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Anti-Pyruvate Dehydrogenase Autoantibodies in Primary Biliary Cirrhosis

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Antimitochondrial antibodies (AMA) may be detected in 95% of patients with primary biliary cirrhosis (PBC). The target autoantigens for the AMA were recently identified as four closely related metabolic enzymes located in the mitochondria. We have purified the pyruvate dehydrogenase (PDH) enzyme from bovine heart, showing that all PBC sera reacted with a 74-kd band. PDH was utilized to establish an ELISA assay for detecting the relevant antibodies. One hundred twelve of 120 sera from patients with PBC (95%) reacted with the PDH but none of the 201 control sera, including normal subjects and a panel of sera from other patients with liver diseases, showed similar reactivity. In 77% of the PBC sera the anti-PDH antibody isotype was identified as a combination of IgG and IgM, while in 18% only IgM was detected. In 5% of the sera the isotype was confined to IgG. PBC sera specifically inhibited the PDH enzyme activity. The enzyme inhibition correlated with the anti-PDH antibody titers. Thus, PDH seems to be one of the major target epitopes for AMA observed in sera of patients with PBC.

KEY WORDS: Antimitochondrial antibodies; pyruvate dehydrogenase; primary biliary cirrhosis; autoimmunity; autoantibodies.

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic progressive liver disease characterized by inflammatory damage to the small intra hepatic bile ducts. Clinical, serological, and histological features support an autoimmune etiology (1), including the association with other autoimmune diseases, the marked predilection for women,[6] the predominance of T lymphocytes in the infiltrates in the hepatic lesions, defects in the function and regulation of T-suppressor cells, hypergammaglobulinemia, and the presence of antimitochondrial antibodies (AMA). The latter are disease specific and can be detected in the sera of more than 95% of the patients with PBC (2).

Antimitochondrial antibodies recognize groups of mitochondrial polypeptides designated M-2, with molecular weights of 70, 56, 52, and 46 kd as determined by immunoblotting (3–5). M-2 autoantigens were identified as four closely related metabolic enzymes located within the mitochondria termed 2-oxo acid–dehydrogenase complexes: pyruvate dehydrogenase (PDH), branch chain 2-oxo acid dehydrogenase (BCOADH), 2-oxoglutarate dehydrogenase (OGDH), and protein X (6–9). Each complex consists of multiple copies of three catalytic components termed E1, E2, and E3 which catalyze consecutive steps in the overall oxidative decarboxylation of the respective 2-oxo acid substrates (10).

In some reports, inhibition of enzyme activity by PBC sera has been shown (11, 12). Yet the relationship among the autoantibody reactivity, the catalytic activity of the enzymes involved, and the autoimmune pathogenesis of the disease is still unclear. A biochemically purified preparation of pyruvate dehydrogenase (PDH) was used to study

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[6]Abbreviations used: AC, alcoholic cirrhosis; ADH, acid dehydrogenase; AMA, antimitochondria antibodies; BCOADH, branch-chain 2-oxo acid dehydrogenase; BSA, bovine serum albumin; CAH, chronic active hepatitis; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; OGDH, 2-oxoglutarate dehydrogenase; PBC, primary biliary cirrhosis; PBS, phosphate-buffered saline; PBSTG, PBS–0.05% Tween-20, 1.5% gelatin, pH 7.4; PDH, pyruvate dehydrogenase; PIC, postinfectious cirrhosis; PNC, postnecrotic cirrhosis; PSC, primary sclerosing cholangitis; SDS, sodium dodecyl sulfate; 2OADH, 2-oxo acid dehydrogenase complexes.

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the diagnostic specificity of M-2-related autoantibodies in a large group of PBC patients.

MATERIALS AND METHODS

Subjects

Sera from 173 patients and 148 age- and sex-matched healthy subjects (blood bank donors) were obtained and stored at −20°C. One hundred twenty patients had PBC, 23 had primary sclerosing cholangitis (PSC), and 7 had chronic active hepatitis (CAH). Twenty-one had cirrhosis: 5 alcoholic (AC), 4 postinfectious (PIC), 3 postnecrotic (PNC), and 7 cryptogenic. One patient had bile duct carcinoma and another one had glycogen storage disease. All diagnoses were based on standard clinical biochemical and histological criteria (13). The patients with PBC also had undergone diagnostic liver biopsy.

