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Susceptibility to experimental biliary atresia linked to different hepatic gene expression profiles in two mouse strains
Susceptibility to Experimental Biliary Atresia is linked to
Different Hepatic Gene Expression Profiles in Two Different Mouse Strains

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Abstract

Aims: Aim of this study was to compare hepatic gene expression during the development of experimental biliary atresia in two different mouse strains.

Methods: Balb/c mice and C57Black/6 (Black/6) mice were infected with RRV postpartum, clinical signs of BA and survival were noted. Liver sections were assessed for CD3, CD4 and CD8 expression, and the hepatic virus load was determined. Second, mice of both strains were sacrificed three days after infection. Isolated hepatic RNA was subjected to gene expression analysis using Affymetrix Gene Chip MOE 430 2.0.

Results: The incidence of BA was significantly lower in Black/6 mice compared to Balb/c mice (13.5% vs. 67%, p<0.05). The mean virus titers were higher in mice with BA compared to mice without BA. Different gene profiles three days after virus infection were noted, with differential expression of 201 genes, including those regulating apoptosis, nucleic acid binding, transport function and particularly the immune response (CCL2, toll-like receptor 3 (TLR 3), CD 14 antigen, CXCL 10 and CXCL 11). This correlated with a significant increase of CD4 positive cells only in Balb/c mice with BA compared to healthy mice (13.5 vs. 5.0; p<0.05). Black/6 mice did not exhibit any significant increase of CD3 or CD4 leukocytes despite cholestasis.

Conclusions: The different susceptibility to experimental BA was associated with an increase of CD4 T-cells in the liver of Balb/c mice, which is linked to different gene profiles at the onset of bile duct obstruction.

Key words: Biliary atresia, rhesus rotavirus, T cells, microarray, CCL2, CXCL 10, CXCL 11.
INTRODUCTION

Biliary atresia (BA) is a rare cholestatic disease of unknown etiology that affects young infants and shows an incidence of 1 out of 18,000 live births in Europe and 1 out of 9,000 in Japan (1). The first therapeutic option is a timely performed portoenterostomy. However, the majority of patients suffer from a progressive inflammatory process, which leads to complete destruction of the extra- and intrahepatic biliary system followed by end-stage liver cirrhosis. Hence, BA is the leading indication for pediatric liver transplantation worldwide (2, 3). To understand the pathogenesis of the disease and improve the outcome of BA patients, research has focused on the inflammatory process in liver and bile ducts, in which several factors are remarkably elevated, such as activated CD4 and CD8 T-cells, TNF alpha, IFNγ and other proinflammatory T\(_{H1}\) cytokines (3-8). By the time of diagnosis, however, the disease has already reached an advanced state, characterized by the complete obstruction of the extrahepatic bile ducts with impaired bile flow and fibrosis or cirrhosis of the liver. Therefore, studies in humans focusing on the trigger mechanism of BA are limited due to the paucity of liver and availability of bile duct tissue for research. One infectious animal model has been developed, in which newborn Balb/c mice exclusively show the experimental BA phenotype after infection with rhesus rotavirus (RRV). This model allows the analysis of the inflammatory reactions in liver and bile ducts at early steps in the development of bile duct atresia. Furthermore, inbred mouse strains have been shown to have a different susceptibility for the development of experimental BA, suggesting that Balb/c mice have an immunological gap responsible for disease progression. Aim of this study was to identify key genes responsible for the BA phenotype by comparing the transcriptomes between two mouse strains with different susceptibilities to BA at an early time point after virus infection before bile duct occlusion. Differences in the virus titration and the clinical course of infected mice were analyzed, and variations in the hepatic gene response assessed by comparative microarray assays were correlated to variances in the hepatic inflammatory reaction.
METHODS

Animals
Rotavirus-free pregnant Balb/c mice and C57Bl6 (Black/6) mice were purchased from Charles River Lab. (Sulzfeld, Germany). Animals were kept pathogen-free in laminar-flow cages with a 12 hour dark-light cycle. All procedures were in compliance with the national regulations for protection of animals (permit no. 03/693) and were performed under supervision of the responsible veterinarian.

Rhesus Rotavirus
As previously described, the MMU 18006 strain of Rhesus Rotavirus was titered in MA-104 cells and expressed as plaque forming units (pfu/mg) (9, 12). To determine the incidence of experimental BA, newborn mice were injected intraperitoneally with 20 µl of saline solution containing either 1x10^6 pfu/ mg of RRV or no virus within 24 h of birth. Animals that died during the first 48 h were excluded from further analysis. The survivors were weighed and checked daily for icterus of the non-fur-covered skin, acholic stools and bilirubinuria (Bilugen-Test\textsuperscript{TM}, Boehringer Mannheim, Germany).

