Serological diagnosis of autoimmune hepatobiliary diseases

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ABSTRACT

Autoimmune hepatobiliary diseases (AIHBD) comprise autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis and the overlap syndromes. Early diagnosis and treatment of AIHBD are essential for the prevention of the high morbidity, and mortality, that is, otherwise, associated with untreated patients. Screening for AIHBD relies heavily on the use of serological tests for the detection of serum autoantibodies that associate with the diseases. Understanding these tests, and the results produced, is important for the efficient diagnosis/exclusion of these diseases. In this review, we discuss the various tests available in the clinical immunology laboratory for screening of AIHBD and comment on the significance of the results produced by each test. We hope that this review will highlight this group of autoimmune diseases and lead to more efficient and earlier diagnosis, and treatment, of patients with AIHBD.

Autoimmune-hepatobiliary diseases (AIHBD) include autoimmune hepatitis types 1 and 2 (AIH-1/2), primary biliary cirrhosis (PBC), autoimmune cholangitis (AC), primary sclerosing cholangitis (PSC) and a combination of these diseases, referred to as the overlap syndromes.[1,2] Early diagnosis and treatment of AIHBD are essential for the prevention of the high morbidity, and mortality, that is otherwise, associated with untreated patients. Screening for AIHBD relies heavily on the use of serological tests, which are based on the detection of serum autoantibodies that are associated with these diseases. These auto-antibodies include antinuclear antibodies (ANA), smooth muscle antibodies (SMA), liver kidney microsomal antibodies (LKM-1), liver cytosole-1 (LC-1) antibodies, soluble liver antibodies (SLA) and mitochondrial antibodies (AMA).[1,3,4] A number of serological tests are available for the detection of these autoantibodies. However, since the tests are associated with varying degrees of sensitivities and specificities, under, or over, interpretation of the results produced can lead to delayed, or even incorrect, diagnosis of these diseases. Therefore, for efficient diagnosis of AIHBD, good knowledge of the different serological tests, and the results produced is required. In the present review, we describe these tests in details and comment on the significance of the results produced. We hope that this information will lead to a more efficient and early diagnosis of these important diseases.

Smooth muscle antibodies. Smooth muscle antibodies are found in approximately 80% of patients with AIH-1 (50% as the sole autoantibodies, while in 35% in combination with ANA).[4,5] Presence of SMA, particularly at high titres (normally 1:80 in adults and 1:40 in children) are taken as a strong predictor of the disease.[4,6,7] Smooth muscle antibodies exist as a heterogeneous group of antibodies that are directed against the different components of the smooth muscle structure, which include F-actin, vimentin, G-actin and tubulin. However, only F-actin antibodiesare

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specifically associated with AIH-1, whereas other antibodies (which tend to be of low titres and of the IgM isotype) can be associated with a variety of other conditions including viral infections (Epstein Barr virus [EBS], hepatitis B and C viruses [HBV, HCV]), rheumatic diseases, Crohn's disease, cancer, hypercholesterolemia, as well as being found in 10% of normal individuals.[8-10] Detection of SMA is performed by an indirect immunofluorescence (IIF) assay, using multiblock tissues (consisting of rat stomach, liver and kidney tissues) as substrate. Presence of SMA is manifested by fluorescence staining of the stomach mucosa, intraglandular fibres coursing the mucosa, blood vessels, submembrane actin of hepatocytes, renal tubular and mesangial tissues.[6,11] The IIF-test is highly specific (90%) and sensitive (80%) for detection of anti F-actin antibodies, particularly when it stains the renal tubules and mesangial tissue.[6] Since the F-actin SMA associated with AIH-1 are of the IgG isotype, while SMA associated with other conditions tend to be of the IgM isotype, anti-IgG antibodies need to be used in the IIF-assay. Enzyme-linked immunosorbent assays (ELISA) can be used for detection of the specific anti F-actin antibodies. However, since the sensitivity and the specificity of this assay for F-actin antibodies is very much less than that associated with the IIF-test, their use for screening would seem inappropriate. However, ELISA-assay can be used as a confirmatory test for the IIF-results; although negative results would not exclude presence of anti F-actin antibodies, and thus, a diagnosis of AIH-1.

