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Differential Expression of Hepatic Fibrosis Mediators in Sick and Spontaneously Recovered Mice with Experimental Biliary Atresia¹

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Abstract

Background—Hepatic fibrosis leading to cirrhosis is the major morbidity in patients with biliary atresia (BA). This fibrosis is due to an imbalance in extracellular matrix (ECM) breakdown and deposition. We have previously demonstrated increased mRNA expression for inhibitors of ECM breakdown without increased expression for mediators of ECM deposition in our animal model of BA by d 14. However, only a mild degree of hepatic fibrosis was seen at this time. We hypothesized that expression patterns for these proteins may change once more significant fibrosis had been established, and added resuscitation to the model to improve survival. Interestingly, we found that some mice spontaneously recovered at later time points with resuscitation, and thus compared expression for inhibitors of ECM breakdown and deposition in sick and recovered mice to determine the differences.

Methods—Newborn Balb/c mice received an intraperitoneal injection 1.0×10^6 fluorescence forming units of rhesus rotavirus 24h after birth. Mice were monitored daily for weight gain, development of jaundice, acholic stools, and bilirubinuria. Fifty μ L/g of 5% dextrose in normal saline were subcutaneously injected daily to each mouse starting on d 7 until sacrifice. Mice that survived past d 14 were sacrificed at d 21 after saline or RRV infection. Livers were then harvested post-injection d 21 for histologic and immunohistochemical analysis. RNA expression of known mediators of fibrosis was evaluated using quantitative real-time PCR. Protein expression was assessed using ELISA. Weights and normally distributed data were compared using Student's *t* test. Histologic findings were compared using Fisher's exact test. Comparisons of gene expression and skewed data were performed by the Mann-Whitney U test. Statistical significance was assigned to any *P* value less than 0.05.

Results—Daily resuscitation resulted in a 35% (24/68) survival rate to d 21 in our model. Mice that recovered were significantly heavier than those that remained ill on d 14 (6.15 ± 1.16 versus 4.94 ± 0.82 , *P* = 0.02) and 21 (7.31 ± 1.41 versus 4.14 ± 0.53 , *P* < 0.001) despite the fact that there was no difference between the groups with respect to weight on d 7 (4.29 ± 0.90 versus 3.89 ± 0.81 , *P* = 0.32). We found that all (10/10) animals that displayed clinical signs of biliary atresia on d 21 had

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moderate or severe histologic findings, while only one (1/9) of the recovered animals had liver abnormalities at sacrifice ($P < 0.001$ versus sick group). We also found that the sick mice had statistically significant median fold-increases of mRNA expression for TIMP-1 (31.9 *versus* 9.1, $P = 0.041$), TIMP-4 (88.1 *versus* 1.8, $P = 0.022$), and MMP-7 (51.8 *versus* 11.9, $P = 0.006$) compared with those that recovered. There was a trend toward decreased mRNA expression for PAI-1, which did not reach statistical significance (median 27.7 *versus* 2.19, $P = 0.066$). Increased protein expression for TIMP-1 and PAI-1 were also found in the sick group. The mRNA expression for the fibrillar collagens, fibronectin-1, connective tissue growth factor, snail-1, TIMP-2 and -3, and MMP-2 and MMP-9 was not different in the sick and recovered groups 21 d after RRV infection, and was not elevated from baseline gene expression.

Conclusions—With resuscitation added to the animal model of BA, some mice spontaneously recover while others progress to more significant hepatic fibrosis. Mice with hepatic fibrosis have a continued increase in mRNA expression of TIMP-1, TIMP-4, and MMP-7, with a trend toward increased mRNA expression of PAI-1 on d 21. Protein levels for TIMP-1 and PAI-1 were also increased in the sick mice. Recovered mice display mild to no hepatic parenchymal disease and a normal pattern of mRNA expression for the mediators of fibrosis tested. No increase in mRNA expression for the mediators of ECM deposition was found in either group. These data further support the notion that inhibition of ECM breakdown alone is sufficient to induce hepatic fibrosis. Modulation of this process may be a putative target for preventing liver injury in patients with BA.

