figure 1 – The effect of RRV and TUCH in vivo in the murine model of BA and in vitro in MA104 and mCL cells

Pups were infected within the first 24 hours of birth with $1.5 \times 10^6$ ffu/pup with RRV or TUCH. RRV injection leads to symptoms of cholestasis in mice between 6–15 days (A), and to decreased survival (B). The onset of cholestasis was very mild and there was 100% survival in mice infected with TUCH. Total number of animals at the time of injection of RRV= 20 and TUCH= 16. During the course of the study, all mice injected with TUCH showed mild symptom, remained healthy and survived. In RRV injected mice, 18 out of 20 animals had died by day 16. (C) Within cholangiocytes RRV bind to and replicated almost 100 fold higher as compared to TUCH but in contrast both the strains almost bind and replicate in a similar fashion in MA104 cell.
Figure 2 – Gel electrophoresis of dsRNA from the reassortants

dsRNA extracted from the 22 single-gene reassortants were separated by SDS-PAGE and silver stained to reveal migration rates of the gene segments. The dashed circles and squares illustrate RRV genes shift on the TUCH background. The solid circles and squares illustrate TUCH genes shift on the RRV background.
Table 2 – Signs of biliary obstruction, mortality rates, and replication competent virus in bile ducts of pups after infection with reassortants

<table>
<thead>
<tr>
<th>Reassortant</th>
<th>Symptoms (%)</th>
<th>Mortality (%)</th>
<th>Infectious Virus in Bile Ducts (x10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RRV</td>
<td>100.0</td>
<td>80.0</td>
<td>29.5 ± 4.5</td>
</tr>
<tr>
<td>RT(VP1)</td>
<td>100.0</td>
<td>100.0</td>
<td>9.0 ± 2.1 *</td>
</tr>
<tr>
<td>RT(VP2)</td>
<td>100.0</td>
<td>85.7</td>
<td>8.4 ± 3.3 *</td>
</tr>
<tr>
<td>RT(VP3)</td>
<td>58.4 ** *</td>
<td>20.8 *</td>
<td>4.5 ± 1.0 *</td>
</tr>
<tr>
<td>RT(VP4)</td>
<td>0 *</td>
<td>0 *</td>
<td>1.1 ± 0.1 *</td>
</tr>
<tr>
<td>RT(NSP1)</td>
<td>85.0</td>
<td>85.0</td>
<td>9.4 ± 3.2 *</td>
</tr>
<tr>
<td>RT(VP6)</td>
<td>95.0</td>
<td>55.0</td>
<td>6.3 ± 0.9 *</td>
</tr>
<tr>
<td>RT(VP7)</td>
<td>91.7</td>
<td>87.5</td>
<td>7.7 ± 1.9 *</td>
</tr>
<tr>
<td>RT(NSP3)</td>
<td>89.5</td>
<td>89.5</td>
<td>18.7 ± 4.7 *</td>
</tr>
<tr>
<td>RT(NSP2)</td>
<td>76.9</td>
<td>53.9</td>
<td>19.9 ± 6.4 *</td>
</tr>
<tr>
<td>RT(NSP4)</td>
<td>95.2</td>
<td>81.0</td>
<td>8.5 ± 1.3 *</td>
</tr>
<tr>
<td>RT(NSP5)</td>
<td>95.5</td>
<td>59.1</td>
<td>5.8 ± 1.4 *</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reassortant</th>
<th>Symptoms (%)</th>
<th>Mortality (%)</th>
<th>Infectious Virus in Bile Ducts (x10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TUCH</td>
<td>0</td>
<td>0</td>
<td>2.9 ± 0.6</td>
</tr>
<tr>
<td>TR(VP1)</td>
<td>0</td>
<td>0</td>
<td>0.5 ± 0.0 *</td>
</tr>
<tr>
<td>TR(VP2)</td>
<td>0</td>
<td>0</td>
<td>0.7 ± 0.2 *</td>
</tr>
<tr>
<td>TR(VP3)</td>
<td>88.9 ** *</td>
<td>5.6</td>
<td>12.5 ± 4.4 **</td>
</tr>
<tr>
<td>TR(VP4)</td>
<td>100.0 **</td>
<td>88.2 **</td>
<td>25.9 ± 4.0 **</td>
</tr>
<tr>
<td>TR(NSP1)</td>
<td>27.8</td>
<td>0</td>
<td>4.7 ± 0.6</td>
</tr>
<tr>
<td>TR(VP6)</td>
<td>68.8 **</td>
<td>0</td>
<td>10.4 ± 2.7 **</td>
</tr>
<tr>
<td>TR(VP7)</td>
<td>0</td>
<td>0</td>
<td>0.2 ± 0.0 *</td>
</tr>
<tr>
<td>TR(NSP3)</td>
<td>84.2 **</td>
<td>5.3</td>
<td>8.7 ± 2.7 **</td>
</tr>
<tr>
<td>TR(NSP2)</td>
<td>43.5 **</td>
<td>0</td>
<td>2.2 ± 0.6</td>
</tr>
<tr>
<td>TR(NSP4)</td>
<td>43.8 **</td>
<td>0</td>
<td>7.6 ± 0.6</td>
</tr>
<tr>
<td>TR(NSP5)</td>
<td>52.6 **</td>
<td>0</td>
<td>5.5 ± 1.4</td>
</tr>
</tbody>
</table>

