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TOPIC HIGHLIGHT

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Autoimmune liver serology: Current diagnostic and clinical challenges

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Abstract

Liver-related autoantibodies are crucial for the correct diagnosis and classification of autoimmune liver diseases (AiLD), namely autoimmune hepatitis types 1 and 2 (AIH-1 and 2), primary biliary cirrhosis (PBC), and the sclerosing cholangitis variants in adults and children. AIH-1 is specified by anti-nuclear antibody (ANA) and smooth muscle antibody (SMA). AIH-2 is specified by antibody to liver kidney microsomal antigen type-1 (anti-LKM1) and anti-liver cytosol type 1 (anti-LC1). SMA, ANA and anti-LKM antibodies can be present in de-novo AIH following liver transplantation. PBC is specified by antimitochondrial antibodies (AMA) reacting with enzymes of the 2-oxo-acid dehydrogenase complexes (chiefly pyruvate dehydrogenase complex E2 subunit) and disease-specific ANA mainly reacting with nuclear pore gp210 and nuclear body sp100. Sclerosing cholangitis presents as at least two variants, first the classical primary sclerosing cholangitis (PSC) mostly affecting adult men wherein the only (and non-specific) reactivity is an atypical perinuclear antineutrophil cytoplasmic antibody (p-ANCA), also termed perinuclear anti-neutrophil nuclear antibodies (p-ANNA) and second the childhood disease called autoimmune sclerosing cholangitis (ASC) with serological features resembling those of type 1 AIH. Liver diagnostic serology is a fast-expanding area of investigation as new purified and recombinant autoantigens, and automated technologies such as ELISAs and bead assays, become available to complement (or even compete with) traditional immunofluorescence procedures. We survey for the first time global trends in quality assurance impacting as it does on (1) manufacturers/purveyors of kits and reagents, (2) diagnostic service laboratories that fulfill clinicians' requirements, and (3) the end-user, the physician providing patient care, who must properly interpret test results in the overall clinical context.

Key words: Autoantigen; Autoimmune hepatitis; Autoantibody; Primary biliary cirrhosis; Primary sclerosing cholangitis; Liver disease

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INTRODUCTION

The presence of autoantibodies plays a central role in the diagnosis and classification of autoimmune liver diseases (AiLD)[1,2], but their nature and significance remain challenging in regard to pathogenesis. Such antibodies discriminate between distinct subtypes of the AiLD and facilitate diagnosis of the overlap syndromes[3]. AiLD represent a broad range of disorders that can affect one or the other of the two cellular components, namely hepatocytes in autoimmune hepatitis (AIH), and cholangiocytes in primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and the autoimmune hepatitis/sclerosing cholangitis overlap syndrome of childhood, designated as autoimmune sclerosing cholangitis (ASC)[4], and discussed elsewhere in this issue.

Antibody to nuclei (ANA) and/or to smooth muscle (SMA) characterizes type 1 AIH (AIH-1) and antibody to a liver kidney microsomal constituent (anti-LKM) defines patients with type 2 AIH (AIH-2)[5]. Usually the two patterns of serology are mutually exclusive, but in the rare cases in which they coexist, the disease features resemble those of AIH-2[6]. ASC is a third form of AiLD

which is similar clinically, histologically and serologically to AIH-1, but is associated with radiological changes of sclerosing cholangitis[7]. SMA, ANA and to a lesser extent anti-LKM can be found in post-transplantation *de novo* AIH[8]. The presence of anti-mitochondrial antibodies (AMA) with a specificity for the E2 subunit of the pyruvate complex (PDC-E2), and certain PBC-specific ANA, characterise PBC[1,9]. Perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA) is the most frequent antibody reactivity in primary sclerosing cholangitis (PSC)[1,3], but *per se* has low specificity for diagnosis.

HISTORICAL NOTES ON AUTOIMMUNE LIVER SEROLOGY

The evolution of knowledge on AIH is discussed in another article in this issue. Here we provide a brief historical survey of the serological tests currently used by diagnostic laboratories.

Anti-nuclear antibody (ANA)

Serum antibodies with specificity for cell nuclear antigens were first described by Miescher *et al* in 1954[10] following the discovery of the lupus erythematosus (LE) cell by Hargraves and colleagues[11] and the recognition that the LE cell phenomenon was related to a serum factor reacting with nuclear antigens, subsequently termed "antinuclear factor" (ANF), and later antinuclear antibody (ANA). Deoxyribonucleic acid (DNA) and deoxyribonucleoprotein (DNAP) were identified in 1957 as "ANF" target antigens[11,12] and it was further shown that antibodies responsible for the LE-cell phenomenon reacted with DNA and gave a "homogenous" pattern of nuclear staining by immunofluorescence[13]. In 1956 a positive test for LE cells in blood was reported in young women with a chronic liver disease then called chronic active hepatitis (CAH), leading to the designation of "lupoid hepatitis", an early label for what is now known as AIH-1[14,15]. Testing for ANF/ANA by immunofluorescence (IFL) supplanted the cumbersome LE cell test in the early 1960s.