Keywords

biliary atresia; metalloproteinases; plasminogen-activator inhibitor; tissue inhibitor of matrix metalloproteinases

Introduction

Hepatic fibrosis leading to cirrhosis and eventual liver transplantation is the major morbidity in patients with biliary atresia (BA), and this fibrosis may be due in part to an imbalance in extracellular matrix (ECM) breakdown and deposition [1,2]. We have previously demonstrated increased mRNA expression for inhibitors of ECM breakdown without increased expression for mediators of ECM deposition on d 7 and 14 in our animal model of BA, suggesting that inhibition of remodeling precedes collagen deposition in this model [3]. However, only a mild degree of hepatic fibrosis was seen at these time points after rhesus rotavirus (RRV) inoculation, and we speculated that expression patterns may differ once more significant fibrosis had been established. To achieve this aim, we added saline and dextrose resuscitation to our model in an attempt to extend the survival of the animals to 21 d. Surprisingly, we found that a significant number of mice spontaneously recovered by the later time point, and thus we were interested in comparing expression for inhibitors of ECM breakdown and deposition in sick and recovered mice to evaluate the disparity. Spontaneous recuperation of mice in this model has been previously reported [4], but prior to this study there has been no characterization of the differences between mice that remain ill and those that convalesce. We hypothesized that increased mRNA expression for inhibitors of ECM breakdown would continue with possible increases in the expression for mediators of ECM deposition in the mice that remained ill, but not in the clinically improved mice. Furthermore, we surmised that if there was increased expression of mediators for ECM deposition, the cells most likely responsible in the liver, the hepatic stellate cells (HSC), would show activation in the sick sub-group.

Methods

Experimental Design

The experimental protocol was approved by the Institutional Animal Care and Use Committee at the New York University School of Medicine. Balb/c mice were bred using a polygamous breeding system. After spontaneous vaginal delivery, neonates received an intraperitoneal injection with 1.0×10^6 fluorescence forming units (ffu) of RRV of serogroup 3 (diluted in 0.02 mL of saline) within 24 h of birth. The mice were returned to their mothers, maintained in their normal environment, and housed in a room with a standard 12-h dark-light cycle. The mice were weighed daily and examined for the development of icterus in their skin not covered with fur, the development of acholic stools, and the development of bilirubinuria. Fifty $\mu\text{L/g}$ of 5% dextrose in normal saline were subcutaneously injected daily to each mouse starting on d 7 until sacrifice. Mice that survived past d 14 were sacrificed at d 21 after RRV infection. The livers were then harvested for RNA and protein isolation, and histologic and histochemical analysis. Weights were compared using Student's *t*-test after confirmation of normally distributed data in each group using the one-sample Kolmogorov-Smirnov test. Statistical significance was assigned to any *P* value less than 0.05.

Virus

We obtained commercially available RRV strain MMU 18006, which was then cultivated in MA-104 cells (embryonic African green-monkey) using minimal essential medium with Earle's salts and 10% fetal calf serum. Penicillin 100 IU/mL and streptomycin 100 $\mu\text{g/mL}$ were added to prevent bacterial contamination. The virus was isolated, concentrated, and then titrated into aliquots to be injected into the mice.

Histology and Immunohistochemistry

For hematoxylin and eosin (H and E), trichrome, and immunohistochemical staining, a specimen of liver from each mouse was isolated, fixed in formalin, and imbedded in paraffin. Each block of paraffin was cut in 5 random sections and stained or probed. The interpretation of the sections was performed by a pediatric pathologist (MAG) who was blinded to the treatment groups. The H and E sections were graded into one of four groups: normal liver, or mild, moderate, or severe disease. Immunostaining was performed with an antibody to detection protein expression of α -smooth muscle actin (Novus Biological, Inc., Littleton, CO) to assess HSC activation [5]. The immunohistochemistry (IHC) was graded into one of two groups: expression or no expression. Comparisons between the sick and recovered groups was carried out using χ^2 analysis or Fisher's Exact test if the expected frequency of any cell was less than 5. Statistical significance was assigned to any *P* value less than 0.05.

