Specificity of antinuclear antibodies in primary biliary cirrhosis

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Abstract

Objective—To study the specific autoantibodies against nuclear antigens in patients with primary biliary cirrhosis (PBC).

Methods—Sera from 21 patients with PBC were tested for antinuclear antibody (ANA) by indirect immunofluorescence on human epithelial (HEp)-2 cells, and for antibodies to various nuclear antigens by enzyme linked immunosorbent assay (ELISA) using different purified proteins or recombinant proteins as antigens.

Results—ANA detected in 10 of 21 patients (48%) with PBC included five anti-centromere antibody (ACA), two speckled, two homogeneous and one nuclear dot staining. ACA were present in 24% of PBC patients. By ELISA, anti-histone antibodies were detected in 81% of PBC patients, anti-ssDNA antibodies in 71% and anti-dsDNA in 10%, anti-topoisomerase-1 antibodies in 24%, anti-Sm/RNP antibodies in 24%, anti-La-48(SS-A) antibodies in 21%, and anti-Ro-60(SS-A) and anti-Ro-52(SS-A) antibodies in 30% and 25%, respectively.

Conclusions—The high frequencies of various antibodies directed against intracellular proteins and nucleic acids in patients with PBC suggests that PBC is a multisystem autoimmune disease which is similar to other systemic autoimmune diseases.

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Primary biliary cirrhosis (PBC) is a chronic idiopathic liver disease characterised by the destruction of intrahepatic bile ducts, portal inflammation and scarring, and eventually leading to liver failure. The aetiology of PBC is unknown, but evidence supports an autoimmune process.¹ A common feature of autoimmune diseases is a humoral immune response manifested by the presence of autoantibodies targeted against intracellular proteins and nucleic acids.² Antimitochondrial antibodies (AMA) are a hallmark of PBC and are detected in up to 95% of patients with PBC.^{1 3 4} In contrast, antinuclear antibodies (ANA) in patients with PBC are less common. Anticentromere antibodies (ACA) and other nuclear antibodies have been reported in patients with PBC.⁵⁻⁸ With the introduction of new autoantigens which can be purified from tissues or from recombinant proteins, a greater number of specific autoantibodies have been

detected by a more sensitive immunoenzymatic assay.² It was the aim of this study to determine the specificity and frequency of ANA in the sera of patients with PBC as detected by immunofluorescence and by enzyme linked immunosorbent assay (ELISA) using different purified proteins or recombinant proteins as antigens.

Patients and methods

SERA

Sera were collected from 21 patients with PBC and 20 healthy controls. During the study, the number of patients with PBC was increased to a total of 24; results for the additional three patients are included where available. The diagnosis of PBC was based on the presence of all of the following: (a) evidence of continuous liver disease based on a history of biochemical abnormalities for more than three months; (b) serum alkaline phosphatase concentrations greater than three times normal; (c) positivity for AMA; (d) a diagnostic or compatible liver histology.⁹ The control group of age and sex matched healthy, asymptomatic individuals was drawn from hospital staff. All sera were collected at Chung Shan Medical and Dental College Hospital and National Taiwan University Hospital, Taiwan.

IMMUNOFLUORESCENCE TEST FOR AMA AND ANA
Sera were tested for AMA by indirect immunofluorescence of frozen sections of mouse kidney and stomach. ANA were detected using human epithelial (HEp)-2 cells (Antibodies Inc. CA, USA) as described previously.¹⁰ AMA and ANA were considered positive at a 1/20 dilution and 1/80 dilution, respectively.

IMMUNODIFFUSION

Immunodiffusion was performed using extractable nuclear antigen of rabbit thymus (lyophilised extract from Pel-Freez Biologicals, Rogers, AR, USA) and human spleen according to the methods of Tan.¹⁰

ELISA

ELISA was performed according to the method of Rubin *et al.*¹¹ All sera were assayed at a dilution of 1/200. Briefly, Microwell plates were coated overnight at room temperature with 100 µl/well of 5 µg/ml antigens in 50 mmol/l Na₂CO₃/NaHCO₃ buffer, pH 7.2. Wells were blocked with gelatin, washed with

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phosphate buffered saline (PBS)-Tween and sequentially incubated with human sera (1:200 dilution) and peroxidase conjugated goat antihuman immunoglobulin. Substrate solution containing 2,2' azino-di-(3-ethyl-benzthiazolin-6-sulphonic acid) 1 mg/ml and 0.005% hydrogen peroxide in 0.1 mol/l McIlvaine's buffer was used for the peroxidase reaction.

ANTIGENS

Native DNA (Calbiochem, La Jolla, CA, USA) was bound to microtitre plates precoated with methylated bovine serum albumin and digested with S1 nuclease to remove any denatured regions. Denatured DNA was prepared by boiling and quick cooling and was absorbed to plate at 2.5 µg/ml.

Total histones were also obtained from Calbiochem.¹¹ Purity was greater than 95% as judged by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Histones were diluted to 2.5 µg/ml in PBS for adsorption to plates.

Sm and Sm/RNP antigens were purified from rabbit thymus powder (Pel-Freez) sera characterised as anti-Sm only, anti-Sm/RNP, and anti-RNP only, were identified as reported previously.¹²

Recombinant proteins of Ro(SS-A) and La(SS-B) for use in the ELISA were kindly provided by Dr Edward K L Chan and Eng M Tan, W M Keck Autoimmune Disease Center,

The Scripps Research Institute, La Jolla, CA, USA. The recombinant proteins rRo-52(SS-A) (at a dilution of 1:16 000) and rRo-60(SS-A) (1:1000 dilution) were for the 52 kDa and 60 kDa Ro(SS-A) antigens, respectively. The recombinant protein rLa-48(SS-B) was for the 48 kDa La(SS-B) antigen and was used at 1:8000 dilution.

