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The Western immunoblotting pattern of anti-mitochondrial antibodies is independent of the clinical expression of primary biliary cirrhosis

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Abstract

Anti-mitochondrial antibodies are the serological markers of primary biliary cirrhosis. We analysed the detailed anti-mitochondrial antibodies patterns to see whether the immunological specificities detected at the time of the diagnosis correlate with the histological, clinical and immunological expression of the disease. One hundred and thirty primary biliary cirrhosis patients were studied at the time of presentation/diagnosis. Anti-mitochondrial antibodies reactivity was dissected and evaluated by Western immunoblotting with bovine heart submitochondrial particles as antigenic source. Six different Western immunoblotting patterns have been identified with the following hierarchy: pattern A (anti-PDC-E2 + anti-E3BP, 38.5%), pattern B (anti-PDC-E2 + anti-E3BP + anti-OGDC-E2, 20.8%), pattern C (anti-PDC-E2 + anti-E3BP + anti-BCOADC-E2 + anti-OGDC-E2, 13.1%), pattern D (anti-PDC-E2 + anti-E3BP + anti-BCOADC-E2, 6.9%), pattern E (anti-BCOADC-E, 6.1%) and pattern F (anti-mitochondrial antibodies negative primary biliary cirrhosis, 14.6%). The different patterns were neither associated with peculiar clinical, biochemical, histological and immunological features nor with the Mayo Risk Score. The anti-mitochondrial antibodies pattern at presentation is independent of the stage of the liver disease; therefore, the Western immunoblotting characterisation of anti-mitochondrial antibodies does not seem to be helpful in identifying the clinical, biochemical or histological expression of primary biliary cirrhosis at the time of the diagnosis.

Keywords: Anti-mitochondrial antibodies; Primary biliary cirrhosis; Western immunoblotting

1. Introduction

Anti-mitochondrial antibodies (AMA) represent the serological hallmark of primary biliary cirrhosis (PBC), a slowly progressive cholestatic liver disease [1]. AMA are detectable even decades before the onset of the biochemical and clinical manifestations [2]. Nine AMA subtypes have been previously identified (anti-M1 to anti-M9) but only anti-M2 appears to be PBC-specific. AMA anti-M2 subtype is directed towards a multienzyme mitochondrial complex called 2-oxo-acid-dehydrogenase complex (2-OADC) [3]; the three members of 2-OADC are pyruvate-dehydrogenase complex (PDC), 2 oxo-glutarate-dehydrogenase complex (OGDC) and the branched-chain 2-oxo-acid-dehydrogenase complex (BCOADC), known for their important functional role in eukaryotic metabolic pathway. Each complex consists essentially of three subunits called E1, E2 and E3. The major mitochondrial autoantigens have been identified and are represented by the E2 subunit of the pyruvate-dehydrogenase complex (PDC-E2) [4], the E2 subunit of branched-chain 2-oxo-acid-dehydrogenase complex (BCOADC-E2) [5], the E2 subunit of oxo-glutarate-deydrogenase complex (OGDC-E2) [6], the E1 subunit and the E3-binding protein of PDC (PDC-E1 and E3BP, respectively) [7–9]. The PDC-E2 and E3BP are cross-reactive, possibly because they share homologous epitopes [3], while OGDC-E2 and BCOADC-E2 are recognised by a distinct subpopulation of autoantibodies, which can

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be found either isolated or in combination with other reactivities.

Different assays (indirect immunofluorescence, IFL; ELISA; Western immunoblot, W-IB) and diverse antigenic sources (recombinant or native) are currently employed for AMA detection. In PBC patients, additional autoreactivities targetting nuclear antigens such as sp100 and gp210 appear to be PBC-specific [10,11] and have been recently associated with more active and severe disease [12–14].

In this study, we analysed autoreactivities against the major mitochondrial autoantigens (E2 subunit of PDC, BCOADC, OGDC and E3BP) to see whether the different AMA patterns at the time of the diagnosis are correlated with biochemical, clinical, immunological or histological features, and may help the clinician to estimate the clinical expression and the histological stage of the disease.