Real-time Polymerase Chain Reaction (RT-PCR)

Standard methods for quantitative real time polymerase chain reaction (qRT-PCR) have been published elsewhere and were used for each mouse in both the sick and recovered groups [6]. Power SYBR green PCR Master mix (Applied Biosystems, Foster City, CA) and an ABI 7900HT Sequence Detection System was used to measure a threshold cycle (C_T) value for each sample with the following profile: 1 cycle of 50 C for 15 s, 95 C for 10 min, 40 cycles of 95 C for 15 s and 60C for 1 min. After amplification was complete, a final melting curve was performed according to the dissociation protocol of the ABI 7900HT instrument. RNA content from each sample was normalized to its eIF4B and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) content. Relative quantitation of gene expression was based on the comparative C_T method. Comparisons of gene expression between the two groups were performed by the Mann-Whitney U test. Statistical significance was assigned to any *P* value less than 0.05.

Enzyme-Linked Immunosorbent Assay (ELISA)

At the time of sacrifice, a portion of the liver was harvested for protein extraction. Organ homogenates were prepared by extraction with Tris buffered saline containing 1% Triton X-100 on ice. Homogenates were stored at 4°C overnight, and the next day, were again centrifuged, and the supernatant was collected. Total protein was then measured using bicinchoninic acid (Sigma Aldrich, St. Louis, MO). ELISA was performed for tissue inhibitor of metalloproteinase (TIMP)-1 and plasminogen activator inhibitor-1 (PAI-1) using commercially available kits following the product instructions. The kit for TIMP-1 was obtained from RayBiotech, Inc. (Norcross, GA) and for PAI-1 from Innovative Research (Novi, MI). The total protein levels for TIMP-1 were compared using Student's *t*-test after confirmation of normal distribution in each group using the one-sample Kolmogorov-Smirnov test. The distribution for PAI-1 protein levels were skewed, and thus compared using the Mann-Whitney U test.

Results

Animal Model

The addition of daily resuscitation with 50 μ L/g of 5% dextrose in normal saline resulted in a 35% (24/68) survival rate to d 21 in our model. Five of these mice were not included in this study as we elected to extend their study to 28 d. Of those that survived to 21 d, nearly 50% (9/19) recovered as evidenced by return of normal colored stool, disappearance of bilirubin in the urine, and weight gain compared with d 14. All mice that recovered (9/9) gained weight between d 14 and 21, while all but one mouse (9/10) that remained ill lost weight between these time points. The one sick mouse that gained weight during the period between 7 and 14 d gained only 0.2 g, which was far less than the average weight gain for the recovered mice (1.16 g). Mice that recovered were significantly heavier than those that remained ill on d 14 and 21, despite the fact that there was no difference between the groups with respect to weight on d 7 (Table 1).

Histologic and Immunohistochemical Evaluation

Since there were clearly two distinct group based on clinical parameters and weight gain, we next performed routine H and E and trichrome evaluation to evaluate the liver pathology in the sick and recovered mice. We found that all (10/10) animals which displayed clinical signs of biliary atresia on d 21 had moderate or severe histologic findings, including proliferation of bile ducts, significant degrees of fibrosis, and variable amounts of necrosis (Fig. 1). One (1/9) of the recovered animals had moderate liver abnormalities at sacrifice ($P < 0.001$ versus sick group), and the rest showed mild disease only (Fig. 1). In order to determine the level of HSC activation in these two groups, we further evaluated the liver for the presence of α -smooth muscle actin, which has been shown to be a reliable method for assessing stimulation of HSC [5]. We found that none of the recovered mice displayed any immunoreactivity to α -smooth muscle actin while all of the sick mice had demonstrable staining (Fig. 2). However, the areas of immunopositivity were mostly surrounding focal regions of necrosis and not reliably associated with the areas of fibrosis.

Mediators of Fibrosis

Since we had previously demonstrated significant up-regulation of tissue inhibitor of metalloproteinase (TIMP)-1 and -4, plasminogen activator inhibitor-1 (PAI-1), and matrix metalloproteinase (MMP)-7 and -9 mRNA expression at 14 d after RRV inoculation [3], we sought to determine the levels of mRNA expression of these mediators and other known intermediaries in fibrosis pathways in our sick and recovered mice. We found that the sick mice had statistically significant median fold-increases in mRNA expression of TIMP-1 (31.9

versus 9.1, $P = 0.041$), TIMP-4 (88.1 *versus* 1.8, $P = 0.022$), and MMP-7 (51.8 *versus* 11.9, $P = 0.006$) compared with those that recovered (Fig. 3). There was a trend toward decreased mRNA expression for PAI-1, but it did not quite reach statistical significance (median 27.7 *versus* 2.19, $P = 0.066$). The mRNA expression for the fibrillar collagens, fibronectin-1, connective tissue growth factor, snail-1, TIMP-2 and -3, and MMP-2 and MMP-9 was not different in the sick and recovered groups 21 d after RRV infection, and was also not elevated from baseline gene expression.