Smooth-muscle autoantibody (SMA)

Antibodies binding to smooth muscle of rat stomach were initially detected in serum samples of patients with liver diseases by Johnson *et al*, in 1965[16]. The presence of SMA in patients with AiLD was confirmed by Whittingham *et al*[17]. Patients with non-AiLDs were reported as seronegative for SMA and, notably, also negative were patients with SLE. The antibody was often found in association with ANA, which was already a known marker of AIH, and tended to fade with steroid induced remission. Bottazzo *et al*[18] reported that the SMA staining arterial vessels (V), glomerular mesangium (G) and fibers surrounding the kidney tubules (T), responsible for the VGT pattern, was confined to an aggressive form of hepatitis now known to be AIH-1. The antigenic moiety mainly but not exclusively responsible for SMA activity in what in the 1970s was called CAH was identified as filamentous (F) actin[19-21].

Liver kidney microsomal antibody (anti-LKM)

Cytoplasmic antibodies in "CAH" were described in the laboratory of Deborah Doniach[22,23] whose group first used the expression anti-liver kidney microsomal (anti-LKM) antibodies[24]. "Microsomal" is something of a misnomer as "microsomes" are the *in vitro* equivalent of particles of the endoplasmic reticulum wherein the antigen is located. Other nosological entities in which anti-microsomal antibodies were evident included drug induced hepatitis, leading to the use of LKM1, LKM2, LKM3 to designate the different immunofluorescent patterns, which reflect the different targeted autoantigens[25]. The ability of anti-LKM1 antibodies to define a second serological type of AIH, i.e. AIH type 2, was proposed by Homberg *et al*[26]. Three groups independently identified cytochrome P450 IID6 (CYP2D6) as the molecular target of anti-LKM1 antibodies[27-29]; the group of Alvarez[27] was the first to publish its data in the form of a full-length paper.

As mentioned, other LKM antibody patterns were subsequently described. LKM2 antibodies were recognised in patients with hepatitis induced by tienilic acid[24], a uricosuric diuretic withdrawn from clinical use in 1980 and Rizzetto's group described LKM3 antibodies in a proportion of cases of chronic hepatitis D infected patients[30]. In contrast to anti-LKM1 and LKM2 antibodies, anti-LKM3 stained human exocrine pancreas and thyroid. Anti-LKM2 reacted with CYP2C9 and anti-LKM3 with uridine diphosphate glucuronosyl transferases (UGT)[25]. A fourth type of LKM antibodies recognising CYP1A2 and CYP2A6 has been described in patients with AIH associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)[31]. The IFL pattern of the antibody is indistinguishable from that of anti-LKM1. An anti-liver microsomal antibody (anti-LM) staining the centrolobular hepatocytes but not the kidney and which recognises CYP1A2 has been described in dihydralazine-induced hepatitis and in a few cases of AIH[32-34].

Liver cytosol antibody (anti-LC1)

Anti-LC1 were originally described in association with anti-LKM1, or in isolation, by Martini *et al* in patients with AIH-2[35]. Lenzi *et al* have also found anti-LC1 antibodies in 14% anti-LKM-1 antibody positive patients suffering from chronic hepatitis C virus infection[36]. The enzyme formiminotransferase cyclodeaminase (FTCD) has been identified as the molecular target of anti-LC1 antibodies[37,38].

Mitochondrial antibody (AMA)

The first indication that PBC could be an autoimmune disease was obtained in 1958 when the serum of a woman with PBC was found to contain high titres of complement-fixing antibodies directed to tissue homogenates[39], that later, by absorption studies, were shown to be absorbed by a rat liver mitochondrial fraction[40]. A breakthrough for the clinical hepatologist was the observation in 1965 by Walker, Doniach,

Roitt and Sherlock that human tissue sections rich in mitochondria give a characteristic immunofluorescence pattern when they are incubated with sera from patients with PBC but not with controls which, in that study, included patients with extra-hepatic bile duct obstruction, drug induced cholestasis and viral hepatitis[41]. In 1967, Berg *et al*[42] demonstrated that PBC sera reacted *in vitro* with a trypsin-sensitive mitochondrial antigen that was named M2 antigen, in contrast to M1, the target of anti-cardiolipin antibody. Subsequently Berg developed a nomenclature based on the types of anti-mitochondrial reactivity that spanned M3-M9, but this is no longer used. The M2 antigen was located at the inner surface of the inner mitochondrial membrane of all mitochondria tested[42-45]. The target antigens of M2 were identified in the 1980s as components of the 2-oxo-acid dehydrogenase complexes, the predominant target being the E2 subunit of pyruvate dehydrogenase complex, as judged by molecular cloning[46,47]. PBC-specific AMA were later shown to recognise other enzymes of the 2-OADC, including the E2 subunits of branched chain oxoacid dehydrogenase complex (BCOADC), the oxoglutarate dehydrogenase complex (OGDC) and the PDC-E3 binding protein[1,48].

Antibodies against soluble liver antigen/liver-pancreas antigen

Two autoantibodies, anti-soluble liver antigen (SLA) and anti-liver-pancreas (LP), both described in AIH by two independent German groups, have been shown to target the same antigen, hence the current name of anti-SLA/LP antibodies[49-51]. The LP antigen has first been reported by Berg's group in the supernatant of liver and pancreas homogenates[50]. The SLA antigen was described by Manns and colleagues in 1987 as a component of the supernatant of liver and kidney homogenates[49]. Anti-SLA antibodies detected by a competitive ELISA were then proposed as markers of a third type of severe AIH seronegative for the conventional AIH-1 autoantibodies[49].

