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Immunoglobulin deposits in liver tissue from infants with biliary atresia and the correlation to cytomegalovirus infection

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Abstract

Purpose: The aim of this report was to study the amount and distribution of immunoglobulin deposits in liver biopsies from infants with biliary atresia (BA) and correlate the results to the cytomegalovirus (CMV) infection status.

Methods: Stored liver biopsies from 18 patients with BA and from 6 control patients without liver disease were immunohistochemically stained to detect IgG and IgM deposits. The intensity of the immunoglobulin staining was evaluated by a semiquantitative scoring scale. Ongoing CMV infection was defined as the detection of CMV-IgM in serum and/or the isolation of CMV in the urine and was noted in 9 of the patients with BA.

Results: When analyzing the immunoglobulin deposits on the hepatocellular canalicular membrane the intensity score for IgM deposits was significantly higher in biopsies from patients with BA infected with CMV than in those without. No canalicular staining was detected in control biopsies.

Conclusions: The results support the possibility that immunologic mechanisms are of importance in the pathogenesis of BA and that a CMV infection may trigger such mechanisms.

The etiology of biliary atresia (BA) is still unknown [1]. Several different viral infections, including reovirus type 3, rotavirus group C, and human papilloma virus, have been associated to the disease [2-4]. We have recently described an association between ongoing cytomegalovirus (CMV) infection and BA [5]. Furthermore, the percentage of mothers with CMV-IgG detected in serum was higher in the cholestatic group than in the control group [5]. By analyzing CMV-DNA on stored Guthrie cards, we have also found that a majority of the CMV-infected cholestatic infants were not viremic at birth [6]. The results from other studies on the importance of CMV infection in the etiology of BA are contradictory [7-11]. Considering the data, which suggest an immunologic component in the pathogenesis of BA [12-15], a mechanism involving both CMV infection and immunologic events could be hypothesized. We therefore analyzed the presence and the amount of

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immunoglobulin deposits, as measured by immunostaining intensity, in liver biopsies from patients with BA and correlated these findings to the CMV status.

2. Methods

2.1. Patients

Eighteen patients with BA born between 1988 and 1995 and followed up until April 2003 were included (Table 1). All patients were clinically investigated at our tertiary referral center for pediatric hepatology. The mean age at the time of liver biopsy and viral investigation was 57 days (range, 30-105 days) and the mean follow-up time was 5.7 years (range, 0.3-12 years). The endpoint of follow-up was liver transplantation and/or death. The diagnostic criteria for BA were as follows: (1) hepatobiliary scintigraphy with a good uptake to the liver but no excretion to the gut within 24 hours; (2) histologic pattern on needle biopsy suggesting extrahepatic biliary disorder; and (3) final confirmation of the diagnosis at laparotomy with intraoperative cholangiogram [16]. All patients were subsequently subjected to the portoenterostomy (Kasai) procedure.

At follow-up the patients were divided into 3 outcome groups (Table 1). In group 1 (4 patients) there was minor liver disease or none, in group 2 (3 patients) there was chronic compensated liver disease, and in group 3 all patients were liver transplanted and/or dead (11 patients).

All patients were previously included in a study analyzing the importance of viral infections in the etiology of BA [5]. In the present study, signs of ongoing CMV infection, that is, the detection of CMV-IgM in serum and/or the isolation of CMV in urine were noted in 9 of 18 patients.

Cytomegalovirus-IgG was analyzed in maternal serum collected at the time of the investigation of the infant, and it was detected in the serum from 14 of 15 examined mothers to patients with BA (Table 1).

2.2. Controls

Liver biopsies from 6 patients were used as controls. In 5 of them the biopsy material was collected at autopsy which was performed because of perinatal death. All 5 were born at term, the cause of death was bacterial infection in 3 and uncertain in 2. The sixth control patient was a 3-month-old infant who had liver biopsy performed because of prolonged unconjugated hyperbilirubinemia.

