

# Autoantibodies against subunits of pyruvate dehydrogenase and citrate synthase in a case of paediatric biliary cirrhosis

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## Abstract

**In a newborn girl with a history of congenital liver damage, histological examination of a liver biopsy sample taken during the seventh week of life revealed incipient destruction of bile ducts. Very high titres of antimitochondrial antibodies were later detected in the plasma. As the hepatic injury tended towards fibrosis, the histological diagnosis became primary biliary cirrhosis. Autoantibodies against E<sub>1α</sub>, E<sub>2</sub>, and E<sub>3</sub> subunits and protein X component of pyruvate dehydrogenase complex, and against citrate synthase were detected on western immunoblotting in a 1 in 1000 dilution of the patient's serum. The patient died of her illness at 11 years of age. In liver specimens obtained at autopsy human immunoglobulin deposition was detected on the surface of almost all hepatic cells by immunohistology. As there is a physical and functional interaction between pyruvate dehydrogenase and citrate synthase within the mitochondria, the presence of autoantibodies against certain proteins in the patient suggests that in this form of the disease the molecular recognition and then the autoimmunisation process could be directed against a mitochondrial enzyme cluster containing both pyruvate dehydrogenase and citrate synthase.**

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Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease characterised by progressive inflammatory obliteration of the intrahepatic bile ducts ultimately leading to cirrhosis.<sup>1-3</sup> Immune tolerance against certain self proteins in the disease is decreased; this is characterised by high titres of antimitochondrial antibodies.<sup>2,3</sup> Abnormal expression of mitochondrial antigens on the cell surface can also have a pathogenic role in the development of PBC.<sup>4</sup>

Up to 99% of "classic" adult PBC patients possess very high titres of autoantibodies directed against a family of antigens, termed M2, that are associated with the inner mitochondrial membrane.<sup>5</sup> These include typically the E<sub>2</sub>, E<sub>1α</sub>, and E<sub>1β</sub> subunits of pyruvate dehydrogenase (PDC), and protein X copurifies with the PDC E<sub>2</sub> subunit. The clinical presentation and natural history of PBC is quite vari-

able, showing heterogeneity of the disease; autoantibodies against other mitochondrial antigens are present.<sup>4</sup> As PBC in childhood is rare, the paediatric forms have not been characterised. In a paediatric patient reported here the liver disease fulfilled the clinical and histological criteria of PBC. Circulating autoantibodies were successfully detected against subunits of PDC, and the known physical association of PDC and citrate synthase (CS)<sup>6-8</sup> prompted us to search for autoantibodies against isolated CS in the patient's serum.

## Case report

The female patient was born from an uncomplicated pregnancy at 41 weeks of gestation; birth weight was 2600 g. Hepatomegaly (4 cm increase over normal) was present after birth; early in the first week of life jaundice developed and blood conjugated bilirubin increased to 15-50 μmol/l. Ultrasound showed a cyst in the lower left quadrant of the abdominal cavity. In the seventh week of life a tumour of approximately 3 cm diameter was removed by abdominal surgery, and a piece of the enlarged liver, which had a yellowish colour, was excised. Retrograde cholangiography was performed; the bile ducts were gracile, but there was no blockage in the biliary tree. Histological examination of the liver showed incipient biliary cirrhosis with signs of biliary duct destruction; the excised tumour was a necrotising benign cyst with a fibrotic coat surrounded by mononuclear cells. The histological diagnosis was confirmed by repeated liver biopsies; the liver disease showed slow progression and fibrosis became more and more pronounced. The presence of antimitochondrial antibodies (AMA) in plasma was first detected when the patient was six years old; high titres were subsequently found during two further investigations but regular follow up was technically impossible. Oesophageal varicosity developed and the patient died at 11 years of age in hepatic coma following rupture of the oesophageal varices.