Antigen

Purification of 2-oxo acid dehydrogenase complexes (2OADH) from bovine heart was performed as described by Stanley and Perham (14). The enzyme preparation was dissolved in 50 m*M* 3-[*N*-morpholino] propanesulfonic acid (MOPS) (pH 7.0) containing 3 m*M* EDTA, 2 m*M* dithiothreitol (DTT), and 11% Triton X-100 and stored at 4°C.

Electrophoresis and Western Blot

Protein samples of 2-oxo acid dehydrogenase preparation were dissolved in sample buffer containing 2% SDS, 2% β-mercaptoethanol, 10 m*M* Tris–HCl, pH 6.8, and 2% glycerol. Samples were subjected to SDS–polyacrylamide gel electrophoresis on 10% slab gels by the method of Laemmli (15). After electrophoresis, the proteins were transferred onto nitrocellulose sheets. Transfer was carried out in a Polyblot transfer system (ABN) at a constant current of 2.5 mA/cm^2 for 1 hr.

Individual lanes of nitrocellulose carrying the enzyme proteins (7 μg) were incubated for 1 hr in PBS, pH 7.4, containing 5% low-fat milk powder and 1% BSA (Sigma) to block nonspecific binding. After three washings with PBS containing 0.05% Tween-20 and 1% BSA, the strips were incubated with sera at a dilution between 1:200 and 1:5000 in the same buffer. Following further washings the strips were incubated for 1 hr with peroxidase-conjugated rabbit anti-human IgG or IgM (Sigma). Finally 4-chloro-1-naphthol (Sigma) and H_2O_2 were added to visualize antigen bound antibodies.

ELISA

A purified PDH preparation was diluted to a concentration of 5 μg/ml in carbonate coating buffer (pH 9.6) and incubated in 96-well polystyrene microtiter plates for 24 hr to effect coating. One hundred microliters of serum diluted 1:200 in PBS–0.05% Tween-20, 1.5% gelatin, pH 7.4 (PBSTG) were incubated for 30 min at 37°C in each well. After four washings, the microtiter plates were incubated at 37°C for 30 min with 100 μl of alkaline phosphatase-conjugated anti-human (Sigma) Ig, diluted 1:3000. The plates were then washed with PBSTG and the substrate (5 m*M* *p*-nitrophenylphosphate) was added and incubated at 37°C for 1 hr. Optical density at 405 nm was read in a Titertek Multiscan reader. The cutoff point for positive titer was defined as control mean + 3 SD.

The influence of modification at the enzyme catalytic site on antibodies binding to PDH was assayed by ELISA. After coating microtiter plates with the enzyme antigen, the coated plates were incubated for 30 min at room temperature with *N*-ethylmaleimide (2 m*M*), which is an irreversible inhibitor of the SH group, or with dithiothreitol (DTT) (2 m*M*), an antioxidant which reduces the disulfide bond of the cofactor lipoic acid. After preincubation the plates were washed and the ELISA was performed as described.

Enzyme Activity Assay

Pyruvate dehydrogenase complex activity was assayed as described by Broun and Perham (16) with a slight modification. The assay was carried out at 30°C with a final volume of 2 ml of potassium phosphate buffer (2.5 m*M*, pH 8.0) containing nicotinamide adenine dinuclotide (NAD^+) (2.5 m*M*), thiamine pyrophosphate (0.2 m*M*), $MgCl_2$ (1 m*M*), CoA (0.13 m*M*), and sodium pyruvate (2 m*M*). The assay was started by addition of the enzyme and the reaction was followed by the increase in absorbance at 340 nm.