Anti-asialoglycoprotein receptor antibodies

Attempts to identify antigens specifically expressed on the hepatocyte surface which could serve as self targets in AiLD have led to the description of a crude liver extract preparation known as the liver specific protein (LSP) and its major component, the asialoglycoprotein receptor (ASGPR)[52,53]. ASGPR, also designated as hepatic lectin, is a type II transmembrane glycoprotein. It is the only known liver-specific autoantigen, and is constitutively expressed on the hepatocellular membrane.

RECOMMENDATIONS FOR AUTOANTIBODY DETECTION BY IMMUNOFLUORESCENCE (IFL)

IFL is the main technique for the screening of autoantibodies diagnostically relevant to liver disease. The methodology is practically unchanged from that introduced by Weller and Coons in 1954[54]. It uses unfixed, air-dried, tissue sections which are incubated with a test serum potentially containing an antibody. After removing unbound serum by washing, a fluorochrome labelled second antibody, raised in animal and specific for human immunoglobulins, is applied to detect the first tissue-bound antibody[55]. Specific patterns can then be recognised using an ultraviolet microscope. A consensus statement in 2004 from the Committee for Autoimmune Serology of the International Autoimmune Hepatitis Group (IAIHG) provided guidelines on how to test for autoantibodies relevant to AIH and concluded that indirect IFL on fresh sections of multi-organ (liver, kidney, stomach) from rodents (usually rat) should be the first line screening[55]. The recommendations of the Committee include detailed guidelines for the preparation of substrate, application of the test serum samples, optimal dilution of samples and fluorochrome-labelled revealing agents, selection of controls and identification of diagnostically relevant staining patterns[55]. The use of the three tissues enables the simultaneous detection of virtually all the autoantibodies relevant to liver disease, namely SMA, ANA, anti-LKM1, AMA and anti-LC1[55]. The first serum dilution recommended for autoantibody detection (before titration) is for adults 1:40, and for children 1:20 for ANA and SMA and 1:10 for anti-LKM1 in children[55].

Autoantibodies detected by IFL and their reactants

ANA: This autoantibody is readily detectable as nuclear staining in all the three tissues of the composite substrate. On the liver it is also possible to identify different patterns, the homogenous being typical of AIH-1[55]. A clearer definition of the different ANA patterns seen in PBC is best achieved by the use of the human larynx epithelioma cancer cell line (HEp-2) because these cells have large nuclei, and the mitotic phase of these cells permits the easy detection of anti-centromere antibodies (ACA) because they stain the chromosomes of cells in mitosis[56,57]. HEp-2 permit ready detection of the IFL patterns called multiple nuclear dot (MND) and rim-like membranous (RLM) typical of PBC[58,59]. Anti-MND stains 5-20 dots of variable size, distributed all over the nucleus but sparing the nucleoli[58]. The pattern can be confused with that of ACA but anti-MND do not stain the chromosomes of cells in mitosis whereas ACA do so[58]. Moreover, the dots of ACA are all of the same size while those of MND vary in size and number between individual cells[58]. In addition to homogenous ANA, speckled and nucleolar patterns are seen in AIH, and to a lesser extent in PBC, but are not disease-specific.

SMA: SMA of the VGT pattern is considered specific for AIH-1, though some 20%-40% of patients with AIH-1 do not have it[55]. SMA can also be detected, always by IFL, using fibroblasts or HEp-2 cells. The VGT pattern corresponds to the microfilament staining of isolated fibroblasts and represents a cable pattern across the cell[18]. Both patterns have been termed "anti-

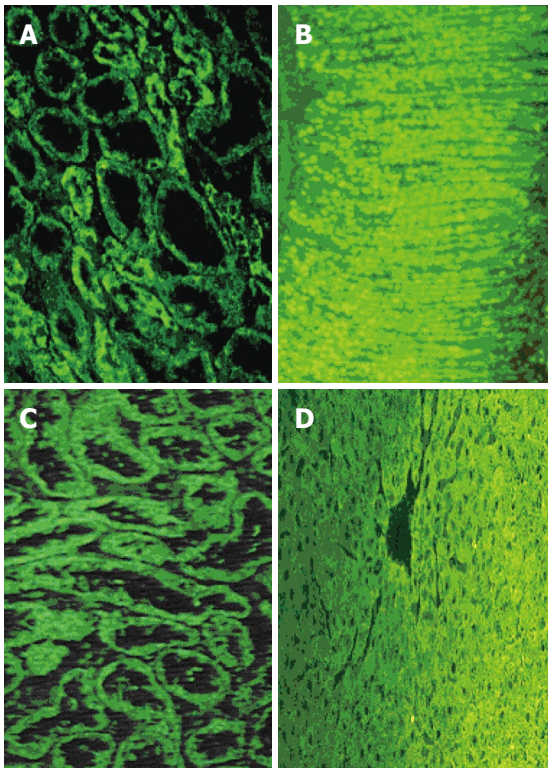

Figure 1 Immunofluorescence of anti-mitochondrial (**A** and **B**), and anti-liver kidney microsomal antibody (anti-LKM1) (**C** and **D**). AMA stain (**A**) stronger the smaller, distal tubules while anti-LKM1 the proximal tubules of the rat kidney (**C**). These specificities are frequently misdiagnosed, especially when only the kidney substrate is used and the sections do not contain both proximal and distal tubules. Thus, the use of rat stomach (**B**) and liver (**D**) is strongly recommended to prevent misinterpretation; AMA characteristically stain the gastric parietal cells while anti-LKM1 stain the rat liver but not the stomach.

actin" though there is no molecular proof as yet that actin is indeed the only or indeed the main target of VGT SMA.