## HISTOCHEMISTRY

Cryostat sections of liver specimens were studied according the guidelines generally followed in our laboratory. A piece of liver was used for haematoxylin-eosin staining; other sections used for immunostaining were fixed in cold acetone for five minutes and then were dried at room temperature. The blocks were stored at -20°C, and before use the slides were rehydrated in a phosphate buffered saline (PBS)

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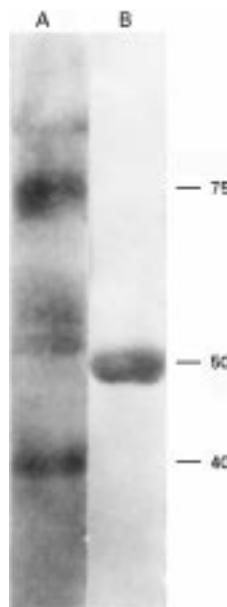
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**Figure 1** Autoantibodies against PDC and CS. Lane A, from the top:  $E_2$  (apparent molecular weight 70–74 kDa), protein X (50–56 kDa),  $E_3$  (55 kDa), and  $E_{1a}$  (41 kDa) subunits of PDC. Lane B: western blotting for CS (50 kDa) showed citrate synthase antibody in the serum.

solution (136 mM NaCl and 3 mM KCl in 10 mM phosphate buffer, pH 7.4). For detection of human immunoglobulin deposits the sections were incubated with peroxidase conjugated antihuman immunoglobulin for one hour at room temperature, and after washing the colour reagent 3-amino-9-ethylcarbazol was applied. The sections were covered with a mixture of nine parts glycerin and one part PBS after haematoxylin nuclear counter staining, and were examined by light microscopy. Two types of control were used: sections of the patient were tested with antimouse immunoglobulins; and the reaction described above with antihuman immunoglobulin was performed on liver from an adult alcoholic patient who had died of cirrhosis.

#### WESTERN BLOT ANALYSIS

The PDC was prepared from pig heart as described,<sup>8,9</sup> and its purity was checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Isolated PDC (5  $\mu$ g) and CS (1  $\mu$ g) was electrophoresed through a 10% polyacrylamide gel by the method of Laemmli.<sup>10</sup> Proteins were electroblotted on to nitrocellulose paper using a standard method,<sup>11</sup> and the paper slices were incubated with serum from the patient diluted to 1/1000. The human IgG bound to proteins on the membrane was reacted with alkaline phosphatase conjugated protein A (1  $\mu$ g/ml), and was visualised using 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium as substrate.<sup>11</sup>

#### Results

Western blot analysis with purified PDC and 1/1000 diluted serum from the patient showed four bands (fig 1, lane A). From the top, an intensive reaction was seen at 74 kDa, which corresponded to the  $E_2$  subunit of the enzyme (apparent molecular weight 70–74 kDa). Below this two adjacent bands were detected which probably corresponded to the protein X and  $E_3$  subunits of the enzyme (apparent molecular weights 50–56 and 55 kDa, respectively). At the bottom the fourth band at approximately 40 kDa was the  $E_{1a}$  subunit of PDC (apparent molecular weight 41 kDa). The same dilution of serum from the patient gave intensive immunoreaction with pure CS (apparent molecular weight 50 kDa, fig 1, lane B).

The usual histology of a liver biopsy specimen (taken at six years of age) showed fibrotic degeneration (fig 2A). In the fibrotic portal tracts lymphoid aggregates with characteristic loss of bile ducts and with ductular proliferation along the portal tract border were seen. The periportal hepatocytes were separated by mononuclear inflammatory cells and showed “feathery” degeneration with notable cholestasis and some Mallory bodies.

On the surface of almost all hepatic cells of the liver specimen obtained at autopsy notable, diffuse binding of the peroxidase labelled antihuman immunoglobulins was detected (fig 2B), shown by the development of a red colour from the chromogen substrate immunostaining. Several liver cells were swollen and

vacuolised. The patient’s liver did not show binding of antimouse immunoglobulins (fig 2C), and the antihuman immunoglobulin reaction was not present in liver from the patient with alcoholic cirrhosis (fig 2D).