2.3. Detection of IgG and IgM antibodies in liver biopsies

Liver biopsies were obtained for diagnostic purposes, either as needle biopsies according to Menghini or as wedge biopsies at the time of Kasai procedure. After fixation with 10% buffered formalin and routine evaluation, they were stored and retrospectively examined for the present study.

Table 1 Eighteen infants with BA: the CMV status of infants and mothers at the time of first investigation, intensity scores of immunohistochemical stainings on liver biopsies, and the long-term prognosis (mean follow-up time, 5.7 years; range, 0.3-12 years)

Sex (m/f)	Infant ongoing CMV[a]	Infant CMV-IgG	Maternal CMV-IgG	Intensity score, canalicular membrane[b]		Long-term prognosis[c]
				IgM deposits	IgG deposits	
m	Yes	+	+	2	1	3
f	No	+	+	1	2	2
m	No	−	−	2	2	3
m	No	+	+	1	1	3
m	No	−	nd	1	2	3
m	No	+	+	1	1	2
f	No	+	+	1	2	3
f	Yes	+	+	1	1	3
m	Yes	+	nd	2	2	1
f	No	+	+	1	2	1
m	Yes	+	+	2	1	1
f	Yes	+	+	3	2	3
m	Yes	+	+	2	2	3
f	No	+	+	1	2	3
m	No	+	+	1	1	1
m	Yes	+	+	2	2	3
m	Yes	+	+	1	1	2
f	Yes	+	nd	1	1	3

nd indicates not done.

[a] CMV-IgM detected in serum and/or CMV isolated in the urine.

[b] 0 = No staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining.

[c] 1 = None or minor liver disease; 2 = chronic compensated liver disease; 3 = liver transplanted and/or dead.

Table 2 The diagnosis and signs of ongoing CMV infection correlated to intensity scores for IgM and IgG staining on the canalicular membrane of the hepatocyte in biopsies from 18 patients with BA

Ongoing CMV infection	No. of patients	Intensity score, canalicular membrane[a]							
		IgM				IgG			
		0	1	2	3	0	1	2	3
Yes[b]	9	0	3	5	1	0	5	4	0
No[b]	9	0	8	1	0	0	3	6	0

[a] 0 = No staining; 1 = moderate staining; 2 = intense staining; 3 = very intense staining.

[b] $P = .042$ for the comparison of IgM intensity scores between CMV-IgM-positive and CMV-IgM-negative patients (Mann-Whitney U test). Same comparison for IgG intensity score was nonsignificant.

Immunohistochemical staining was performed to investigate the presence of IgM and IgG antibodies. Formalin-fixed, paraffin-embedded tissue samples were sectioned, deparaffinized in xylene, treated with H_2O_2 in methanol, rehydrated through a series of graded alcohols, and washed in phosphate-buffered saline (PBS). Treatment with 0.1% pronase in PBS for 5 minutes at room temperature and nonspecific blocking with PBS containing 5% bovine serum albumin preceded immunostaining. The sections were incubated with rabbit antihuman IgM or IgG antibodies (Dakopatts, Glostrup, Denmark) at final dilutions of 1:500 and 1:2000, respectively. After washing, the specimens were incubated with a biotin-conjugated swine antirabbit antibody, followed by a horseradish peroxidase–conjugated AB complex (Dakopatts). The presence of IgM or IgG immunoglobulins was detected using the DAB substrate (Sigma). The sections were counterstained with hematoxylin, washed, dehydrated, and mounted before examination in a light microscope. As a negative control for the staining procedure, a section from each biopsy was treated as above except for omitting the rabbit antihuman IgM and IgG antibodies.