Anti-LKM1: Anti-LKM1 brightly stains the third portion of the proximal renal tubules and the cytoplasm of the hepatocytes but it spares cells of the gastric mucosa[55]. Anti-LKM1 is a frequently undiagnosed autoantibody, being commonly misinterpreted as AMA[1,60]. AMA is extremely rare in pediatric patients and PBC is extremely rare in childhood[61,62]. So, when AMA is reported in a child with clinical and histological characteristics of AIH, the serological report is almost certainly incorrect.

AMA: The confusion between AMA and anti-LKM1 occurs because both autoantibodies stain the renal tubules, though with a pattern different to a trained eye and readily appreciated when the kidney tissue section contains both distal and proximal tubules (Figure 1). AMA stains strongly the mitochondria-rich distal tubules which are smaller than the proximal tubules stained by anti-LKM1 antibodies. AMA also stains the gastric parietal cells within the stomach, which are spared by anti LKM1, whereas AMA stains hepatocytes much less brightly than does anti-LKM1. The analysis therefore of the three-tissue substrate should allow a correct serological interpretation. Some serodiagnosticians claim a utility of HEp-2 cells for recognition of AMA which gives a "string of pearls" pattern of cytoplasmic staining. Unfortunately interpretative problems are still frequent especially in those laboratories where only kidney is used as substrate, and particularly when the tissue is poorly oriented. Advice on how to orient and cut the kidney has been issued by the Autoimmune Serology Committee of IAIHG[55].

Anti-LC1: This antibody stains the cytoplasm of hepatocytes with a zonal distribution within the liver, being particularly abundant on perivenous hepatocytes and the renal tubules. In most cases, however, anti-LC1 is obscured by the simultaneous presence of anti-LKM1[35,36]. Anti-LC1 can be also detected by gel diffusion techniques such as double dimension immunodiffusion and counter immunoelectrophoresis, techniques in which the cytosol of liver homogenate is used as antigen and the test serum is run with a positive control[63].

ANCA: ANCA is detected by indirect IFL using neutrophils as substrate and can give a cytoplasmic (c-ANCA) or perinuclear (p-ANCA) pattern[64,65]. The pattern of p-ANCA is an artifact caused by the ethanol fixation of the neutrophils which leads to the migration of some positively charged cytoplasmic antigens to the negatively charged nuclear envelope, so giving the characteristic perinuclear fluorescence staining. An atypical p-ANCA staining, unaffected by ethanol fixation, gives a perinuclear staining subtly different from the classical p-ANCA. It recognizes components of the nuclear envelope and has been described, especially in patients with PSC[66]. In view of the location of the antigen, some groups are now describing these antibodies as perinuclear anti-neutrophil nuclear antibodies (p-ANNA)[67,68].

AUTOANTIGENS OF LIVER-RELATED AUTOANTIBODIES

Nuclear antigens

No single AIH-1-specific nuclear antigen has been identified so far. A number of nuclear molecular targets has been detected, including centromere, histones, double-stranded DNA, chromatin, and ribonucleoprotein complexes with no single pattern or combination thereof being characteristic of AIH[3], although most typical is a homogenous pattern attributable to anti-chromatin.

Smooth muscle antigens

SMA giving the "anti-actin" IFL pattern has long been considered highly diagnostic for AIH type 1, its target deemed to be F-actin (noting that purified actin is a monomer G-actin, which is polymerized in the presence of ATP)[3,20,55,69]. The advent of commercial kits using highly purified F-actin as target has provided the opportunity both to test the molecular specificity of the SMA giving the IFL actin pattern and to assess the diagnostic performance of antibodies directed to molecularly pure F-actin (anti-FA)[70-75]. In Granito and

Villalta's studies, the IFL anti-actin pattern was strongly associated with AIH-1 and so was anti-FA, this latter being marginally more sensitive[70,74,75]. When disease specificity of the two reactivities was analysed the IFL pattern was found to be highly specific, being absent or extremely rare in diseases other than AIH-1. In sharp contrast, anti-FA was detectable in patients with viral hepatitis, PBC, primary sclerosing cholangitis, AIH-2 and celiac disease[70,74,75]. In a paper by Frenzler, positivity for anti-FA was found in some 75% of patients subsequently diagnosed as having AIH-1 but also in 24% non-AIH patients[71]. In an attempt to address the relatively high non-specificity of the molecular assay, Villalta *et al* performed a receiver operating curve (ROC) analysis, from which they deduced for this assay a cut off point giving a specificity similar to IFL: the cut off point had to be increased from the 30 arbitrary units (AU) suggested by the manufacturer to 53 AU[75]. At this cut-off point the specificity of the molecular assay was indeed comparable to that of IFL, but the sensitivity dropped by more than 10% below that of IFL.