#### Discussion

The chronic and progressive liver disease of the female patient reported here fulfilled the criteria for a diagnosis of PBC: histology of the liver showed the abnormalities usually seen in PBC; high titres of AMA were present in the circulation; and autoantibodies were present against subunits of PDC. In addition to these findings commonly observed in “classic” adult PBC, the disease also exhibited some unusual features. Firstly, the onset was extremely early. Secondly, in addition to autoantibodies against the  $E_2$  and X component of PDC (present in 96% of antimitochondrial antibody positive adult patients<sup>4</sup>), antibodies were also detected against  $E_{1a}$  (present in 53% of adult patients) and  $E_3$  (occasionally present only in 1/100 but not in the conventional 1/1000 diluted serum<sup>4,12</sup>) subunits of PDC. Thirdly, autoantibodies against CS were also present; this has not been previously reported. Finally, deposition of human immunoglobulin was detected on hepatocytes.

In adult PBC patients the autoantibodies are directed against a family of antigens, termed M2, that are associated with the inner mitochondrial membrane.<sup>5</sup> The  $E_2$  subunit of PDC is the major autoantigen of the M2 cluster,<sup>4,5</sup> but it has been recognised that other rarely observed proteins belonging to the clusters can also be involved in the autoimmunisation process.<sup>4,13</sup> As discussed above, in addition to the usual  $E_2$  subunit, autoantibodies were also detected against other subunits of PDC, showing that in this disease other unusual proteins were involved in the immunoreaction. During the next steps of examination, our working hypothesis was initiated by the intramitochondrial enzyme-enzyme association concept.<sup>14,15</sup> As part of the metabolon concept, several lines of evidence suggest association between functionally related enzymes in the inner membrane matrix space compartment.<sup>14,15</sup> Kinetic and physical evidence has been presented for an interaction between PDC and CS.<sup>6–8</sup> We postulated that perhaps not PDC alone, but a supramolecular mitochondrial enzyme cluster containing PDC and CS (and maybe other functionally related mitochondrial enzymes) could be the antigens. It is known that citrate synthase alone is a poor antigen (PA Srere, personal communication), but clustering with other mitochondrial enzymes such as PDC (forming a several million molecular mass enzyme cluster) may increase its antigenic property. If this supramolecular enzyme cluster is the antigen, it was reasonable to assume that autoantibodies could be formed against CS in the patient. We could detect circulating autoantibodies against CS, suggesting that the interactive PDC-CS complex participated in the immune processes as components of a mitochondrial cluster.