In a second set of experiments, mice were infected as described above, and animals with clinical signs of murine BA were sacrificed and prepared under a dissecting microscope 10 to 14 days after RRV infection. Controls were sacrificed after 10-14 days. Additionally, three Balb/c mice and two B6 mice were sacrificed three days after RRV infection for the microarray analysis. Explanted livers were harvested for further examination. Specimens were paraffin embedded after formalin fixation or snap frozen and stored at -80°C for cryo-sections for immunochemistry (CD3, CD4 and CD8) and for virus titration according to the pfu/mg technique as described elsewhere (9, 12). Additionally, the brains of all animals were snap-frozen and harvested for titration of the neurotropic RRV. For RNA isolation, the livers were snap-frozen in liquid nitrogen after embedding in RNAlater (Quiagen, Hilden, Germany).
Histology of paraffin and frozen liver sections

Liver specimens were deparaffinized and microdissected (5µm) for hematoxylin and eosin (H.E.) staining according to standard protocols, and analyzed by light microscopy. TissueTek O.C.T. (Baxter Scientific) embedded liver cryostat sections (5µm) were air-dried and fixed in acetone. CD3, CD4 and CD8 lymphocytes were phenotyped by the alkaline phosphatase antialkaline phosphatase (APAAP) technique. The slides were incubated with the following primary monoclonal antibodies for 30 min: KT-3 (Serotec, Wiesbaden, Germany) for CD3, L3T-4 (Dianova, Hamburg, Germany) for CD4 and Ly-2 (Pharmingen, Heidelberg, Germany) for CD8. After TBS-Tween (0.05% Tween 20, Saerva, Heidelberg, Germany) washes and incubation with a bridging Ab (rabbit anti-rat, 30 min, Dako, Hamburg, Germany), the APPAP complex was applied. This procedure was repeated twice. Fast blue (Sigma, Munich, Germany) served as substrate for alkaline phosphatase. All sections were counterstained with hemalaun, sealed with glycergel (Dako), and visualized using a Leica microscope (200x). Ten representative fields of view were counted and the median was estimated.

DNA Microarray Hybridization and Analysis

A GeneChip probe array consists of a number of probe cells where each probe cell contains a unique probe. Probes are tiled in probe pairs as a Perfect Match (PM) and a Mismatch (MM). The sequence for PM and MM are the same, except for a change to the Watson-Crick complement in the middle of the MM probe sequence. A probe set consists of a series of probe pairs (11-20) and represents an expressed transcript. The signal value is calculated from the combined, background-adjusted, PM and MM values of the probe set. It represents the amount of transcript in solution. Total RNA was isolated from liver tissue using Qiagen RNAeasy Kit (Qiagen, Hilden, Germany), and the quality and integrity was assessed by running all samples on an Agilent Technologies 2100 Bioanalyzer (Agilent Technologies; Waldbronn, Germany). For biotin-labelled target synthesis starting from total RNA, reactions
were performed according to the manufacturer’s protocol (Affymetrix; Santa Clara, CA). Samples were hybridized to an identical lot of Affymetrix MOE430 2.0 for 16 hours. After hybridization, the Gene Chips were washed, stained with SA-PE, and read using an Affymetrix Gene Chip fluidic station 400 and scanner GCS3000.

In addition, a statistical non parametric Wilcoxon Rank Test was used to estimate the reliability of each probe set measurement. Analysis of microarray data was performed by Affymetrix GCOS 1.2 software using MAS5 algorithm for signal intensity calculation. For normalization all array experiments were scaled to a target intensity of 150, otherwise using the default values of GCOS 1.2. Differentially expressed genes were determined using Array Assist 5.0 software suite (Stratagene, La Jolla, CA, USA). Genes, whose expression intensities do not exceed 100 were called as background and excluded from further analysis. The entire dataset was stored at NCBI’s GEO database server using a MIAME compliant format and is accessible under GSE13245.

**Quantitative Real-time RT-PCR**

To validate the array results, we performed quantitative RT-PCR on selected genes using the individual liver samples. RNA was isolated with the RNeasy kit (Qiagen, Hilden, Germany) following cDNA synthesis using the Superscript II Reverse Transcriptase (Invitrogen). Quantitative real-time RT-PCR was performed in a LightCycler480 System (Roche) using the ProbeMaster kit from Roche. For quantization we used the UPL Probe Library from Roche. Relative mRNA levels were determined by using included standard curves for each individual gene and further normalization to RPS9 (relative gene expression value).

