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Biliary Atresia and Cytomegalovirus Infection: A DNA Study

G.P. JEVON* AND J.E. DIMMICK

Department of Pathology, Children's and Woman's Health Centre of British Columbia and University of British Columbia, 4480 Oak Street, Vancouver, BC, Canada V6H 3V4

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ABSTRACT

The cause of extrahepatic biliary atresia (EHBA) is undetermined in most instances, but an infectious agent is widely suspected. Cytomegalovirus (CMV) infection has been associated with intrahepatic bile duct destruction and paucity, raising the question of its role in EHBA. We identified 12 children in the past 5 years with biliary atresia and examined the bile duct biopsy. These showed acute/chronic inflammation and epithelial degeneration. CMV inclusions were not identified. We used in situ hybridization and the polymerase chain reaction (PCR) for CMV-DNA on formalin-fixed, paraffin-embedded tissue. All samples showed the presence of amplifiable DNA using β-globin primers. No biopsy tissue showed CMV DNA using specific probes and primers. The absence of demonstrable CMV DNA by in situ hybridization and PCR in EHBA biopsies implies that it is unlikely that this virus has any major role in the pathogenesis of this condition.

Key words: cytomegalovirus, biliary atresia, polymerase chain reaction

INTRODUCTION

Extrahepatic biliary atresia (EHBA) is thought to be an acquired condition, but in most instances, the etiology is undetermined. A small number of cases may be associated with chromosomal errors such as trisomy 18 or developmental anomalies, including the laterality sequence (polysplenia syndrome) and abnormalities of cardiac, gastrointestinal, and urinary systems, or with intestinal malrotation, preduodenal vein, or situs inversus [1].

While the cause of this entity is likely to be multifactorial, an infectious agent (as yet unidentified) is widely suspected in most instances [2]. The rarity of biliary atresia in stillborns and neonates, the presence of inflammatory changes in the biliary tree soon after birth, and its persistence following portoenterostomy argue in favor of this. Some investigators regard EHBA and paucity of intrahepatic bile ducts as a disease spectrum in which the principal process is bile duct destruction [3]. Most pathology reports describe epithelial degeneration of the extrahepatic ducts, mural inflammation, and fibrosis, while quantitative studies show a progressive loss of intrahepatic bile ducts despite portoenterostomy [2,4]. Other morphologic studies have noted lymphocytic infiltration of bile duct epithelium, and phenotypic characterization of the mononuclear inflammatory cell infiltrate is consistent with a viral etiology [5].

Congenital or acquired infantile cytomegalovirus (CMV) infection may involve intrahepatic bile ducts. Other investigators have drawn attention to the morphologic similarities between CMV hepati-

*Corresponding author

tis and the hepatitic changes associated with EHBA in some instances [6]. CMV may also infect endothelial cells, resulting in an ischemic vasculopathy, which is also suspected in the pathogenesis of EHBA [7]. CMV infection has been associated rarely with intrahepatic bile duct paucity and is described in stillborns with disseminated congenitally acquired CMV infection [8,9].

Past serologic studies have not provided conclusive evidence for hepatitis A or B virus, Epstein-Barr virus, or reovirus 3, while the role of CMV in biliary atresia has not been fully investigated [2,10,11]. This study concerns the results of our in situ hybridization (ISH) and polymerase chain reaction (PCR) analyses for CMV DNA in biopsies of atretic bile ducts obtained at the time of portoenterostomy.

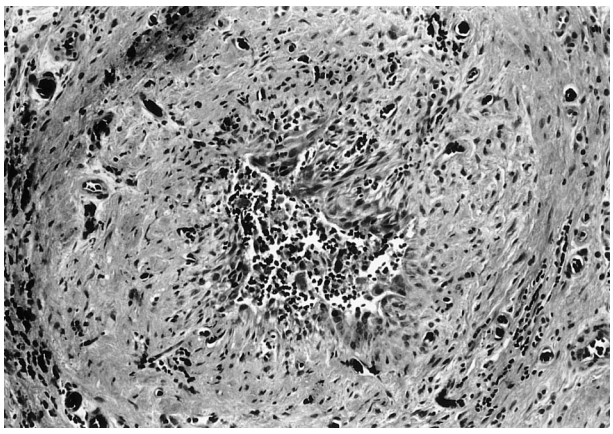

Figure 1. An atretic bile duct with epithelial necrosis, chronic inflammation, and fibroblast proliferation used in this study for CMV DNA (H and E, original magnification, ×100).

MATERIALS AND METHODS

We identified 12 children with biliary atresia confirmed at the time of portoenterostomy and a biopsy of the extrahepatic bile duct between 1988 and 1995 by a computer search of British Columbia's Children's Hospital surgical pathology records. The histology was reviewed specifically to note the type of inflammation, changes to the bile duct epithelium, and the presence of CMV inclusions by examining the hematoxylin and eosin (H and E)–stained slides. A single, representative, formalin-fixed, paraffin-embedded tissue block was identified for ISH and PCR studies in each case (see Fig. 1).