2. Patients and methods

2.1. Study population

One hundred and thirty consecutive patients with a diagnosis of PBC according to published criteria [15], referred to our Department between 1997 and 2002, were studied. Viral, obstructive, metabolic and drug aetiologies were ruled out using appropriate tests and careful medical history. AMA was detected by routine indirect immunofluorescence in 94 (72.3%) patients, as previously reported in details [16]; 17 patients were reactive only with PBC-specific mitochondrial polypeptides by W-IB, but were negative by IFL; and 19 (14.6%) patients were negative for AMA both by IFL and W-IB, thus were diagnosed as AMA-negative PBC. A liver biopsy obtained within 3 years since the diagnosis was available in 105 (80.7%) patients: 68 (64.7%) had histological evidence of initial disease (stages I/II), whereas 37 (35.3%) showed a more advanced disease (stages III/IV). The Mayo Risk Score [17] and the International Autoimmune Hepatitis Group score [18] were calculated for each patient.

2.2. Control population

Twenty patients with primary sclerosing cholangitis and 50 blood donors were tested as pathological and normal controls by IFL and W-IB assays.

2.3. Indirect immunofluorescence

Sera diluted 1 in 40 in phosphate buffered saline (PBS) were tested on snap-frozen sections of rat liver, kidney and stomach. A fluorescein-conjugated secondary antibody directed against human immunoglobulins (anti-human polyvalent immunoglobulins IgA, IgG, IgM FITC conjugate, Sigma ImmunoChemicals, St. Louis, MO) was used diluted 1:100 in PBS. The patterns of reactivity were assessed under a fluorescence microscope (Orthoplan, Leitz, Wetzlar, Germany).

2.4. Western immunoblotting

Serum samples were drawn at the time of presentation/diagnosis and were stored at −20 °C until use. In addition to routine indirect immunofluorescence, all sera were tested by W-IB using as source of antigens a preparation of submitochondrial particles extracted from bovine heart. Briefly, mitochondrial proteins (1 mg/ml) were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis in 10% mini gels (Mini-Protean II System, Bio-Rad Laboratories, Richmond, CA) and transblotted onto nitrocellulose filters, which were then incubated in PBS containing 5% skimmed milk (blocking solution) for 1 h at room temperature. The filters were then cut into strips, and each strip was incubated with serum sample diluted 1:500 in blocking solution for 90 min in gentle agitation at room temperature. After incubation, the strips were washed three times in PBS containing 0.1% Tween 20 and then incubated for 1 h at room temperature in blocking solution containing the secondary antibody peroxidase conjugate rabbit anti-human IgG, IgA and IgM diluted 1:2000 (Dako, Copenhagen, Denmark). After further washing, the colorimetric reaction was developed with 4-chloro-1-naphtol for 10 min at room temperature.

2.5. Anti-nuclear reactivities

Reactivity against recombinant or purified sp100 and gp210 was assessed using commercially available ELISA kits (IMTEC, Berlin, Germany), according to manufacturer's instructions, as previously reported [14].

2.6. Absorption test

To assess the specificities of the W-IB reactivities, three representative AMA-positive sera (100 μl diluted 1:1000 in PBS) were separately incubated with 100 μg of purified recombinant fusion protein (kindly provided by Professor E. Gershwin, Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis, USA) expressing the three major mitochondrial epitopes (PDC-E2, BCOADC-E2 and OGDC-E2) [19] overnight at 4 °C, with gentle agitation. Antibody–antigen complexes were separated by ultracentrifugation (100,000 × *g* for 30 min) and the recovered supernatants were tested in IFL and W-IB assays.

2.7. Statistical analysis

The comparison of categorical variables was performed using chi-square and Fisher's exact test when applicable. The comparison of continuous variables between the different groups (pattern A through F) was performed with the Kruskal–Wallis test. A probability (p) value less than 0.05 was considered significant. Statistical analysis was performed using GraphPad InStat version 3.0a for Macintosh, GraphPad Software, San Diego, CA, USA, and StatView 5.0.1 for Macintosh, SAS Institute Inc., Cary, NC, USA.