Inhibition of pyruvate dehydrogenase activity was performed by preincubation of the enzyme preparation with PBC sera or control sera. Sera diluted 1:40 in 200 μl of potassium phosphate buffer (100 m*M*, pH 7.0) were incubated with 5 μl of enzyme preparation (activity, 1 U/ml) for 30 min at

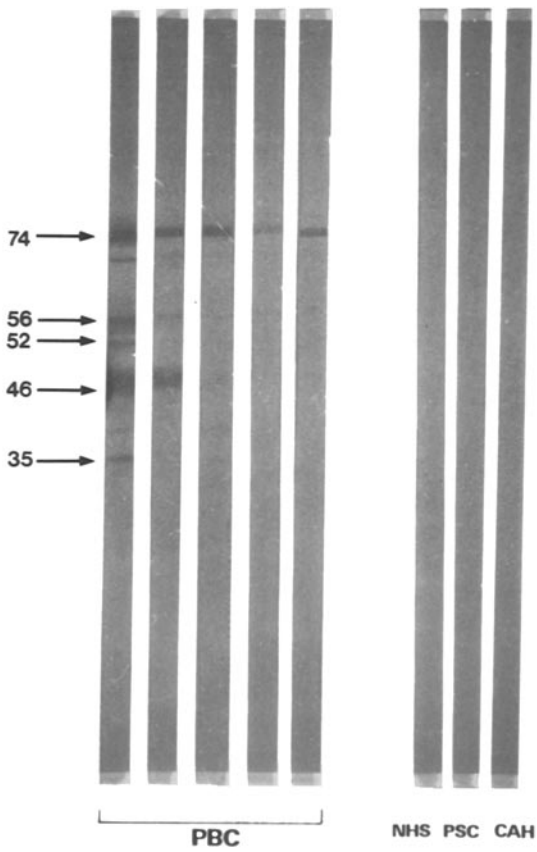

Fig. 1. Typical reaction of sera from patients with PBC, chronic active hepatitis (CAH), primary sclerosing cholangitis (PSC), and normal human serum (NHS) with 2OADH polypeptides by immunoblotting. Arrows indicate molecular weights of the proteins.

4°C. After preincubation, 2 ml of reaction mixture was added and enzyme activity was assayed as previously described.

RESULTS

Resolution of PDH multienzyme complex from bovine heart on SDS–polyacrylamide gel electrophoresis and immunoblotting showed that PBC sera reacted with four main M-2 antigens of 74, 56, 52, and 46 kd (Fig. 1). All PBC sera assayed (N = 23) reacted with the polypeptide of 74 kd. Some of the PBC sera reacted, in addition, with other polypeptides, either 46, 52, or 56 kd.

None of the 70 normal sera or sera from patients with other diseases, including 5 with primary sclerosing cholangitis, 4 with chronic active hepatitis, and 4 with cirrhosis, reacted with these proteins, indicating the specificity of these PBC-related antigens.

The molecular weights of these proteins are compatible with that of the dihydrolipoyl transacylase (E2) component of PDH (74 kd), 2-oxo glutarate dehydrogenase (52 kd), and BCOADH (46 kd) complexes and protein X (56 kd). One serum reacted, in addition, with a 35-kd peptide whose identity is unknown but which may be a breakdown product of the 74-kd protein (17).

The diagnostic specificity of anti-PDH autoantibodies was evaluated by the ELISA method, using wells coated with purified PDH complex preparation. Serum samples from 120 patients with PBC and 201 control sera were analyzed. One hundred twelve of the PBC sera were found to be positive (95%), while none of the control samples from healthy people (n = 148), patients with primary sclerosing cholangitis (n = 23), or patients with other liver diseases (n = 30) had raised levels of autoantibody reactivity (Fig. 2).

The anti-PDH antibody isotypes were examined in 40 randomly selected PBC patients; 77% of them had the combined IgM and IgG isotype, 18% had only the IgM isotype, and 5% exhibited only IgG reactivity. Immunoblot analysis of sera specific for the different isotypes showed that they reacted with the same M-2-specific polypeptides. In general, the titer of the anti-PDH antibodies in the sera which exhibited both isotypes was higher then the titer of antibodies in the sera which had either IgM or IgG isotypes alone.

The enzyme preparation exhibited a pyruvate dehydrogenase activity as measured by the increase in absorbance of nicotinamide adenine dinucleotide (NADH) at 340 nm. This enzymatic activity was inhibited following preincubation of the enzyme with sera from patients with PBC, but not with normal sera or sera from patients with other liver diseases such as PSC and CAH (Fig. 3).