Using a standardized semiquantitative scoring system for the intensity of the IgG and IgM staining, respectively, the biopsies were independently evaluated by 3 of the authors (BF, SW, NP). Predefined cellular locations of staining were studied, that is, the bile duct epithelium, the canalicular membrane of the hepatocyte, the sinusoidal membrane, and the sinusoidal lumen. For each location the intensity of the staining was evaluated on a scale from 0 (no staining) to 3 (very intense staining). The average of the scores given by the 3 investigators was considered to be the final score. When examining the biopsies the investigators had no information on the diagnosis nor the CMV status of the patient corresponding to each biopsy.

2.4. Statistical analysis

The Mann-Whitney U test was used for the comparison of staining scores between different groups.

2.5. Ethical considerations

The patient study was approved by the local ethics committee at Huddinge University Hospital. The control patients were investigated after obtaining informed consent from the parents.

3. Results

3.1. Patients

The most consistent staining pattern appeared on the hepatocytic canalicular membrane in biopsies from the

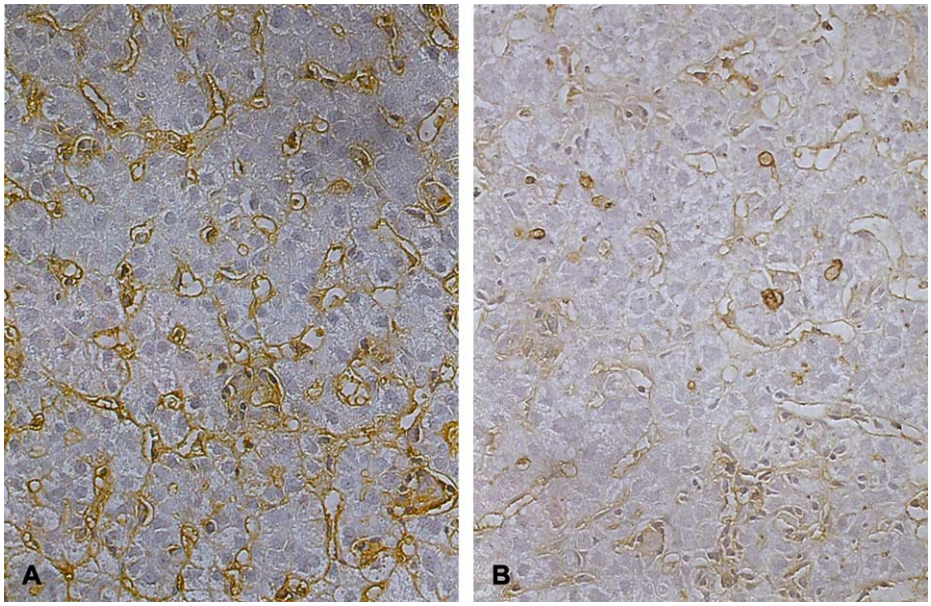

Fig. 1 Immunohistochemical staining for IgG (A) and IgM (B) in liver tissue from a patient with extrahepatic BA. Both canalicular and sinusoidal staining is demonstrable. Final magnification, ×173.