The results obtained with the IFL and molecular assays overlap considerably, but by no means completely, with several instances of positivity with one test and not with the other[72,73,75]. With the availability of highly purified F-actin the question as to whether the antibody responsible for the anti-actin IFL pattern is directed against actin could be tested directly[70-75]. Three anti-SMA positive sera containing both reactivities were absorbed with solid phase F-actin: the reactivity against F-actin was abolished (absorbed out) but that giving the fluorescent pattern was unaltered in two of the 3 sera and reduced, but not abolished, in the third[72]. In summary, detection of the IFL anti-actin pattern continues to provide to date the best specificity/sensitivity compromise[55]. The antibody responsible for the IFL "actin" pattern targets, in addition to actin, molecules other than actin[3,72]. The question arises as to whether to maintain the tradition, and with it the term of "anti-actin" for the antibody recognised in IFL, or whether to call it anti-micro filament (MF) pattern as suggested by the Serology committee of the IAIHG[55].

LKM1 antigen

While the target antigens of ANA and SMA certainly need better molecular definition, that of anti-LKM1 in AIH-2 has been clearly identified as the microsomal enzyme cytochrome P450IID6 (CYP2D6)[5,26-28]. Its identification has enabled the establishment of assays based on the use of recombinant antigens which have proven useful in solving diagnostic uncertainties between AMA and anti-LKM1[1,60,76]. Such ELISAs, however, are not always able to detect anti-LKM1 antibodies in patients with chronic hepatitis C virus infection whereas IFL and radioligand assays can do so possibly because of their ability to identify conformational epitopes undetectable by ELISA[77-80]. Short CYP2D6 peptides used as antigenic preparations perform less well than those using full-length CYP2D6 and their diagnostic use is limited.

LC1 antigen

ELISAs for detection of antibodies to FTCD, the target of anti-LC1, have been developed and used in diagnostic laboratories and their diagnostic and clinical relevance is under investigation[37,38].

SLA/LP and ASGPR

Progress has been made in the definition of other autoantibodies frequently present in AIH but undetectable by IFL including antibodies against SLA/LP[51,81-85] and ASGPR. Most of anti-SLA/LP positive patients are also positive for ANA, SMA or anti-LKM1, but occasionally anti-SLA is present in isolation and, in this case, its detection is of diagnostic importance[81,86]. The identification of the molecular target of anti-SLA/LP antibodies as the UGA serine tRNA-associated protein has led to the development of ELISA or dot-blot assays increasingly replacing the conventional inhibition ELISA originally used for anti-SLA antibody detection[51,83]. Recent studies investigating the exact role of this protein have shown that SLA/LP is a selenocysteine synthase but how the biosynthesis of selenocysteine may relate to the pathogenesis of AIH is not known[87].

Anti-ASGPR antibody detection requires either purified or recombinant antigen. The lack of disease-specificity and the difficulty in developing a reliable molecular based assay for the detection of anti-ASGPR has limited its wider applicability in diagnostic practice.

Mitochondrial antigens

The most recent advance in the immunodiagnosis of AMA is the availability of an ELISA using the triple MIT3 hybrid antigen preparation, developed in the Gershwin laboratory. This preparation contains all three immunodominant mitochondrial antigenic epitopes, namely PDC-E2, BCOADC-E2 and OGDC-E2[88]. Although assays based on MIT3 are reported to give positive results for PBC sera that test negative for AMA by conventional IFL techniques[89,90], IFL testing for AMA should remain the screening procedure.

PBC-specific nuclear antigens

As mentioned above, major target antigens of PBC-specific ANA have been identified. These include the nuclear body speckled 100 kDa (sp100), promyelocytic leukaemia (PML), and small ubiquitin-like modifier (SUMO) proteins corresponding to the MND pattern, and proteins within the nuclear pore complex (anti-NPC) including the 210 kDa glycoprotein (gp210) and the 62 kDa nucleoporin (NUP62), the major target antigens of anti-RLM antibodies and responsible for the RLM pattern[58,59,91]. New immunoassays testing autoantibodies to sp100, PML, gp210 and NUP62 have been developed using short peptides, polypeptides or full-length proteins as targets, but they have not been fully evaluated nor standardized[91-98]. They may be of diagnostic assistance, especially in those cases where it is difficult to interpret the IFL staining patterns due to concurrent autoantibody reactivities or in true AMA-negative PBC cases[1,92,99,100]. We note also the presence

of ACA reactivity in the combined PBC/CREST disease. Assays to detect multiple reactivities (multiplex) and to provide a full autoimmune serological profile of relevance to PBC are being developed[89]. At present, a lack of guidelines for the detection of PBC-specific autoantibodies by scientific bodies responsible for the standardization of autoimmune serological tests is a significant handicap and perpetuates uncertainties on which are the clinically relevant tests (see below).

Atypical p-ANCA (pANNA) antigens

These are under current investigation[66-68]. The original description of a 50 kDa neutrophil-specific nuclear protein of the nuclear pore complex as the target antigen recognised by 90% of atypical p-ANCA from patients with PSC was followed by a study from the same group suggesting that the identity of the antigen is tubulin beta chain 5 (TBB5)[101]. However, when using the molecular target for their detection anti-TTB5 antibodies were found not only in PSC but also in other AiLDs.