The degree of enzyme inhibition by PBC sera correlated with the titer of antibodies as measured by ELISA. It is possible that the inhibitory activity of PBC sera on PDH activity may reflect autoantibody binding to the active site of the enzyme—the lipoyllysine residue. We therefore modified the catalytic site of the enzyme in order to test for changes in autoantibody binding. After PDH was bound to microtiter plates, the enzyme was treated with 2 mM N-ethylmaleimide, which causes irreversible modification of the free thiol group of the lipoic acid, or with 2 mM dithiothreitol, which reduces the disulfide bond (Fig. 4).

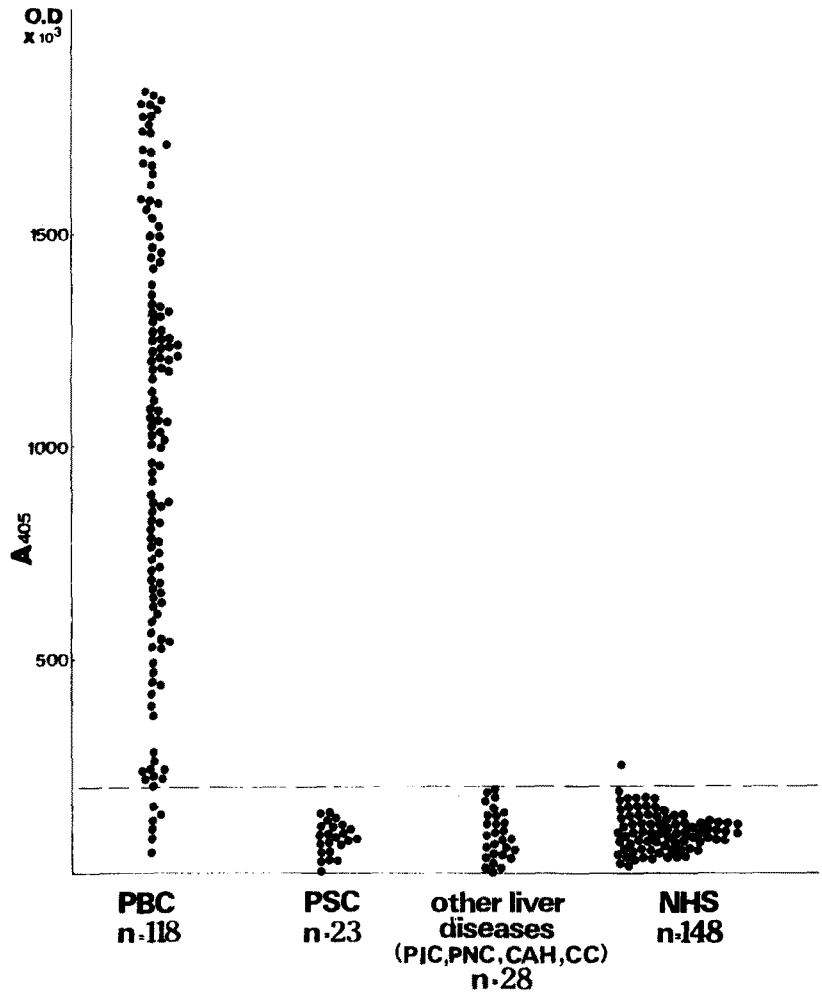

Fig. 2. Clinical diagnostic specificity of anti-2OADH antibodies by ELISA. The upper limit of normal (mean + 3 SD) is shown by the dashed line. PIC, postinfectious cirrhosis; PNC, postnecrotic cirrhosis; CAH, chronic active hepatitis; CC, cryptogenic cirrhosis.

Preincubation of the enzyme antigens with *N*-ethylmaleimide decreased the binding of sera from PBC patients to PDH by approximately 20% relative to controls ($P < 0.001$). In contrast, preincubation of PDH with the antioxidant DTT enhanced the binding of autoantibodies by the same order of magnitude ($P < 0.002$).

DISCUSSION

PBC autoantibodies recognize PDH complex which is central to cellular energy metabolism. Similar to M-2 autoantigens, many other cellular autoantigens have been found to participate in the universal and essential biological functions of the cell (18). Such examples including the splicing of precursor mRNA by Sm and U_1RNP antigens, DNA replication by proliferating cell nuclear antigen (PCNA), DNA topoisomerase I and RNA polymerase I, antigens which have been shown to be involved in DNA replication and transcription (18). Against these autoantigens, autoantibodies can be found in sera of patients with rheumatic diseases. Furthermore, antibodies found in patients with polymyositis and dermatomyositis recognize several species of tRNA synthetases involved in protein synthesis, while anti-neutrophil cytoplasm antibodies, specifically associated with Wegener's granulomatosis, were found to be directed against lysosomal enzymes such myeloperoxidase, elastase, and other proteinases (19). The interactions detailed above also were found to affect the enzymatic and functional activity of the bound species.