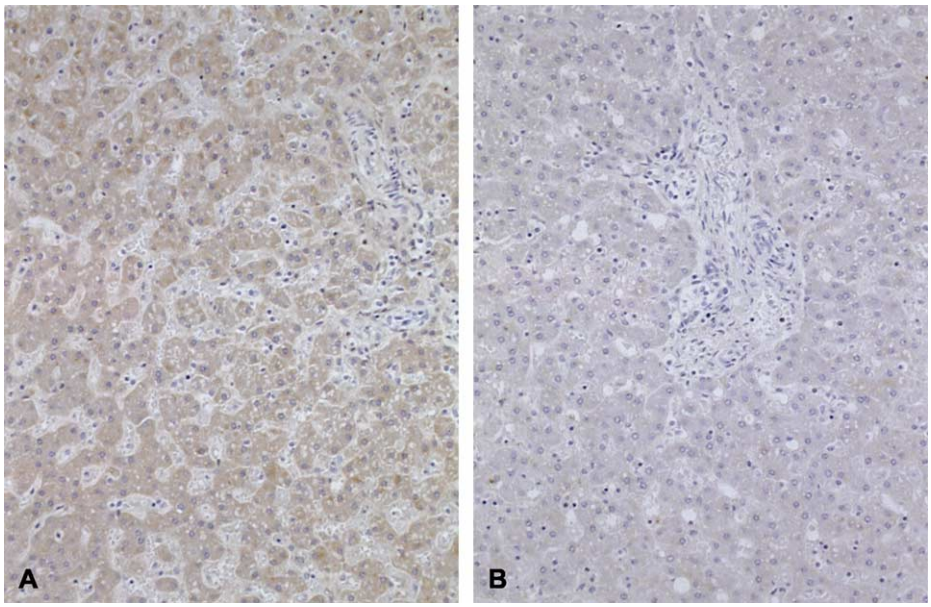

Fig 2 Immunohistochemical staining for IgG (A) and IgM (B) in liver tissue from a control patient without liver disease. No specific canalicular or sinusoidal staining is demonstrable. Final magnification, ×173.

patients with BA, and this structure was therefore more carefully evaluated (Tables 1 and 2). Fig. 1 shows a representative immunohistochemical staining of high intensity for IgM and IgG deposits on the canalicular membrane compared with controls. In the sinusoidal lumen and on the sinusoidal membrane, staining was abundant in all biopsies but lacked any specific pattern. No staining was detected on the bile duct epithelium in any of the biopsies. No staining at all was seen in any of the biopsies when the rabbit antihuman IgM and IgG antibodies were omitted in the staining procedure.

When analyzing immunoglobulin deposits on the canalicular membrane, the intensity score for IgM staining was significantly higher in biopsies from patients with signs of ongoing CMV infection than in those from patients without such signs (Table 2). No significant differences between the 2 groups were seen in IgG staining intensities (Table 2). No differences were seen when the staining intensities were correlated to the maternal CMV status.

The combined staining intensity score (IgM + IgG) in biopsies from patients in outcome group 3 did not differ from that of patients in groups 1 and 2 ($P = .69$). However, it was noted that 4 of 5 patients with a combined score of 4 or more were in group 3 (Table 1).

3.2. Controls

Of 6 controls, 4 had minor histologic changes in the liver, with small hemorrhages, secondary to asphyxia. However, none had any signs of cholestasis, bile duct proliferation, or inflammation in the liver. Virological data, including serology and CMV-DNA analysis by PCR in white blood cells and placenta, were only available for 3 of 5 perinatally dead patients. None of the 3 babies had signs of ongoing CMV infection. However, CMV-DNA was detected by PCR from the placenta of one of the mothers.

The 2 other controls had normal liver histology. This included the 3-month-old infant with prolonged unconjugated hyperbilirubinemia, for whom no virological data were available.

In 5 of the 6 control biopsies no staining was identified at all (Fig. 2). In the biopsy from the 3-month-old patient, weak staining was detected around the sinusoidal lumen, but no staining was detected on the canalicular membrane.

4. Discussion

In the present study we found an association between ongoing CMV infection and the amount of immunoglobulin deposits on the canalicular membrane of the hepatocyte. Such deposits were only seen in biopsies from patients with BA and not in those from control patients. In an earlier study, Hadchouel et al [15] found immunoglobulin deposits on the extrahepatic bile duct remnants from patients with BA and suggested that this could be a sign of progressive immunologic injury in the development of an acquired obstructive cholangiopathy. It was rightly pointed out that these findings might represent secondary events, perhaps after the cholestatic development rather than constituting primary etiologic factors [15]. Similar questions may be raised concerning our results. However, in this study we demonstrated that there was a difference in staining intensity depending on the CMV status of the patient. This result may indicate a role for CMV infection in the pathogenesis of BA and thus suggests that the immune deposits may not necessarily be simply secondary to cholestasis.