* Groups of ≥ 10 pups per strain were injected within 24 hours of birth with 1.5 x 10^6 ffu of rotavirus and monitored for symptoms of BA for 21 days. A subset of these mice had their bile ducts harvested on day of life seven.

b Percentage of mice expressing two or more signs of biliary obstruction.

c Mortality rate over 21 days.

d Bile Ducts were harvested seven days post-infection and the amount of virus determined by focus-forming assay is expressed as mean ffu/ ml/mg tissue weight ± standard errors.

** p<0.05 significantly lower than RRV (panel A), TUCH (panel B)

* p<0.05 significantly higher than RRV (panel A), TUCH (panel B)

n= 15 to 20 pups for monitoring and 6 to 10 pups per strain for viral content in bile ducts.
Figure 3 - Histology of the liver and extra-hepatic bile ducts in RRV, TUCH and saline injected mice

RRV induces a massive inflammation of liver with remarkable portal expansion at 7 days containing primarily lymphocytes (top panel). In contrast TUCH injected mice do not show any liver inflammation similar to that of saline controls (top panel). N = 15–20 mice in each group; scale bar (left upper portion of each panel): 100 µm. In the lower panel injection of RRV leads to an obstruction of the lumen by inflammatory cells and by sloughed epithelial cells by day 7, while the TUCH and saline injected show normal epithelium and unobstructed lumen at 7 days. Scale bar: 100 µm. All the slides were stained by H&E.
Figure 4 - Histology of liver and extra-hepatic bile ducts in selected RRV reassortant injected mice

\( R_T^{(VP2)} \) and \( R_T^{(VP3)} \) induces a massive inflammation of liver with remarkable portal expansion at 7 days similar to parent strain RRV but \( R_T^{(VP4)} \) injected mice do not show any liver inflammation (top panel). \( N = 15–20 \) mice in each group; scale bar (left upper portion of each panel): 100 µm. In the lower panel injection of \( R_T^{(VP2)} \) leads to an complete obstruction of the lumen similar to parent strain RRV while the \( R_T^{(VP4)} \) injected mice showed unobstructed lumen at 7 days. \( R_T^{(VP3)} \) showed an intermediate phenotype with only a partially obstructed bile duct. Scale bar: 100 µm. All the slides were stained by H&E.
Figure 5 - Histology of the liver and extra-hepatic bile ducts in selected TUCH reassortant infected mice

$TR^{(VP2)}$, $TR^{(VP3)}$ induced very mild inflammation of liver while $TR^{(VP4)}$ injected mice showed massive inflammation with remarkable portal expansion at 7 days similar to RRV (top panel). $N = 15–20$ mice in each group; scale bar (left upper portion of each panel): 100 $\mu$m. In the lower panel injection of $TR^{(VP4)}$ leads to an complete obstruction of the lumen while the $TR^{(VP2)}$, $TR^{(VP3)}$ injected mice showed unobstructed lumen at 7 days. Scale bar: 100 $\mu$m. All the slides were stained by H&E.
Table 3 – In vitro binding and viral yield after infection of cholangiocytes with reassortants