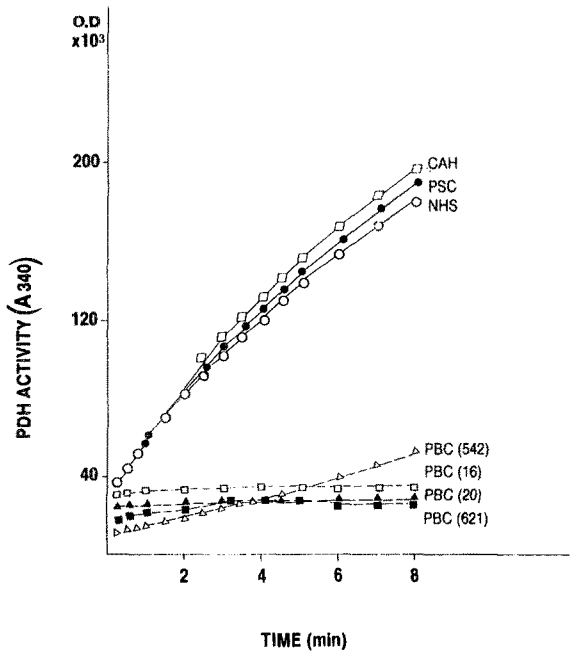

Fig. 3. Inhibition of PDH enzyme activity by sera from patients with PBC, chronic active hepatitis (CAH), and primary sclerosing cholangitis (PSC) and normal human serum (NHS). The enzyme preparation of 2-oxo acid dehydrogenase complex (2OADH) was incubated for 30 min at 4°C with various sera. After preincubation the reaction mixture was added and the enzyme activity was determined by increase in the absorbance of NADH at 340 nm.

In the current study we report on the generation of a simple ELISA method to detect antimitochondrial (PDH) antibodies. This ELISA was employed in a very large number of patients with PBC, in which its specificity and sensitivity was shown. It should be emphasized that in previous studies, only relatively small numbers of patients were analyzed.

We have shown the PBC specific antibodies bind to PDH and inhibit their enzymatic activity. On the other hand, reagents which change the catalytic activity of the enzyme may interfere with antigen recognition by the antibodies.

Disulfide bridges play an essential part in the catalytic mechanism of the PDH complexes. For example, the cofactor lipoic acid covalently binds to lysine residues in the E_2 subunit, carries the acyl group by thiol ester linkage, and shuttles between oxidized and reduced forms.

The autoepitope on PDH E_2 contains the lipoil group in the reduced form, since DTT enhanced, and NEM decreased, the binding activity of PBC antibodies to the enzyme. About 20% of the binding activity of PBC sera was changed by reducing or blocking the SH groups at the active site, when the catalytic activity was completely blocked by antibody binding. The change of only 20% may reflect the fact that the autoepitope is larger than the active site and includes more determinants beside the SH sites on E_2. This finding agrees with the data of Flannery *et al.* (20), who found that the lipoic acid is not the only target antigen for PBC antibodies.

Like the interaction between PDH and PBC antibodies, other active sites or functional domains of cellular enzymes have been shown to be affected by their respective autoantibodies. Thus, human autoantibodies inhibit DNA replication in an *in vitro* analysis of PCNA-dependent DNA replication (21) and autoantibodies to tRNA synthetase from patients with polymyositis inhibited the function of the enzyme in an *in vitro* assay system (22). Recently, Ludemann *et al.* (23) showed that the IgG fraction from the sera of patients with Wegener's granulomatosis affects the enzymatic activity of proteinase 3, the purported target antigen of antineutrophil cytoplasmic antibodies.