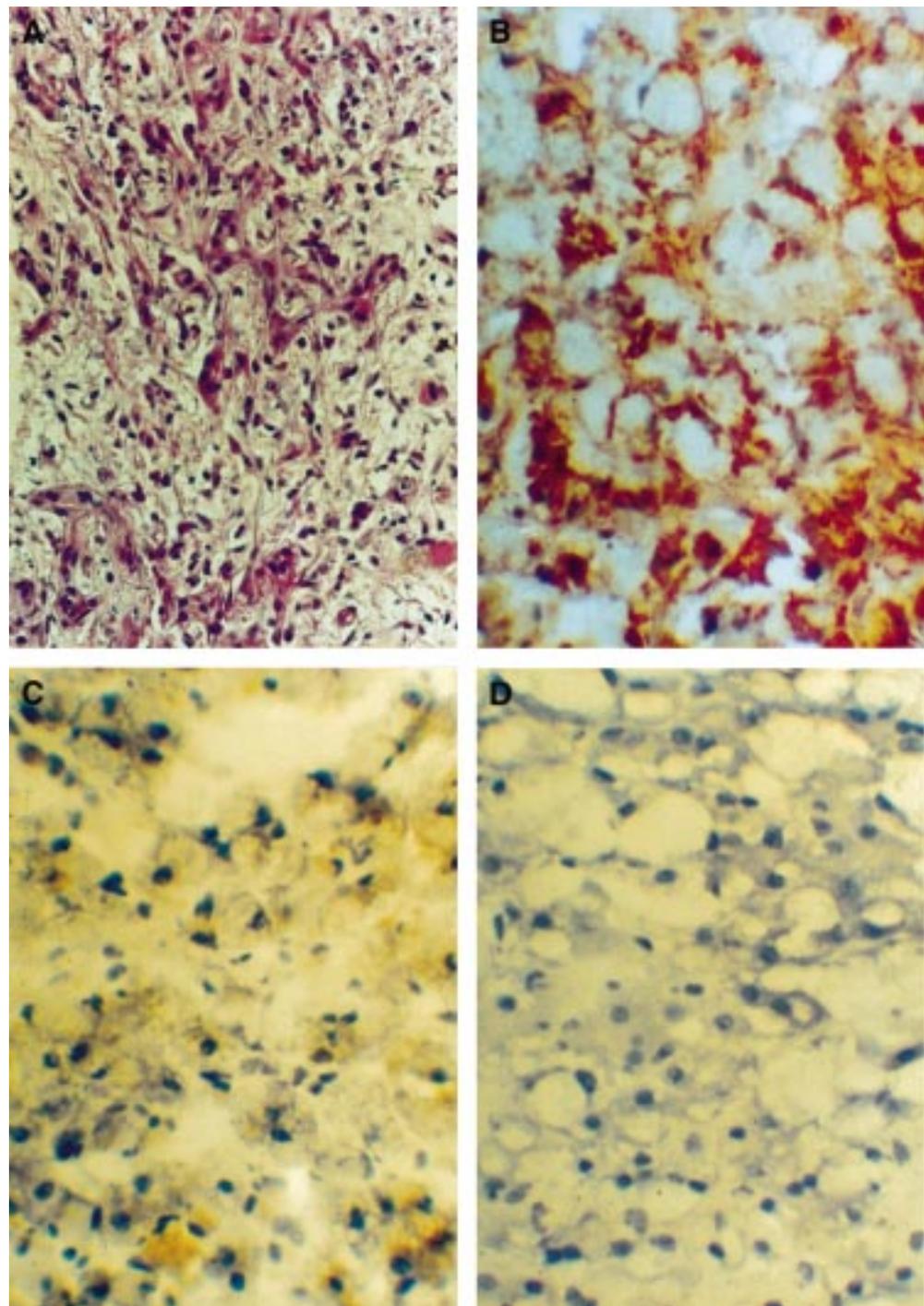


Figure 2 (A) In needle biopsy samples signs of biliary cirrhosis were prominent at six years of age (haematoxylin–eosin staining). (B) Following immunostaining of the liver there was intensive reaction with antihuman immunoglobulin predominantly on the surface of the hepatocytes. (C) Hepatocytes did not show binding of antimouse immunoglobulins (haematoxylin nuclear staining background; the yellow areas are from the bile pigments). (D) The hepatocytes of an adult patient who had died of alcoholic cirrhosis did not bind human immunoglobulins.

In “classic” PBC not only does the immune system show abnormal tolerance to self peptides of mitochondrial origin, but it is also primed to reject cells having abnormally expressed mitochondrial peptides on the cell surface<sup>4</sup>: in adult PBC these are the cells of the intrahepatic bile ducts. In the present case, immunostaining of the liver obtained at autopsy revealed intensive immunoglobulin deposition on almost all cells of the liver, showing that in this paediatric case of PBC, hepato-

cytes were also involved in the immunoreaction. In other words, in this paediatric case of PBC not only the protein composition, but also the intrahepatic distribution of the immunoclusters showed differences from that of the adult type.

It is proposed that in adult PBC the initial stimulus for antibody production is most frequently a chronic urinary tract infection,<sup>4</sup> and during the development of PBC, autoantibodies against the antimicrobial immunodomi-

nant epitopes cross react with certain conserved sequences expressed on the surface of the cells. In the present case we had no direct evidence for this possibility: the mother had no infection during pregnancy, and there were no clinical and laboratory signs of transplacental microbial invasion. However, as a possible explanation, tissue removed from the abdominal cavity of the patient was a necrotising, partially degenerated cyst which could represent the primer target for the immunisation process during intrauterine development of the patient, as the self proteins were liberated from the immunologically hidden intracellular localisation during destruction of the cells which later formed the cyst. Nevertheless, a simple coincidence of the cyst and PBC in the same patient cannot be ruled out.

The real incidence and natural history of PBC in childhood is unknown; due to insufficient data the existence of a rare, but relatively well circumscribed paediatric liver disease group cannot be ruled out. After this first report further studies on similar patients are required to delineate whether the autoimmune liver disease described here represents a special paediatric form of PBC, or can be regarded as a separate entity.

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