**Primer sequences**

5’ RPS9: atccgccaacgtcacatta

3’ RPS9: tcttcactcggcctggac
5' CXCL1:  tttgtatattagggtgaggacat
3' CXCL1:  gcgttgtgaccatacaatatgaa
5' CXCL10:  gccgcgtctatttctgc
3' CXCL10:  tcetctgcccgcgctcete
5' CXCL11:  gctgtgagatgaacaggaa
3' CXCL11:  ccctgttgaacataaggaagc
5' CCL2:  catccacgtgttggtcga
3' CCL2:  gatactctggctgtgaatgagt
5' CD14:  aaagaaactgaagcctttctcg
3' CD14:  agcaacaagccaagcacaac
5' Caspase9:  gacctgcagtccctccttct
3' Caspase9:  gcttccccgaggaagttaaaa
5' TLR3:  gatacagggattgcacccata
3' TLR3:  tcccccaaaggagttacattaga

Statistical analysis

Statistical evaluation of the BA incidence and survival rate was performed using SPSS 15.0 (SPSS Inc., Chicago, IL). Data of lymphocyte expression are expressed as means +/- SEM. The Mann-Whiney Rank Sum Test was performed to compare the distribution of lymphocytes and the virus titration. P-value of <0.05 was considered to be statistically significant.
RESULTS

Black/6 mice have a significantly lower incidence of biliary atresia compared to Balb/c mice.

A total of 55 newborn Balb/c mice were infected with RRV postpartum and, of these, 37 animals developed experimental biliary atresia (67%). A total of 37 Black/6 mice were infected, and 5 developed BA (13.5%) as shown in figure 1a. The difference in the incidence of BA was statistically significant (p<0.05). No significant difference in the overall survival was observed, as shown in figure 1b. All control mice injected with saline solution remained healthy during the three week study period (Balb/c, n =19; Black/6, n =10).

The viral load was similar in diseased Black/6 mice and Balb/c mice.

The mean virus titer measured in livers and brains was higher in mice with the BA phenotype compared to mice without BA. However, no statistically significant difference in the hepatic virus titers was observed between mice with BA from both strains, as shown in Table 1. Black/6 mice with BA showed a significantly higher median viral load in the brain compared to Balb/c animals (10^6 versus 10^5 pfu/mg, p<0.05).

Mice with BA showed inflammation of porta hepatic and bile duct proliferation

HE stained liver sections from Black/6 and Balb/c mice with the BA phenotype showed inflammation of the liver parenchyma, signs of cholangitis, and proliferation of intrahepatic bile ducts; no major differences were detected between both mouse strains. Moderate inflammation of the liver and extrahepatic bile ducts was observed in RRV-infected mice, which were otherwise healthy.

To further investigate the molecular mechanisms underlying the development of BA after RRV infection in Balb/c mice, we conducted a transcriptome analysis. We compared three individual expression profiles from RRV-infected Balb/c mice against 2 individual expression
profiles from RRV-infected Black/6 control mice using the Affymetrix GeneChip MOE 430 2.0. Of a total of 45,101 analyzed transcripts, 201 transcripts showed significant differential expression (p < 0.05), and the average fold change was greater than threefold. The complete list of the differentially regulated genes is provided in Supplement Table 1. The genes were functionally classified by matching the gene list to Gene Ontology terms using the GO database. Furthermore, we tested the corresponding gene list for the specific enrichment of a particular GO term compared to a random situation. The results of this GO term enrichment study are summarized in Supplement Table 2. We identified several interesting GO terms at a significance level of 95% (p<0.05). We observed marked up-regulation of genes regulating the immune response (p<0.0001; 45 genes), cell adhesion (p<0.05; 21 genes), cell differentiation (p<0.05; 25 genes), cell cycle (p<0.05; 15 genes) and cell surface (p<0.0001; 14 genes). Here, we focused on CCL 2, CXCL 1, CXCL 10, CXCL 11, CD14 and TLR3, as these factors play an important role during the immune response (Tab. 2). These chemoattractants are involved in the mediation of inflammatory liver diseases, particularly in the recruitment and homing of T-cells. The two most up-regulated genes were Serpina3K, a cysteine peptidase inhibitor, and H2-D1 (histocompatibility 2, D region locus 1), which encodes for a MHC class I protein involved in the presentation of foreign antigens to the immune system.