DIAGNOSTIC RELEVANCE OF LIVER-RELATED AUTOANTIBODIES

ANA, SMA, anti-LKM1, AMA and p-ANCA should be determined in all patients with biochemical, clinical and/or histological features suggestive of AiLD[3,5]. Autoantibody titres usually vary during the course of the disease. Hence seronegativity or low autoantibody titres on a single test cannot exclude the diagnosis of AiLD and repeat tests may allow autoantibody detection and correct disease classification. Conversely, the presence of autoantibodies even at high titres in the absence of any other clinical and laboratory features suggestive of AiLD is insufficient to make a diagnosis though a patient with high titre autoantibodies needs to be seen at regular intervals. Titres of ANA, SMA and LKM1 antibodies contribute in calculating the IAIHG diagnostic score for patients with a probable or definite diagnosis of AIH[5]. IFL titres of > 1:80 attract a +3 score; 1:80 a +2 score and 1:40 +1 score. A negative score of -4 is given to cases with hepatitic features but detectable AMA at a titre of $\geqslant$ 1:40; such mixed serology points to "overlap syndrome", discussed in another article in this issue. In children, titres of 1:20 for ANA or SMA and 1:10 for anti-LKM1 are sufficient to support the diagnosis of AIH if accompanied by other suggestive features[5,55].

In AIH-1, ANA alone are present in 15% of patients, SMA alone in 35%, and ANA and SMA co-occur in 60%[3]. In the 5% or so of cases negative for these reactivities, anti-SLA/LP may be positive. In AIH-2 at presentation anti-LKM1 and/or anti-LC1 antibodies are positive in more than 90% of patients[25,35,36,63]. In PBC, AMA are detectable in more than 95% of patients and disease-specific ANA occur in 30%-70% of PBC patients according to different reports[9,58,59,100]. In PSC, atypical p-ANCA are present in up to 90% of patients but this reactivity also occurs in AIH (up to 70%) and PBC (5%), as well as frequently in patients with inflammatory bowel disease[66-68,102,103]. In what is termed "*de novo*" AIH and in post-liver transplant patients, ANA, SMA, AMA and anti-LKM have been reported, at varying frequencies[85,94]. A diagnosis of AIH-2 is strongly supported by seropositivity for anti-LKM1 and/or anti-LC1, particularly in the absence of viral hepatitis C[5]. For PBC, the presence of AMA is one of the three widely accepted diagnostic criteria[9].

Autoantibody positivity is part of the criteria used for the diagnosis of AiLD, though it is not diagnostic on its own. Elevated titres and certain patterns carry significant diagnostic connotations.

We are aware of various reports that, at first sight, might appear prejudicial to the diagnostic utility of liver-related autoantibodies[104]. Thus ANA and/or SMA are reported in PBC, PSC, *de novo* AIH, chronic viral hepatitides B, C and D, acute liver failure, drug-induced hepatitis, non-alcoholic steatohepatitis, alcohol-induced liver disease, hepatocellular carcinoma, and also in a variety of non-liver related diseases. Hence, the diagnostic significance of antibody positivity depends on the associated clinical features[3], as well as the level of reactivity. Anti-LKM1 and anti-LC1 are reported in a proportion of adult (0%-6%) or pediatric (0%-11%) cases with chronic hepatitis C infection[36,105-107]. AMA are present (expectedly) in patients with AIH/PBC overlap syndrome, and also in chronic hepatitis C virus infected patients[1], and most recently were described in patients with acute liver failure[108]; AMA occur also in various rheumatological disorders which may co-exist with PBC notably Sjögren's syndrome and systemic sclerosis[1,48,108-111] and are described in non-liver related conditions with asymptomatic recurrent bacteriuria in women, pulmonary tuberculosis and leprosy[112-114]. However we would submit that in the index disease (AIH or PBC) the frequency and titre of the relevant liver-related autoantibody is substantially higher than for the contrast disease.

Anti-ASGPR antibodies are found particularly in AIH-1 (approximately 90%) but are also present in patients with PBC (14%), chronic hepatitis B and C (7%) and alcoholic hepatitis (8%)[3,52,115]. Anti-SLA antibodies can be found in occasional seronegative AIH patients i.e. those who are negative for ANA, SMA or anti-LKM-1. Anti-SLA antibodies are also frequently present (up to 50%, depending on the sensitivity of the method used) in typical cases of AIH-1 and AIH-2, and also in ASC[86]. Their high specificity for AiLD is has been questioned by reports of anti-SLA being present in some 10% of chronically infected HCV patients[115]. More recently, anti-SLA antibodies have been described in 22% of patients with acute liver failure (ALF)[111]. Since in most cases of ALF we do not know the cause, the presence of anti-SLA can either detract from their disease specificity or, alternatively, suggest an autoimmune pathogenesis (or an autoimmune component to the pathogenesis) of ALF. Monitoring of autoantibodies may be useful in the case of AIH as disappearance or sharp decrease of ANA, SMA and anti-LKM1 can be an indicator of response to

immunosuppressive treatment[3,6]. AMA titres do not relate to the stage of PBC and their fluctuation over time does not seem to have pathogenic significance[1,9,116], although "activity" of the PBC process is not as readily measurable as that of AIH. Practically AMA are only tested at presentation to help establish the diagnosis and repeat tests are normally requested only in cases seronegative for AMA at presentation but with clinical or laboratory findings compatible with PBC[1,2,117].