Liver kidney microsomal antibodies. Liver-kidney microsomal antibodies type-1 are found in approximately 3-4% of patients with AIH.[12] Liver-kidney microsomal antibodies type-1, which recognize a 50K cytochrome oxidase P450 2D6 (CYP2D6), are a marker for type-2 AIH (AIH-2). They are found in 80% of patients with the classical form of AIH-2, as well as in 15% of patients with AIH-2 associated with the autoimmune poly-glandular syndrome type-1 (APS-1).[13] Liver kidney microsomal antibodies occur in 6-10% of patients with chronic HCV infection. However, these antibodies react with epitopes different from those recognized by the AIH-2 associated LKM-1 antibodies and tend to be of low titres.[4,14] The association of LKM-1 antibodies with HCV infection seems to be restricted to patients from Southern Europe, with such an association being very rare in other parts of the World.[15-17] Liver-kidney micro-somal antibodies, other than LKM-1, also exist and includes LKM-2 (which recognize the CYP2C9) and LKM-3 (which recognize a 55 KD glucuronyl transferase). Liver-kidney microsomal antibodies type-2 are associated with drug-(tienilic acid)-induced hepatitis, while LKM-3 are found in 5-13%

of patients with AIH-2, occurring alone, or in association with LKM-1.[18-20] In addition, LKM-3 are also found in 13% of patients with chronic hepatitis D (hepatitis B surface antigen positive) and, more recently, have been reported in patients with HCV infection.[2,4] In most patients with HCV/HDV and LKM-1/LKM-3, the viral component predominates and these patients have been designated as chronic viral hepatitis with an autoimmune feature.[2] Detection of LKM antibodies is performed by an IIF-assay using rat multiblock tissues, as substrate. The antibodies produce homogenous IIF-staining of liver tissues, and tissues of the proximal tubules of kidney (the stomach tissue may also be stained in the presence of LKM-3 antibodies as well).[4,20] Typing of LKM antibodies can be achieved by immunoblotting and by the counter current immunoelectrophoresis assay (CCIE). The titre of antibodies does not correlate with the disease activity, and elevation of LKM-1 antibodies can remains high following therapy and normalization of liver biochemistry.[21]

Liver cytosol antibodies. Liver cytosol type-1 antibodies are autoantibodies that bind to a 58-62KD cytosolic liver protein, identified as formiminotransferase cyclodeaminase (FTCD) by one group, and argininosuccinate lyase (ASL) by another.[22,23] These antibodies are found in 67% of patients with AIH-2 (occurring either alone [43%], or in combination with LKM-1 [24%]). Liver cytosole-1 antibodies were initially thought to be highly specific for AIH-2; however, a recent study showed that the antibodies can also be associated with adult, but not pediatric, patients with HCV.[24,25] Detection of LC-1 antibodies is performed by an IIF-assay using rat multiblock tissue as substrate. The antibodies produce a homogenous/granular IIF-staining of liver tissues, with sparing of the pre-venular areas.[24,25] In the presence of LKM-1 antibodies, other detection assays (IB, CCIA) can be used to identify the LC-1 antibodies.[26] The titres of LC-1 antibodies correlate with the disease activity and fall, or disappear, following immunosuppressive therapy, and normalization of liver biochemistry.[21]

Soluble liver antigen antibodies. Soluble liver antigen antibodies are associated with AIH-1.[27] These antibodies recognize a 35-50 KD soluble liver-pancreas antigen (LP) whose identity has remained controversial. The antigen was originally identified as the cytokeratin 8/18.[28] However, recent studies suggest that the antigen is either the glutathione-S-transferase isoenzyme, or the UGA-suppressor tRNA-associated protein.[29,30] Soluble liver antigen antibodies can occur alone, or in combination with ANA and SMA.[31] Soluble liver antibodies were thought to be more specific for AIH-1, as they could not be detected in patients

with AIH-2, PBS, PSC, chronic viral hepatitis, alcoholic liver diseases and non-hepatic autoimmune diseases.[31,32] However, recent study questioned the specificity of these antibodies for AIH-1 showing the presence of SLA antibodies in pediatric patients with PSC.[33] Indirect immunofluorescence-assay cannot detect SLA antibodies; therefore, they carried out radioimmuno assay (RIA), ELISA and radio ligand-assay in detecting these antibodies.