To confirm that increased mRNA correlated with increased protein expression, we performed ELISA for PAI-1 and TIMP-1 in the sick and recovered mice. Similar to mRNA expression, we found an increased total TIMP-1 protein content (ng/mg protein) in sick mice compared with their recovered counterparts (2076 ± 795 *versus* 1398 ± 308 , $P = 0.028$). Although the difference in PAI-1 mRNA expression did not quite reach statistical significance, we also found that sick mice had a median PAI-1 protein level far greater than that for the recovered mice (6.07 *versus* 0.27, $P = 0.003$).

Discussion

The animal model of biliary atresia (BA) has been fairly well characterized [4,7,8]. Intraperitoneal inoculation of Balb/c mice with the appropriate titers of RRV within the first 48 h of life leads to an initial viremia that later clears, however, a resultant infection of the biliary epithelium remains [4,7,8]. The biliary injury leads to honing of lymphocytes and eventual extra-hepatic biliary obstruction, which appears to be interferon gamma mediated since interferon γ knockout mice are immune to the disease [8]. Since hepatic fibrosis is present at the time of diagnosis in many human patients with BA [9], we began to investigate the possible mediators of hepatic fibrosis using the animal model and found increased mRNA expression for TIMP-1, TIMP-4, and PAI-1, which are all inhibitors of ECM degradation [3]. No increased expression of mediators of ECM deposition was noted 14 d after RRV administration and only mild to moderate hepatic fibrosis was present. Therefore, we postulated that inhibition of ECM degradation occurred prior to increased ECM deposition in this model, and that the mediators of increased ECM deposition may be seen once more significant liver fibrosis had been established. Due to the 90% lethality after d 14 in the animal model as described, we added resuscitation with dextrose and saline to the model in hopes of increasing survival in the mice with the wishing that we could further evaluate the interplay between the mediators of ECM degradation and deposition in the animal model of BA to gain better insight into the human condition.

The addition of daily resuscitation with 50 $\mu\text{L/g}$ of 5% dextrose in normal saline resulted in an increase to a 35% survival rate to d 21 in the model. Previous authors have described either complete lethality by 21 d or at best a 10% survival rate [4,8]. Of the mice that survived to 21 d, nearly 50% recovered from their disease as evidenced by return of normal colored stool, disappearance of bilirubin in the urine, and continued weight gain. The increased survival with added resuscitation suggests that some of the mice were succumbing to dehydration from the acute infection and not necessarily from progressive liver disease. The spontaneous recovery data further suggest that some mice have an acute infection that does not result in a permanent biliary obstruction but rather transient obstruction of the bile duct with no subsequent fibrosis. These are clearly limitations of the animal model of BA that must be considered in all future analyses. Some may even argue that the finding of spontaneous recovery suggests that this animal model is not comparable to BA in humans and that the model may be better characterized as one of virally-induced hepatic fibrosis. Spontaneous recovery in this model has been reported previously as an incidental finding [4], but no authors have described these recuperated mice in detail nor recognized their potential importance. Our aim was to evaluate

the recovered mice and compare them with those that remain ill at 21 d to characterize the differences.