In situ hybridization for CMV DNA

Sections (5 μm) were deparaffinized in xylene twice and washed in ethanol. Endogenous peroxidase activity was blocked in 1/10 hydrogen peroxide/methanol solution for 20 min. We used a modification of the in situ hybridization procedure suggested by the manufacturer of the CMV DNA kit (Enzo Diagnostics, Farmingdale, NY). The sections were digested in 0.2% trypsin × 30 min, covered with hybridization solution, coverslipped, heated at 90°C × 5 min, and incubated in a prewarmed humid chamber at 37°C overnight. The slides were washed in 1 × SSC for 30 min at 50°C, their coverslips removed, then washed in TBS. The tissue was covered with streptavidin peroxidase–conjugated reagent (Dimension Labs, Mississauga, Ontario) for 20 min, rinsed, and developed in 50 ml AEC buffer, pH 5.2, with 120 μl hydrogen peroxide for 20 min. These were counterstained with Harris' hematoxylin, immersed in lithium carbonate, and rinsed.

DNA extraction/precipitation

We cut 2–7 sections (10 μm in thickness) for a total tissue area of approximately 1 cm^2, taking care to prevent contamination for PCR DNA amplification [12]. The tissue was dried in a vacuum centrifuge and digested with 0.004 ml Proteinase K (Boehringer Mannheim, 50 mg/ml) in 0.18 ml ABI lysis buffer (Applied Biosystems) and 0.22 ml water in a 60°C waterbath overnight. Two standard phenol/chloroform DNA extractions were performed, and the DNA was precipitated in 0.04 ml 3 M sodium acetate, pH 5.5, and 0.88 ml of 95% ethanol for 30 min in a dry ice/ethanol bath and then centrifuged. The DNA pellet was resuspended in 95% ethanol and centrifuged, then the supernatant was removed and dried completely in a vacuum centrifuge. The DNA was dissolved in 0.05 ml TE on a rocker at 4°C for 48 h and the DNA concentration of samples was calculated using optical densitometry measurements.

PCR for CMV DNA

The CMV and β-globin PCR conditions were the same [13,14]. DNA amplification for each of the

primer sets was performed in a total volume of 50 μl. The reaction mixture consisted of 1 μg DNA, 0.5 M KCl, deoxyribonucleotide triphosphate (dATP, dCTP, dTTP, dGTP; Boehringer Mannheim) and 0.3 μmol of each primer. The mixture was covered with 50 μl mineral oil, and 1 μl (1.5 U) *Taq* polymerase (Perkin-Elmer Cetus) was added. Amplification was performed in a DNA thermocycler (Perkin-Elmer Cetus) under the following conditions: 94°C × 1.5 min, 55°C × 2 min, and 72°C × 2 min for 35 cycles without extension. The DNA products were identified using 2% Nusieve gel, a ϕX174 DNA-*Hae*III Digest ladder (Sigma), staining with ethidium bromide, and photography. Positive CMV DNA controls included the weakest positive serial dilution from a formalin-fixed, paraffin-embedded section of CMV-infected autopsy lung confirmed by immunohistochemistry and a 10-pg DNA dilution of MRC-5 fibroblast culture infected with CMV AD169 (at 1 PFU, fixed and embedded, with DNA extracted/precipitated as above). CMV-negative human DNA and reagent control specimens without template DNA were included in the PCR analysis.

RESULTS

The average infant age at the time of surgery was 86 days (range 44–200 days). The sex distribution was equal (6 males, 6 females). All bile duct biopsies showed chronic inflammation with lymphocytes and plasma cells in the muscular wall or adjacent fibroconnective tissue or surrounding the periductal glands (12/12). Mild to moderate numbers of polymorphonuclear leukocytes (7/12) and eosinophils (5/12) had a similar distribution. The biopsies were characterized by aggregates of loose, immature-appearing fibroconnective tissue (10/12) and dense, hypocellular areas of fibrosis (7/12). Five had extravasated bile within macrophages or fibroconnective tissue. Changes to the bile duct epithelium included intraepithelial poymorphonuclear leukocytes/ulceration (7/12), and degeneration (7/12). The ganglion cells were unchanged (12/12). Viral inclusions were not identified in any of the biopsies.

In situ hybridization studies on representative sections were negative for CMV DNA. This DNA was not detected by PCR in any patient sample of extracted bile duct DNA.

DISCUSSION

It is possible that an infectious, likely viral, agent is responsible for EHBA in early infancy. Previous morphologic studies have implicated CMV infection with bile duct paucity in a small number of cases [8,9]. This virus infects bile duct epithelium and is associated with ductule destruction. In this study we were unable to detect CMV DNA in any of 12 bile duct biopsies using in situ hybridization and PCR.

The bile duct biopsies showed chronic active inflammation, epithelial ulceration, and areas of fibrosis of variable maturity, in keeping with earlier descriptions [2]. These findings are suggestive of a persistent insult, such as an unresolved epithelial infection. Some investigators believe that the accompanying changes in the intrahepatic bile ducts are secondary to the same insult and are not caused by extrahepatic obstruction alone.

EHBA was confirmed at surgery in all patients. The failure to demonstrate CMV DNA does not exclude the possibility of infection by this virus since it may be present in numbers less than the limit of PCR sensitivity. However, such numbers are unlikely to be of clinical significance since we were able to consistently detect CMV DNA from infected fibroblasts in positive controls run simultaneously using ethidium bromide staining and photography. We were able to demonstrate amplifiable DNA using β-globin primers in all samples. Furthermore, a resolved CMV infection may initiate biliary atresia by immunologic, ischemic, or other mechanisms, a possibility not excluded by a negative result.

EHBA is associated with considerable morbidity. The identification of causative factors in children is potentially important to its prevention. However, the absence of demonstrable CMV DNA in bile duct biopsies with chronic and/or active inflammation from infants with biliary atresia by in situ hybridization and PCR implies that it is unlikely that this virus has a major role in the pathogenesis of this condition.

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