Anti-mitochondrial antibodies. Anti-mitochondrial antibodies are associated with PBC.[4,34] The antigen detected by these antibodies is the dihydrolipoamide acyltransferase (enzyme-2 [E2]) component of the pyruvate dehydrogenase complex (PDC). This enzyme is a member of the 2-oxoacid dehydrogenase family of multi-enzyme complexes, which include in addition to the PDC, the 2-oxoglutarate dehydrogenase complex (OGDC) and the branched chain 2-oxoacid dehydrogenase complex (BCOADC). All these enzymes share a common structure, with a multiple repeating E1 (2-oxoacid dehydrogenase) and E3 (dihydrolipoamide dehydrogenase subunit) built around an E2 (and in case of PDC only, E2 and E3 binding protein [E3BP]) core.[35] The AMA associated with PBC are directed against the E2 component of the PDC and occur in 95% of patients with PBC.[4,34] Antibodies to the E1a and E1b component of PDC are present in 40% and 10% of patients, while antibodies to the E2 of OGDC and BCOADC are present in 90% and 50% of patients with PBC. However, antibodies to the E1 components of OGDC and BCOADC enzymes are not detected.[36,37] Detection of AMA is performed by an IIF-assay using multiblock rat tissues, as substrate. Results produced by this assay are highly specific (100%) for the diagnosis of PBC, particularly at high titres. The antibodies produce strong granular IIF-staining of the stomach, the liver and the proximal and distal tubules of kidneys only. This IIF-staining pattern distinguishes AMA from LKM which stain the liver and the proximal tubules of kidneys. The M-2 AMA are found in 95% of patients with PBC, and their presence are taken as a marker of the disease; even in the absence of any clinical signs and symptoms.[3,34,35] Enzyme-linked immunosorbent assays have been developed for the detection of the M-2 AMA. However, unlike the IIF-assay, which is regarded as the gold standard test for the detection of AMA, ELISA-assays have both reduced sensitivities (93%) and specificities (96%) for the M-2 antibodies. Reduced sensitivities have been attributed to loss of conformational epitopes on the E2 antigen, in addition to patients lacking the E2 antibodies (but who otherwise have antibodies to the PDC E1 and E2 of OGDC and BCODAC).[35] The low sensitivities and specificities

associated with ELISA-assays would tend to argue against using such assays in screening for PBC.

Other mitochondrial antibodies. Antimitochondrial antibodies, other than the M2 antibodies, have been reported and classified as M1-M9. The majority of these antibodies are of the IgM isotype.[38,39] While many of these antibodies occur with a variety of clinical conditions; M4, M8 and M9 have been linked to PBC and have been reported to have some prognostic significance for the disease. Detection of these antibodies is carried out by ELISA, immunoblotting and complement fixation assays; although IIF-assay has been used in some studies.[38-40] The existence of these antibodies, and the identity of their respective antigens, is still controversial, as many of the previous data has not been reproduced.[40] This has led some authors to suggest that these antibodies should be set aside until previous data is reproduced and the antigens identified.[35]

Antinuclear antibodies. Antinuclear antibodies are autoantibodies directed against various components of the cell nucleus. These antibodies are associated with a variety of clinical conditions, including hepatobiliary conditions.[35] Antinuclear antibodies are detected by IIF-assay using either mouse liver tissues, or human cell line (Hep-2 cells), as substrates. Antinuclear antibodies produce various nuclear IIF-patterns including the homogenous-, speckled-, rim- and dot-patterns.[41,42] High titres ANA are seen in 70% of patients with AIH-1 (homogenous pattern 34-58%; speckled patterns 21-24%); occurring either alone (35%), or in association with SMA (50%).[5,43] The identity of ANA observed in AIH are unknown. Although typing for individual ANA is not carried out for AIH-1, high titres ANA should normally lead to typing for dsDNA and extractable nuclear antigen antibodies (ENA), in order to exclude the presence of connective tissue diseases. The low affinity dsDNA antibodies that are associated with AIH-1, are not specific for the disease, as they can be associated with a variety of other clinical conditions.[41] However, their presence can be used, as an additional marker, to distinguish AIH-1 from other forms of AIHBD. High titres ANA are normally associated with AIH (titres 1:80 in adults and 1:40 titres in children are taken as significant), whereas low titres can occur with viral (EBV, HCV) hepatitis, as well as in normal elderly people.[5,19] Strong ANA are also detected (as perinuclear membrane- and nuclear dot-staining patterns) in 30-70% of patients with PBC.[3,43] The nuclear rim-pattern is due to autoantibodies binding to the nuclear pore membrane glycoproteins (gp) (including the gp210 and P62) and the integral protein of the inner nuclear membrane (Lamin B receptor).[41,44] Glycoproteins 210 and P62 antibodies detected by

ELISA assays, are found in 25% and 25-47% of patients with PBC. These antibodies have a 100% specificity for the disease.[45,46] Multiple nuclear dot-patterns are due to autoantibodies binding to sp100, gp20 and Colin p80.[44] Sp100 and coilin p80 antibodies are found in patients with PBC and connective tissue diseases, but are not detected in other forms of hepatitis.[35,44] Typing for the individual glycoproteins (by ELISA- and IB assays) is not routinely carried out by most laboratories. Since the titres and patterns of ANA are of significant relevance to the diagnosis of AIHBD, both of these parameters should be reported.