RT-PCR

To validate the array results, we performed quantitative RT-PCR on selected genes.
Both CD3 and CD4 but not CD8 T-cells were significantly increased in Balb/c mice with BA compared to Black/6 mice and controls.

The array data demonstrated an increased expression of genes encoding for chemokines involved in T-cell recruitment. Therefore, we then analyzed the increase of T-lymphocytes in both mouse strains. After RRV infection, there was a clear infiltration of significantly higher numbers of leukocytes in Balb/c mice with BA symptoms with compared to controls and symptom-free Balb/c mice. Most of the infiltrating cells were CD4 positive, with a significant increase of this cell population in diseased Balb/c mice compared to controls (13.5 vs. 5.0; p < 0.05). Black/6 mice did not exhibit any significant increase in CD3 or CD4 leukocyte numbers, despite BA symptoms (Figure 2a, b, c) and we hypothesised that these cells might be neutrophil granulozytes.
DISCUSSION

The basic principle of the murine BA model is threefold: (i) the virus type, (ii) the quantity of virus (recommended $10^5$ or $10^6$ pfu/mg), and (iii) the crucial early time point of infection, i.e. the day of birth (21, 22). Furthermore, as described previously, inbred mouse strains have a different susceptibility to develop the BA phenotype after RRV infection, suggesting that these mice have an immunological gap responsible for disease progression (10, 12). In the current study, we found that Black/6 mice have a significantly lower susceptibility to develop bile duct atresia after RRV infection compared to Balb/c mice (13.5% versus 67%; p<0.05). However, lethality was similar in both groups. The cause of death in Black/6 mice without biliary atresia remains unclear, but our virus distribution data suggest that death might be related to the neurotropic effect of RRV. All animals were administered the same amount of virus, but the highest RRV titers were measured in the brains of Black/6 mice. However, histological data of the brain are lacking. Animals with the clinical picture of BA had a significantly higher virus load in both livers and brains, suggesting that a certain amount of active virus appears to correlate with the incidence of the BA phenotype. Moreover, our study demonstrated differences in the hepatic response of the highly susceptible Balb/c and the nearly resistant Black/6 strain to RRV infection. To identify the cellular events that lead to the development of the clinical symptoms of BA, we evaluated the hepatic gene response three days after virus infection. This time point is characterized by the onset of hepatic inflammation without any signs of cholestasis or atretic changes in the bile ducts, as described previously (11, 12). The significance of the immune response in the pathogenesis of human and murine BA was elucidated in recent studies using microarray analysis of liver and bile ducts of children or mice with BA (4, 11, 14). Based on the gene lists from these studies and the theory that
BA is an immune mediated liver disease, we focused on the genes involved in immune reaction and immunity.

Our microarray analysis showed that susceptible Balb/c mice displayed significantly increased expression of genes encoding chemoattractants, such as CXCL 1, CXCL 10, CXCL 11 and CCL 2. Our results are consistent with those from previous studies using microarray techniques in experimental murine BA by Bezerra and co-workers, as well as by our group (11, 14). Moreover, the upregulation of CCL 2 (monocyte chemoattractant protein-1, or MCP-1) was previously reported in these murine BA studies (11, 14). However, the lack of CCL2 in Balb/c mice has been shown to protect mice against CCl4-induced severe toxic liver damage (Fehler: Referenz nicht gefunden), suggesting a central role of this chemokine in inflammatory liver damage.

The strong expression of these chemoattractants in diseased Balb/c mice was associated with an infiltration of CD3 and CD4 leukocytes, which was not observed in Black/6 mice. The CD4 leukocyte infiltration is in concordance with experimental studies by Mack et al., who demonstrated an increase of predominantly CD4 positive T-cells on day 7 after RRV infection, followed by an increase of CD8 T-cells in the second week (24). This late influx of CD8 cells appears to be crucial for antibody-mediated depletion (19).