Our histologic data from previous experiments revealed that although biliary obstruction is present at 14 d post-inoculation, the degree of fibrosis is still mild. Liver specimens harvested at 21 d revealed more significant biliary ductal proliferation, increased peri-portal fibrosis, some bridging fibrosis, and scattered areas of necrosis throughout the hepatic parenchyma. These histologic findings were confirmed by trichrome staining, and are consistent with hepatic changes that would be expected in animals with continued extra-hepatic biliary obstruction. Conversely, the recuperated animals showed relatively little liver pathology. Nearly all the specimens displayed bile duct proliferation, suggesting the presence of biliary obstruction at an earlier time, but the parenchyma itself showed no fibrosis or necrosis except in two animals, where mild fibrosis and necrosis were seen. The regenerative properties of the liver are well documented [10], and it is possible that both the sick and recovered animals had similar degrees of liver injury on d 14, but that in some animals, when the injury subsided, the liver repaired itself to appear almost normal by d 21. Certainly, when we and others evaluated mice 14 d after RRV inoculation, the liver injury is fairly uniform among the animals, and there has been no suggestion of a subpopulation of mice that are destined to convalesce from the insult. However, when we reviewed the clinical course in our mice, some differences between sick and recovered mice were noted that may have been a clue to which mice are likely to recover. All mice that recovered gained weight between d 14 and 21, while all but one mouse that remained ill lost weight between these time points. Furthermore, mice that recovered were significantly heavier than those that remained ill on d 14 and 21, despite the fact that there was no difference between the groups with respect to weight on d 7. Thus, it appears that some mice, despite having a similar insult due to RRV inoculation, are able to handle the acute infection of their biliary epithelium without sustaining an irreversible injury. A perhaps related situation could be postulated in the human condition where children with the same degree of hepatic fibrosis at the time of diagnosis may have markedly different outcomes after portoenterostomy [9,10], however, spontaneous regression of BA is not seen in humans. In some patients, once biliary drainage has been re-established, the liver injury abates and the native liver can regenerate and function, while in others with a similar degree of injury at the time of portoenterostomy and demonstrable bile flow after the procedure still suffer from an ongoing liver injury resulting in cirrhosis despite adequate biliary outflow [11]. Therefore, determining what factors contribute to the continued liver injury despite the re-establishment of proper biliary drainage could be critically important in improving outcomes after portoenterostomy, and perhaps even treating patients medically with different types of fibrotic liver diseases other than BA.

There is no shortage of purported mediators of injury in the pathogenesis of BA, and newer molecules that have been suggested to be important in the inflammatory and fibrotic stages of the disease are under investigation [12,13]. We have focused on the fibrogenic process and, in particular, TGF- β -mediated pathways since TGF- β has been proven essential in the development and progression of hepatic disease [14,15]. Strategies for inhibiting TGF- β function including the blockade of one of its primary regulators, the integrin $\alpha_v\beta_6$, have been demonstrated to abrogate liver fibrosis after bile duct ligation [16]. We have similarly shown that integrin $\alpha_v\beta_6$ is expressed in the biliary epithelium of both patients with BA and mice after RRV inoculation, and that several downstream mediators of the TGF- β - pathway are up-regulated in the animal model [3]. Specifically, we found that mRNA expression for TIMP-1, TIMP-4, PAI-1, and MMP-7 was significantly upregulated 14 d after RRV infection. No up-regulated expression was noted for the mediators of ECM deposition. We expected that by extending the model out to 21 d, we would start to see increased expression for the mediators of ECM deposition as the fibrosis became more significant. However, that was not the case. We found that the only four mediators with considerable mRNA expression 21 d after RRV inoculation continued to be TIMP-1, TIMP-4, PAI-1, and MMP-7, and no new increased

expression for the mediators of ECM deposition was found. Furthermore, increased levels of TIMP-1 and PAI-1 protein were found in sick mice compared with the recovered mice. The absolute levels of these proteins are widely disparate and therefore difficult to interpret, and a functional assay or selective blockade would be required to ascertain what actual level of inhibition of ECM breakdown is achieved by each mediator. However, it is clear that the increased message is being translated into protein.

We did find some mild activation of HSC using immunoreactivity to α -smooth muscle actin as the indicator of the phenotype, but these cells were not necessarily found in the areas of periportal fibrosis, which further suggests that increased deposition of new collagen may not be as important in the animal model of BA. TIMP-4 expression was the most elevated at 21 d, but very little is known about TIMP-4 and its role in fibrogenesis [17]. The basic action of the TIMPs is to inhibit ECM degradation that is in opposition to those of the MMPs, which is to increase ECM degradation. It is possible that the increased MMP-7 expression is actually a direct result of the sustained inhibition of ECM degradation *via* a feedback loop mechanisms as is suggested by some authors in other models [18]. PAI-1 inhibits plasminogen activator activity, which also impairs ECM breakdown, has been shown to be important in cholestatic liver disease, and may have a synergistic effect with the TIMPs in our model [19]. Therefore, it appears that inhibition of ECM degradation alone is sufficient to induce significant hepatic fibrosis of mice after experimental BA. This paradigm may have precedent in the human condition as patients who were given nonspecific synthetic MMP inhibitors as potential therapy for arthritis developed tendonitis and adhesive capsulitis (fibrosis), necessitating early cessation of the clinical trial [20,21]. It is thus reasonable that strategies aimed at restoring appropriate ECM breakdown rather than preventing new deposition may be most effective in treating patient with BA. It may be, however, that inhibition of ECM breakdown may represent an early step in the final common pathway of fibrotic liver disease and is not related to BA in particular.