Antineutrophil cytoplasmic antibodies (ANCA). Antineutrophil cytoplasmic antibodies are auto-antibodies directed against various component of the neutrophil cytoplasm. These antibodies are detected by an IIF-assay using ethanol fixed human neutrophils, as substrates. Two types of IIF-patterns are detected; a cytoplasmic pattern (C-ANCA) and a perinuclear-pattern (P-ANCA). Cytoplasmic pattern is produced by antibodies binding to neutrophil cytoplasmic proteinase 3, while P-ANCA is due to antibodies binding to a number of cytoplasmic cationic antigens (which localize to the nuclear membrane following ethanol fixation) and include myeloperoxidase (MPO), cathepsin G, lactoferrin, elastase, azurocidin, bacteriocidal-permeability increasing protein. Antineutrophil cytoplasmic antibodies are associated with a variety of clinical conditions including AIHBD.[43,44] Thus, P-ANCA are detected in 40-96% of patients with AIH-1 and in 60-90% with PSC, but not in patients with AIH-2.[46,47] Low titres have been detected in patients with alcoholic and other chronic liver diseases.[47,48] In one study, C-ANCA (Protienase-3) were found in high titres in 56% of patients with HCV infection.[49] However, the latter results need confirmation. Due to the low specificity of these antibodies for AIHBD, testing for ANCA is not normally recommended. However, detection of P-ANCA can be used, as an additional marker, to differentiate between AIH-1 and AIH-2.

Other serological tests used in the diagnosis of AIHBD. In addition to serum autoantibodies, testing for serum immunoglobulins (IgG/IgA/IgM) is also performed when investigating hepatibiliary diseases. High levels IgG are normally associated with AIH-1, however, elevation of IgA alone, or in combination with IgG, can also occur.[7] In AIH-2, elevation of IgG and decreased IgA is normally observed.[50] In contrast, high levels IgM are associated with PBC, while normal IgM is associated with PSC.[51] In the variant syndromes, IgG alone, or in combination with IgM, may be elevated. Finally, since the individual AIHBD is associated with a variety of autoimmune diseases (**Table 1**), testing for other autoantibodies may be required, and the test pattern would be determined by the clinical picture.

Discussion. Autoimmune hepatobiliary diseases include autoimmune AIH-1/2, PBC, AC, PSC and the overlap syndromes. Prompt diagnosis and treatment of AIHBD is of paramount importance, as without treatment, these diseases are associated with high morbidity and mortality. Diagnoses of AIHBD rely heavily on the use of serological testing for serum autoantibodies (ANA, SMA, LKM-1, SLA, LC-1, SLA, AMA and P-ANCA) and serum immunoglobulins, which can be used, in conjunction with the hepatitic/cholestatic and histological pictures, to arrive at the correct diagnosis of a particular disease (**Table 1**). The sensitivities and specificities of these autoantibodies are shown in **Table 2**. Autoimmune hepatitis commonly present as a chronic liver disease and is associated with high morbidity and mortality in untreated patients. Immunosuppressive (mainly corticosteroid) therapy is very effective in halting and reversing the disease in the majority of patients when instituted early on the course of the disease. Diagnosis of AIH is based on the finding of characteristic autoantibodies (ANA, SMA, SLA-1, LKM-1, SLA, P-ANCA) and high serum Igs, with exclusion of other etiologies (viral, drug and metabolic [Wilson's disease, hemochromatosis, 1-antitrypsin deficiency]) of chronic hepatitis. However, since AIH can co-exist with HCV infection, as well as can be induced by certain drugs (minocycline, sulfasalazine, isoniazid), presence of these autoantibodies (particularly at high titres), along with high serum Igs, should be taken as strong indicator of AIH, even in the presence of other etiologies. Autoimmune hepatitis can be further subdivided into type-1 (ANA, SMA, SLA-1, P-ANCA, dsDNA) and type-2 (LKM-1, LC-1), however, this division does not appear to have any significant implication on the treatment and prognosis of either disease.[4,6] Primary biliary cirrhosis is associated with strong AMA (occurring in 95% of patients), ANA and elevated serum IgM. Presence of AMA, particularly in high titres, is highly indicative of PBC, even in the absence of clinical signs and symptoms of the disease.[3,35] Asymptomatic patients with strong AMA need to be monitored, with annual liver functional tests (LFTs), for development of the disease. Appearance of abnormal LFTs should lead to consideration of liver biopsy for confirmation and staging of the disease. Treatment with ursodeoxycholic acid (UDA) may be beneficial when started early.[4] In AMA negative patients, presence of nuclear rim-, or dot-patterns, in combination with elevated serum IgM, would strongly suggest a diagnosis of autoimmune

Table 1 - Autoimmune hepatobiliary diseases and their associated autoantibodies.