Several recent studies in humans have pointed towards an early role of these chemoattractants in the mediation of inflammatory liver diseases (25). Up-regulation of CXCR3, the receptor for CXCL 9 (or Mig), CXCL 10 (or IP-10) and CXCL 11 (or I-Tac), was detected in children with BA (6). Moreover, CXCL-10 and CCL2 were proposed to serve as predictive factors in patients with BA (26, 27) and seem to be prognostic markers for the response to treatment in patients with acute hepatitis (25). Taken together, the available data suggest that an early up-regulation of chemoattractive factors in the liver could lead to a subsequent infiltration of activated inflammatory cells that mediate the tissue damage and ultimately lead to the clinical picture of BA. Recent in vitro studies from Japan demonstrated that biliary epithelial cells
from BA patients produce antiviral products such as IFN beta and MxA on exposure to a synthetic analogue of viral ds-RNA (Poly I:C) with diffuse expression of toll-like receptor 3 (TLR3). This receptor recognizes double-stranded RNA, such as ds-RNA reoviridae, and could be involved as part of the innate immune response in the pathogenesis of human BA. Our data confirmed an up-regulation of TLR3 in the murine model in the early phase after RRV infection, supporting a potential role of this surface receptor in the pathogenesis of BA. CD14, a cell-surface glycoprotein with crucial inflammatory regulation properties, is another factor found highly expressed in the livers of patients with BA. Recently, Shih et al. found an association between the single-nucleotide polymorphism at CD14/-159 and the development of BA, pointing toward the regulation of endotoxin susceptibility as crucial in the development of BA (28). Again, our results supported these findings, as CD14 was up-regulated in susceptible Balb/c mice compared to Black/6 mice.

In summary, we observed a different susceptibility to biliary atresia after RRV infection in Black/6 and Balb/c mice. The development of the clinical signs of BA appears to depend on hepatic virus load and virus distribution. The increased sensitivity of Balb/c mice was associated with an increase of the gene expression of chemoattractants and factors of innate immune activation, as well as an influx of CD3 positive T-cells, particularly CD4, in the liver of Balb/c mice. The early activation of genes regulating the inflammatory response may indicate that these chemoattractants might be promising targets for new treatment strategies in the prevention of biliary atresia in children.

Acknowledgments

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REFERENCES


Legends to the figures

1a The incidence of experimental biliary atresia after RRV infection was significantly higher in Balb/c mice compared to Black/6 mice (67% vs. 13.5%).

1b The survival of both mouse strains were comparable. However, whereas Balb/c mice died from experimental BA, the majority of Black/6 mice died without signs of BA.

2a Balb/c mice showed the highest increase in CD3 T-cells, which was significant in comparison with Black/6 mice and controls.

2b Lymphocytes were predominantly CD4-positive, and a significance increase was found in Balb/c mice with the BA phenotype compared to healthy animals and Black/6 mice. The latter strain did not exhibit an increase of CD4 T-cells.

2c Level of CD8 T-cells was equal in both strains with no significant increase observed in mice with experimental BA. Only Balb/c mice showed a slight increase, although this did not reach statistical significance.

Values represent the mean with SEM (standard error of the mean).
Table 1 Rhesus Rotavirus titre of two mouse strains

<table>
<thead>
<tr>
<th></th>
<th>RRV liver [pfu/mg]</th>
<th>RRV brain [pfu/mg]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Balb/c +BA</td>
<td>1.1x10^5 (10^5 – 10^6) #</td>
<td>1.2x10^5 (10^5 – 10^6) #</td>
</tr>
<tr>
<td>(n=8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Balb/c -BA</td>
<td>1.3x10^3 (10^2 – 10^3)</td>
<td>1.2x10^3 (10^2 – 10^3)</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black/6 +BA</td>
<td>1.2x10^5 (10^5 – 10^6) #</td>
<td>1.4x10^6 (10^5 – 10^6) #</td>
</tr>
<tr>
<td>(n=5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Black/6 -BA</td>
<td>1.3x10^3 (10^2 – 10^3)</td>
<td>1.2x10^4 (10^3 – 10^4)</td>
</tr>
<tr>
<td>(n=10)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Rhesus Rotavirus was detected in the liver and brain of all infected animals, with a significantly higher level in mice with BA compared to mice without signs of BA (#).

Table 2 Most up-regulated genes in Balb/c mice after RRV infection

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Signal log ratio SLR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCL 1 chemokine (C-X-C) ligand 1</td>
<td>3.79</td>
</tr>
<tr>
<td>CXCL 11 chemokine (C-X-C) ligand 11</td>
<td>3.10</td>
</tr>
<tr>
<td>CCL2 chemokine (C-C) ligand 2</td>
<td>2.81</td>
</tr>
<tr>
<td>Cd14 CD14 antigen</td>
<td>2.56</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>2.47</td>
</tr>
<tr>
<td>TLR 3 toll-like receptor 3</td>
<td>1.82</td>
</tr>
<tr>
<td>CXCL 10 chemokine (C-X-C) ligand 11</td>
<td>1.77</td>
</tr>
</tbody>
</table>

Differentially expressed genes were determined using Array Assist 5.0 software suite. These most up-regulated genes were found in this experiment and could be involved in the BA pathogenesis.