Other reports have also suggested the involvement of immunologic mechanisms in the etiopathogenesis of BA. An increased tissue expression of intracellular adhesion molecules and of major histocompatibility complex class II molecules and an association to certain human leukocyte antigen types have been reported in some studies [12-14] but been refuted in a recent one [17]. A theory of a "2-hit mechanism," which would explain both the evidence found for viral infections and for immune-mediated mechanisms in the development of BA, has also been suggested [18]. The results of our study are therefore interesting because an association between ongoing CMV infection and the amount of immune deposits at certain locations was found in patients with BA.

The role of CMV in BA is controversial. Serologic evidence for ongoing CMV infection in patients with BA has been reported by other groups [7,8,11], and we found a significantly higher incidence of such evidence in patients with BA than in age-matched controls [5]. Furthermore, we detected CMV-DNA by PCR in liver tissue from 9 of 18 investigated patients [5]. This is supported by a recent PCR study by Domati-Saad et al [9], but not by Jevon and Dimmick [10], who reported all 12 patients with BA to have CMV-DNA–negative liver biopsies. One methodological difference between these studies was that Jevon et al used formalin-fixed, paraffin-embedded liver biopsies whereas Domati-Saad used frozen liver tissue. It is possible that the latter method may preserve viral DNA better than the former.

It should be noted that the number of control biopsies in the present study was significantly lower than the number of biopsies from patients with BA. However, specific canalicular staining was consistently absent in all control biopsies, suggesting that our findings were indeed representative for noncholestatic infants. Secondly, the age of the controls did not entirely match that of the patients. Still, we would claim that using newborns as controls is adequate, considering the specific pattern of CMV-induced liver disease in this age group as compared with that of older children or adults [19,20]. A further limitation of our study is that the method used to detect immune deposits was not tested for specificity and that it only allows for semiquantitative assessment.

The mechanism by which CMV would cause possible immunologic damage remains to be clarified. One of several suggested mechanisms for such a CMV-induced immune modulation is the formation of CD13-specific autoantibodies, which has been described in bone marrow–transplanted patients with chronic graft-versus-host disease [21]. Interestingly, CD13 is expressed on the canalicular side of the hepatocyte membrane [22]. By using monoclonal antibodies to CD13 in liver specimen from patients with BA, Liu and coworkers recently showed that the staining was specific for the bile canaliculi. Furthermore, in their study, the staining was more intense in biopsies from patients with BA with a poor prognosis compared to those with a good prognosis [23].

Immunologic mechanisms could be of importance not only in the etiology of BA, but also in the later stages of the disease after Kasai operation. Detailed studies on the liver histology in these patients suggest that there is an ongoing destruction of intrahepatic bile ducts over time [24] and that the expression of the macrophage marker CD68 and of intracellular adhesion molecule-1 in the extrahepatic biliary remnants at the time of portoenterostomy correlates to the long-term prognosis [25]. It was therefore of interest in the present study to investigate the relation between the amount of immunoglobulin deposits and the long-term outcome in the patients with BA. No clear association was found, although a majority of patients (4 of 5) with the most intense staining in their biopsies belonged to outcome group 3. The relatively low number of patients in each prognostic group is problematic and may warrant a future study including a larger group of patients with BA. Furthermore, the inclusion of stainings from repeated biopsies, obtained over time from such patients, may give more information.

Cytomegalovirus infection has also been implicated in etiology of intrahepatic forms of neonatal cholestasis [5,7,11,19,20]. It is possible that immunologic mechanisms are of importance in that setting as well. The occurrence of similar, virally induced pathological mechanisms in BA and intrahepatic forms of cholestasis would fit into the Landing [26] hypothesis of infantile obstructive cholestasis as a continuous spectrum of diseases.

In conclusion, our results suggest that CMV infection is one of several possible pathogenetic factors in an immune-mediated series of events leading to the development of BA. Because of the inherent limitations of the present study the results need to be further explored in a larger, prospective investigation.

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