PROGNOSTIC SIGNIFICANCE AND UTILITY OF LIVER-RELATED AUTOANTIBODIES

AIH

Both SMA and ANA tend to lower in titre and even disappear during immunosuppressive therapy in most patients with AIH-1 although neither their titre at diagnosis nor their fluctuations during the disease are thought to predict disease course and outcome[3]. However, in 2002 Gregorio *et al* found a positive correlation between SMA titre and AST levels over time in pediatric AIH-1 cases, suggesting a potential use of these antibodies, together with IgG levels, to monitor disease activity[118]. There are no comparable adult sequential studies; this may be a reason why no correlation has been ascertained. Nevertheless, Czaja and colleagues have suggested that adult AIH-1 patients with antibodies to anti-actin have a disease onset earlier in life, respond less well to corticosteroids and progress to liver failure or require liver transplantation more frequently compared to those without anti-actin antibodies[69]. The presence of antibodies to double stranded DNA (dsDNA) has been associated with higher levels of immunoglobulin G and higher relapse rates during immunosuppressive treatment compared to seronegative cases[119]. Seropositivity for anti-ASGPR in patients with AIH correlates with histological activity with persistence indicating unresponsiveness to immunosuppressive treatment, and re-appearance being highly suggestive of relapse especially after corticosteroid withdrawal[3,52,115,120]. Anti-SLA antibodies denote patients with a more severe course of AIH and a propensity for relapse after corticosteroid withdrawal compared to their negative counterparts[49,81,121,122]. AIH-2 patients with anti-LC1 antibodies have histologically more severe disease compared to those without anti-LC1 antibodies[35,123,124].

PBC

AMA titres do not seem to be associated with disease severity but those of the IgG3 subclass may identify patients prone to develop more severe disease compared to those without AMA-IgG3[116,125]. PBC-specific ANA have been found more frequently in patients with advanced disease in a number of cross-sectional studies. Anti-NPC seropositivity is associated with accelerated progression to advanced disease and death[94,96,100,126-129] and also, ACA may identify patients with more severe PBC according to studies from USA and Japan[96,130]. These data have obvious implications for the clinical management of PBC given that the only accepted index for estimating survival has been obtained and validated in patients with advanced PBC and hence is of limited use in early disease. Thus, anti-NPC and ACA testing may be important for identifying asymptomatic patients with a likely unfavourable disease course. Once PBC has progressed to advanced histological stages, and serum bilirubin levels have become abnormal, anti-NPC determinations do not appear to offer any additional advantage over other prognostic models such as the Mayo risk score.

PATHOGENIC RELEVANCE OF LIVER-RELATED AUTOANTIBODIES

Despite their undoubted clinical relevance in diagnosis and classification of AiLD, the pathogenic role of autoantibodies and the mechanisms through which they may cause liver damage remains a topic for further research, mainly because of the difficulty in discriminating those actively involved in the immunopathogenic cascade, from those secondary to liver cell damage. The mechanism(s) responsible for the induction of liver-related autoantibodies is currently unknown; several possibilities including molecular mimicry and immunological cross-reactivity have been suggested[78,93,106,131-145]. Most liver-related autoantibodies have limited organ specificity and this notion militates against a direct pathogenic role in highly organ-specific autoimmune injury. For antibodies with a pathogenic potential, complement-dependent and/or antibody-dependent cell-mediated cytotoxicity (ADCC) are the likely effectors of damage[131,146].

EMERGING ISSUES: DIAGNOSTIC ACCURACY, QUALITY ASSURANCE AND STANDARDIZATION PROGRAMMES FOR LIVER AUTOIMMUNE SEROLOGY

There are a number of open issues on serum autoantibodies in AiLD. Their diagnostic significance is unquestioned, but problems concerning autoantibody detection and interpretation have not yet been resolved and are not being addressed with sufficient vigour. Several laboratories ignore, for example, the IFL cut-off points recommended by the Committee for Autoimmune Serology of the IAIHG and use their own, thus undermining comparability between different laboratories/centres. Worryingly, the cost per test seems a major reason for arbitrary elevation of cut-off points in routine practice: selecting 1:80 or even 1:160 as a screening dilution expands the number of "negatives" albeit reducing or eliminating the need for re-testing. In patients with AiLD and relatively low autoantibody titres, such as children with AIH, a report that is inaccurately indicative of negativity for autoantibodies can delay diagnosis and, harmfully, defer treatment[76,147]. Hence rigorously performed autoantibody testing may in fact

provide a more economical report than a "false negative" one if such leads the clinician to order additional costly diagnostic procedures.

Additional problems for autoantibody testing especially with IFL are intrinsic to the methodology itself. First, availability of tissue substrate comprised of freshly cut sections from cryostat blocks of unfixed liver, kidney stomach tissue is limited to relatively few specialised laboratories. Second, sections of commercial origin are of variable quality because, to lengthen shelf-life, they are treated with fixatives, which readily result in enhanced background staining[55]. Third, IFL requires highly-trained and experienced personnel, is time-consuming and cannot be automated, resulting in a low throughput and increased personnel costs leading to a significant shift from IFL towards ELISAs or blot assays based on liver-autoantibody profiles; these compared to IFL are less-time consuming, easy to perform and amenable to automation. However, the authors of this review reiterate the recommendations of the Committee for Autoimmune Serology of the IAIHG stating that the current ELISAs should complement but not replace IFL. Either technique has their *pros* and *cons*, and gives answers to different questions, such that results are not directly comparable[148-150]. Most liver-related autoantibodies can be detected by IFL when using a triple rodent tissue. HEp-2 cells can help to differentiate ANA patterns and ethanol-fixed neutrophils can be used for the detection of ANCAs. In contrast, ELISAs give answers for (usually) pre-selected individual autoantibody specificities. While the analytical sensitivity of ELISAs is satisfactory, their specificity varies according to the manufacturer[150] whereas such problems are rather infrequent by IFL testing based on a triple rodent tissue substrate[151].