IMMUNOFLUORESCENCE

When HEp-2 cells were used as substrate, 10 of 21 sera (48%) from patients with PBC produced positive nuclear fluorescence. Of these 10 ANA positive sera, five had ACA (fig 1A), two had homogenous patterns (fig 1B), one showed nuclear dots (fig 1C), and two had speckled patterns (fig 1D). The ACA were characterised by a discrete speckled pattern on resting tissue culture cells and by a distinct pattern on chromosomes of dividing cells. The nuclear dot pattern was a distinct one of two to seven discrete nuclear dots which varied in size and number among individual cells and differed from centromere staining in that they were larger, fewer in number, and not seen in mitotic cells or on metaphase chromosome spreads. Mitochondrial staining of the cytoplasm is evident in figure 1B, C and D.

IMMUNODIFFUSION

Using extracts of rabbit thymus and human spleen as sources of antigen, antibodies to Ro

Figure 1 Patterns of nuclear immunofluorescence on human epithelial-2 cells observed in the patients with primary biliary cirrhosis. A: Anticentromere staining; B: homogenous staining; C: nuclear dot staining; D: fine speckled staining. Typical staining of chromosome in mitotic cells in A; mitochondrial staining of cytoplasm is seen in B, C, and D.

Figure 2 Antibodies to histones, single stranded DNA (ssDNA), double stranded DNA (dsDNA), and topoisomerase-1 (Topo-1) detected by ELISA using purified antigens as substrates and antibodies to the recombinant proteins of the 60 kDa form of Ro(SS-A), the 52 kDa form of Ro(SS-A) and the 48 kDa form of La(SS-B) in patients with PBC.

were found in two of 21 (10%) and antibodies to La in one of 21 (5%) patients with PBC by means of immunological identity with reference sera. One was found to be immunologically distinct from a series of antibodies reacting with other known cellular antigens including Sm, nRNP, Ro, La, topoisomerase-1, Jo-1, proliferating cell nuclear antigen, and PM-Scl.

ELISA

Figure 2 shows the results of using different purified proteins or recombinant proteins as antigens. The normal value of the absorbance was based on the results from 20 normal control subjects. Values greater than mean + 2SD were considered to indicate increased antibodies.

Antibodies to histone were found in 17 of 21 (81%) patients with PBC, antibodies to ssDNA in 15 of 21 (71%), antibodies to nDNA in two of 21 (10%), antibodies to topoisomerase-1 in five of 21 (24%), antibodies to Sm/RNP in five of 21 (24%), antibodies to Ro-60 in seven of 24 (30%), antibodies to Ro-52 in six of 24 (25%), and antibodies to La-48 in five of 24 (21%). Antibodies to native DNA, Sm/RNP, and topoisomerase-1 were detected only in low titre (fig 2).

Discussion

In the present study we looked for the presence of autoantibodies against specific nuclear antigens in the sera of patients with primary biliary cirrhosis by using various purified autoantigens and recombinant proteins as antigens. These autoantigens were chosen because they include common antigens that are recognised by sera from patients with systemic autoimmune diseases such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), and systemic sclerosis (SSc). PBC has been regarded as an autoimmune disease, but there remains some controversy as to whether it is an organ specific or a multisystem autoimmune disease. In this study antihistone

antibody was found in 81% of PBC patients, anti-ssDNA in 71%, anti-La-48(SS-B) in 21%, anti-Ro-60(SS-A) in 30%, anti-Ro-52(SS-A) in 25% and anti-Sm/RNP in 24%. This heterogeneity of ANA in our patients with PBC suggests a heterogeneous immunological background comparable to that in patients with systemic autoimmune disease, and that PBC may be regarded as multisystem autoimmune disease.

The frequency of ANA on HEP-2 cells by immunofluorescence in the present study (48%) was comparable to others reported previously.^{7,8} The patterns of ANA were diverse and included ACA, speckled, homogenous, and nuclear dot staining. Bernstein *et al*⁷ and Cassani *et al*⁸ reported frequencies of nuclear dot staining in PBC as 13% and 17%, respectively, and suggested that this pattern might be a serological marker for PBC. We found nuclear dot staining in one of 21 sera from patients with PBC; an increased incidence of ACA was also found. These two antibodies seem to be unique in patients with PBC.

Antihistone antibodies are generally considered to be related to drug induced lupus. An association between antihistone antibodies and clinical features has also been reported.^{2,13,14} They were found in 81% of sera from patients with PBC in our study—a frequency comparable to that found in drug induced lupus,² in generalised morphea,¹³ and in juvenile chronic arthritis with uveitis.¹⁴ Such a high prevalence of antihistone antibodies in PBC has not been reported previously and its significance in PBC needs further study.

It has been noted that PBC is often associated with Sjögren's syndrome.¹⁵ This study shows that the frequency of anti-Ro-52(SS-A), anti-Ro-60(SS-A), and anti-La-48(SS-B) antibodies in the sera of patients with PBC was 20–30%. This frequency of these autoantibodies in PBC was higher than that reported in a previous study,⁷ and as anti-Ro(SS-A) and anti-La(SS-A) are antibodies specific for Sjögren's syndrome, it provides confirmation that PBC may often associate with Sjögren's syndrome.

Using a sensitive immunoenzymatic assay, we found a distinct profile of antinuclear antibodies in PBC. The varying frequencies of autoantibodies we observed were similar to those associated with other systemic autoimmune diseases such as SLE, SS, and SSc. Further investigation of these associations may improve understanding of PBC and other autoimmune diseases.

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