The *in vitro* inhibitory effect of autoantibody binding described in the above examples may reflect an underlying universal principle in the pathogenic mechanism of autoimmunity *in vivo*. Such a mechanism would suggest that the activity of certain biological molecules is affected or altered by antibody binding. Yet there is no evidence for the autoantibodies penetrating live cells or causing inhibition of intracellular enzyme. The similarity of PBC and other autoimmune conditions with respect to the nature of the autoantigen, the biological effects of antibody binding, and the specific presence of high titers of AMA is typical of that found in other autoimmune diseases. Examples of these include Sjogren's syndrome and scleroderma, in which there is a well-defined intracellular autoantigen found in all tissues and yet the autoimmune damage is restricted to specific tissue system.

Another important feature of autoimmunity in PBC and in other autoimmune conditions discussed here is that the autoimmune response is characteristic of the disease and the autoantibodies are useful as diagnostic markers in clinical medicine. The identification of the M-2 autoantigens provides us with a specific method for detecting and monitoring AMA found in patients with PBC. Thus, we have here assayed large number of sera from patients with PBC and report a sensitivity of 95% and a specificity approaching 100% for the ELISA with anti-PDH antibodies, in the clinical diagnosis of

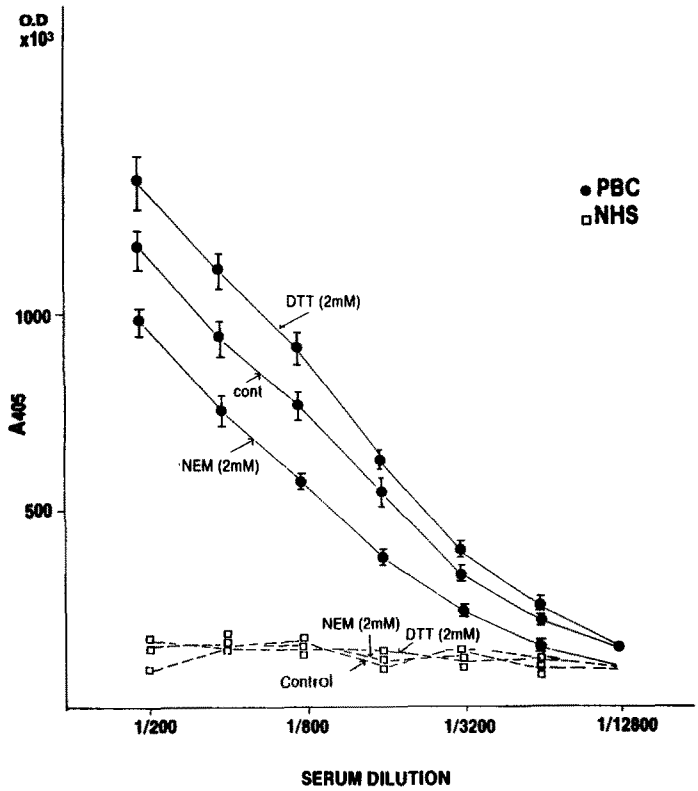

Fig. 4. Effect of dithiothreitol (DTT) and *N*-ethylmaleimide (NEM) on binding activity of PBC serum and NHS to 2OADH. A representative dilution curve of binding activity of PBC serum and normal human serum to 2OADH-coated plates. After 2OADH was bound to microtiter plates, the coated plates were incubated for 30 min at room temperature with 2 m*M* NEM, an irreversible inhibitor of the SH group, or with 2 m*M* DTT, which reduces the disulfide bond. The plates were washed and the binding of PBC serum or NHS to antigen was assayed by ELISA as described under Materials and Methods.

well-documented PBC as confirmed by liver biopsy. The same sensitivity and specificity of AMA were reported by Van De Water *et al.* (24) using recombinant mitochondrial proteins coding for 74- and 52-kd autoantigens.

Since AMA represent a complex and variable family of peptide-specific antibodies, individual PBC patients reacted with different polypeptides of the M-2 antigen family. Moreover, a growing amount of evidence shows that M-2 antigens have shared epitopes as well as specific proteins (20). Thus a mixture of M-2 autoantigens is needed for detecting AMA in PBC patients.

The ease of determining anti-PDH antibodies and the high specificity of the assay may encourage screening for detection of early cases of PBC, which may benefit from an early institution of specific treatment to prevent destruction of small bile ducts (25, 26).

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