In summary, we found that with resuscitation added to the animal model of BA, some mice spontaneously recover while others progress to more significant hepatic fibrosis 21 d after RRV inoculation. Mice with hepatic fibrosis have a continued increase in mRNA expression of TIMP-1, TIMP-4, and MMP-7, with a trend toward increased mRNA expression of PAI-1 on d 21. TIMP-1 and PAI-1 protein levels are also increased in the sick mice. Recovered mice display mild to no hepatic parenchymal disease and a normal pattern of mRNA expression for the mediators of fibrosis tested. No increase in mRNA expression for the mediators of ECM deposition was found after 21 d in either group. These data further the contention that inhibition of ECM breakdown in the liver alone is sufficient to induce hepatic fibrosis, and that modulation of the important mediators in this process may be a putative strategy for preventing on-going liver injury in patients with BA.

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FIG. 1.

Hematoxylin and eosin and trichrome staining of sick and recovered mice 21 d after RRV infection. Newborn Balb/c mice were administered RRV within 24 h of birth. Livers were harvested on d 21. Sick animals (upper panels) displayed the bile duct proliferation (arrows) and hepatic fibrosis (blue on trichrome) that one would expect with biliary atresia. The recovered animals (lower panels) displayed a minor degree of, if any, bile duct proliferation and minimal hepatic fibrosis. A 70 μ measurement bar is included in the right lower aspect of each panel. Magnification $\times 20$.

FIG. 2.

α -Smooth muscle actin immunoreactivity 21 d after RRV infection. Newborn Balb/c mice were administered RRV within 24 h of birth. Livers were harvested on d 21. None of the recovered mice displayed any immunoreactivity to α -smooth muscle actin, while all of the sick mice had demonstrable staining (arrows). However, the areas of immunoreactivity were mostly surrounding focal regions of necrosis and not reliably associated with the areas of fibrosis. A 70 μ measurement bar is included in the right lower aspect of the photomicrograph. Magnification $\times 40$.

FIG. 3.

Day 21 gene expression of potential mediators of fibrosis in experimental biliary atresia. Newborn Balb/c mice were administered RRV within 24 h of birth. Mice were divided into two groups based on their phenotypes, sick and recovered. Livers were harvested on d 21 for analysis. Mice that remained ill had significantly increased levels of mRNA for TIMP-1 and -4, and MMP-7 compared with mice that recovered, whose expression was near baseline. There was also a trend ($P = 0.066$) toward increased expression for PAI-1 mRNA. Expression of mRNA for the fibrillar collagens, fibronectin-1, connective tissue growth factor, snail-1, and the other TIMPs and MMPs evaluated were unchanged from baseline.

TABLE 1
Weights of Mice with Experimental Biliary Atresia at Different Time Points after RRV Inoculation*

Group	D-7 weight (g)	D-14 weight (g)	D-21 weight (g)
Sick (<i>n</i> = 10)	3.89 ± 0.81	4.94 ± 0.82	4.14 ± 0.53
Recovered (<i>n</i> = 9)	4.29 ± 0.90	6.15 ± 1.16	7.31 ± 1.41
<i>P</i> value	0.32	0.02	<0.001

* Newborn Balb/c mice were administered RRV within 24 h of birth. The mice were returned to their mothers, maintained in their normal environment, and housed in a room with a standard 12-h dark-light cycle. The mice were weighed daily and examined for the development of icterus in their skin not covered with fur, the development of acholic stools, and the development of bilirubinuria. Fifty μ L/g of 5% dextrose in normal saline were subcutaneously injected daily to each mouse starting on d 7 until sacrifice on d 21. Data represent the mean $\pm$ the standard deviation.