<table>
<thead>
<tr>
<th>Panel A</th>
<th>Reassortant</th>
<th>Percent Binding (%)</th>
<th>Infectious Virus in Cholangiocytes (x10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RRV</td>
<td>11.9 % ± 0.1</td>
<td>3850.0 ± 449.2</td>
</tr>
<tr>
<td></td>
<td>RRV(VP1)</td>
<td>13.6 % ± 1.6</td>
<td>4568.7 ± 321.7</td>
</tr>
<tr>
<td></td>
<td>RRV(VP2)</td>
<td>11.6 % ± 1.4</td>
<td>3882.7 ± 298.7</td>
</tr>
<tr>
<td></td>
<td>RRV(VP3)</td>
<td>15.3 % ± 3.2</td>
<td>3929.3 ± 291.9</td>
</tr>
<tr>
<td></td>
<td>RRV(VP4)</td>
<td>4.0 % ± 0.5 *</td>
<td>0.8 ± 0.1 *</td>
</tr>
<tr>
<td></td>
<td>RRV(VP6)</td>
<td>15.3 % ± 2.4</td>
<td>5012.0 ± 42.0</td>
</tr>
<tr>
<td></td>
<td>RRV(VP7)</td>
<td>16.2 % ± 0.2 **</td>
<td>4652.7 ± 426.7</td>
</tr>
<tr>
<td></td>
<td>RRV(NSP1)</td>
<td>15.3 % ± 0.6</td>
<td>13.3 ± 1.7 **</td>
</tr>
<tr>
<td></td>
<td>RRV(NSP2)</td>
<td>16.4 % ± 0.6 **</td>
<td>3168.7 ± 172.9</td>
</tr>
<tr>
<td></td>
<td>RRV(NSP3)</td>
<td>17.6 % ± 0.8 **</td>
<td>3313.3 ± 382.0</td>
</tr>
<tr>
<td></td>
<td>RRV(NSP5)</td>
<td>15.6 % ± 0.7 **</td>
<td>4265.3 ± 264.4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Panel B</th>
<th>Reassortant</th>
<th>Percent Binding (%)</th>
<th>Infectious Virus in Cholangiocytes (x10^4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TUCH</td>
<td>5.3 % ± 0.6</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>TR(VP1)</td>
<td>3.1 % ± 0.7</td>
<td>0.8 ± 0.0 *</td>
</tr>
<tr>
<td></td>
<td>TR(VP2)</td>
<td>1.4 % ± 0.2 *</td>
<td>0.3 ± 0.0 *</td>
</tr>
<tr>
<td></td>
<td>TR(VP3)</td>
<td>6.5 % ± 0.6</td>
<td>65.9 ± 0.8 **</td>
</tr>
<tr>
<td></td>
<td>TR(VP4)</td>
<td>12.4 % ± 0.5 **</td>
<td>226.3 ± 50.4 **</td>
</tr>
<tr>
<td></td>
<td>TR(VP6)</td>
<td>5.9 % ± 1.4</td>
<td>10.5 ± 2.0 **</td>
</tr>
<tr>
<td></td>
<td>TR(VP7)</td>
<td>7.2 % ± 1.5</td>
<td>0.2 ± 0.0 *</td>
</tr>
<tr>
<td></td>
<td>TR(NSP1)</td>
<td>6.2 % ± 0.6</td>
<td>13.3 ± 1.7 **</td>
</tr>
<tr>
<td></td>
<td>TR(NSP2)</td>
<td>4.9 % ± 0.8</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>TR(NSP3)</td>
<td>2.9 % ± 0.2 *</td>
<td>20.3 ± 6.3 **</td>
</tr>
<tr>
<td></td>
<td>TR(NSP4)</td>
<td>7.6 % ± 0.7</td>
<td>12.5 ± 2.3 **</td>
</tr>
<tr>
<td></td>
<td>TR(NSP5)</td>
<td>5.3 % ± 0.4</td>
<td>17.4 ± 0.8 **</td>
</tr>
</tbody>
</table>

* Cholangiocyte inoculated with reassortants to determine binding and replication rate.

b Values are expressed as mean percent of virus bound to cholangiocytes ± standard errors.

c Values are expressed as mean ffu/ml ± standard errors in immortalized cholangiocytes.

* d p<0.05 significantly lower than RRV (panel A), TUCH (panel B)

** g p<0.05 significantly higher than RRV (panel A), TUCH (panel B)

n= 6 samples per strain