Disease	Associated autoantibodies	Percentage	Serum Ig	Liver chemistry	Histology	Associated autoimmune diseases
AIH-1	ANA SMA ANA and SMA SLA P-ANCA	15 35 50 30 40-96	IgG/ IgA IgG+IgA	AST+ALT	Interface hepatitis	Thyroid disease, Pernicious anemia, Vitiligo Type-1 diabetes mellitus, alopecia Rheumatoid arthritis, celiac disease Cryptogenic fibrosing alveolitis Glomerulonephritis Autoimmune hemolytic anemia
AIH-2	LKM-1 LKM-LC-1 LC-1	80 45 10	IgG/ IgA	AST+ALT	Interface hepatitis	
PBC	M2-AMA ANA (Rim/Dots)	95 10-44	IgM	ALP+ GT	Cholangitis	Sjögren's syndrome (75%) Thyroid disease (15%) Cryptogenic fibrosing alveolitis (55%) CREST (5%), Scleroderma (15%) Renal tubular acidosis (50%) Arthritis (70%), Reynaud's syndrome (10%)
AC*	ANA (Rim/Dots)		IgM	APL+ GT	Cholangitis	
PSC	P-ANCA		normal IgM	APL+ GT	Cholangtis	Inflammatory bowel disease

*preferred term; AMA negative PBC. AIH - autoimmune hepatitis, PBC - primary biliary cirrhosis, PSC - primary sclerosing cholangitis, AC - autoimmune cholangitis, ANA - antinuclear antibodies, SMA - smooth muscle antibodies, SLA - soluble liver antigen, P-ANCA - peri-nuclear cytoplasmic antibodies, LKM-1 - liver kidney microsomal antibodies-1, LC-1 - liver cytosole-1, AMA - antimitochondrial antibodies, Ig - immunoglobulin, AST - aspartate aminotransferase, ALT - alanine aminotransferase, ALP - alkaline phosphatase, GT - -glutamyl transpeptidase, RF - rheumatoid factor, ATA - antithyroid antibodies, CREST - calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia

Table 2 - Sensitivities and specificities of the various serological tests used in the diagnosis of autoimmune hepatobiliary diseases.

Tests	AIH-1	AIH-2	PBC
SMA			
Sensitivity	80		
Specificity	90		
SLA			
Sensitivity	30		
Specificity	100		
LKM-1			
Sensitivity		80	
Specificity		High	
LC-1			
Sensitivity		67	
Specificity		100	
AMA			
Sensitivity			95
Specificity			100

Tests performed by an indirect immunofluorescence and enzyme-linked immunosorbent assays. AIH-1/2 - autoimmune hepatitis 1/2, PBC - primary biliary cirrhosis, SMA - smooth muscle antibodies, SLA - soluble liver antigen, LKM-1 - liver kidney microsomal antibodies-1, LC-1 - liver cytosole-1, AMA - antimitochondrial antibodies

cholangitis, a variant form of PBC.[43,44,51] Presence of a combination of the above autoantibodies would suggest presence of the overlap syndromes (consisting of AIH and either PBC, AC, or PSC). Accurate diagnosis of these syndromes is important, since they can respond to a combination of immuno-suppressive and UDA therapies.[35] Finally, presence of some of the above autoantibodies (namely LKM-1, LKM-3, ANA) can occur in patients with chronic HCV infection, either as an overlap syndrome (AIH-HCV), or as a true HCV infection with an autoimmune phenomena.[4,52] Establishing the correct diagnosis of these patients is important to allow institution of correct therapies. In patients with positive HCV, monitoring patients during interferon therapy is important for the early detection of development of AIH, and allow prompt institution of immunosuppressive therapy.

In conclusion, detection of autoantibodies is of paramount importance for the diagnosis of AIHBD. Understanding the assays used for the detection of these autoantibodies and the significance of the results produced, is important for the effective diagnosis, or exclusion, of these diseases, thus allowing rapid institution of appropriate treatments.

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