Over the last decade there has been a steady increase in the use of the liver-related autoantibody tests to assist both diagnosis and clinical research into AiLD[55]. This increase has been attributed mainly to the introduction of molecularly based assays for the testing of antibodies to F-actin[70-75], CYP2D6[152] and SLA[83,122,153] in AIH, and for evaluating antibodies to sp100 and gp210 in PBC[92,94-96,154]. Of concern, results for these antibody specificities may be promulgated by laboratories without authentication from externally or independently monitored quality assurance programmes (QAP).

Quality assurance (QA) can occur at three levels. The first is at the level of commercial providers of assay kits, reagents etc who would establish QA "in house" before marketing but who often elect to participate also in QAPs for routine laboratories. The second are the formalised QAPs, run by semi-governmental agencies or other organizations, as described below. The third level, which scarcely exists, involves the end-user, the responsible clinician, who must order tests advisedly with good clinical data and interpret these in the light of the clinical information to make wise evidence-based decisions. Thus it behoves the clinician to become fully aware of the many contributions (and shortcomings) of contemporary diagnostic immunoserology.

Table 1 Laboratories from various countries participating to the UK National External Quality Assessment Service (UK NEQAS)

Country	Number	Country	Number
Austria	3	Latvia	1
Belgium	7	Malaysia	1
Croatia	2	Malta	1
Cyprus	1	New Zealand	3
Denmark	2	Norway	9
Eire	15	Portugal	31
Estonia	1	Republic of Chile	1
Finland	5	Singapore	1
France	29	South Africa	3
Germany	9	Spain	68
Greece	16	Sweden	14
Hong kong	1	Switzerland	7
Hungary	5	The Netherlands	1
Israel	8	Turkey	2
Italy	65	UK	136
Kingdom of Saudi Arabia	1	United Arab Emirates	1
Kuwait	1	USA	3

REPRESENTATIVE QUALITY ASSURANCE PROGRAMMES FOR DIAGNOSTIC SEROLOGY IN LIVER DISEASE

USA

The College of American Pathologists (CAP, www.cap.org) runs survey programmes which allow laboratories to evaluate regularly their autoantibody testing performance. Of relevance to liver, CAP circulates coded anti-M2 AMA, anti-LKM1 and SMA samples for testing. The participating laboratories analyse the sera and return their results for evaluation. In return, each laboratory receives an anonymised report of the performance of all participating laboratories.

UK

A National External Quality Assessment Service (UK NEQAS) (www.ukneqas.org.uk) is responsible for the objective assessment of the performance of autoantibody testing. The UK NEQAS for General Autoimmune Serology incorporates one sample in each of six distributions annually for AMA, anti-LKM1 and SMA. The performance reports of the participating laboratories also provide information on kit suppliers. Participation is not limited to UK but is open to non-UK Countries (Table 1).

Germany

There are currently two regulatory and quality assurance agencies, namely INSTAND (Institut für Standardisierung, www.instandev.de) and DGKL (Deutsche Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, www.dgkl.de). INSTAND circulates twice per year two samples to be tested for AMA, SMA and anti-LKM1 antibody testing. Participants (150) report results quantitatively and semi-quantitatively (from 0-4 to evaluate antibody titre; 0 = negative; 1 = borderline; 2 = low; 3 = middle; and 4 =

high). There is no reference to specific manufacturers but only to test methods and overall percentage of consistent results. DGKL has a similar approach but evaluations are divided on the basis of the methods used and they provide also information in relation to the kits manufacturers. Target values are determined in two reference laboratories.

France

Quality autoantibody assessment in France is organised by the French Health Products Safety Agency (AFSSAPS, Agence Française de Sécurité Sanitaire des Produits de Santé, http://agmed.sante.gouv.fr/). This Agency has the executive responsibility for proposing relevant QAPs to clinical laboratories, whether in the private or in the public sector. An autoantibody detection survey has been running on an annual basis since 1998.

Italy

In Italy there are no formal regulatory and quality assurance programmes with several laboratories participating in the surveys by UK NEQAS or CAP. Recently, a study group has been formed (Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni-FIRMA-www.grouppofirma.com). FIRMA aims to provide guidelines for autontibody testing and to identify and collect sera of different autoantibody specificities that will be available for all of its member institutions.

Finland

Labquality at Helsinki offers twice per year three samples for SMA, AMA and anti-LKM1 assessment. Qualitative target values are determined in a reference laboratory and results are listed according to manufacturer and method. Evaluation reports are confidential.

Australia and New Zealand

QAPs have been established under the auspices of the Royal College of Pathologists of Australasia (RCPA) based on the selection by RCPA of expert organizing groups which distribute batches of sera to diagnostic laboratories that voluntarily elect to participate (www.rcpaqap.com.au). Diagnostic laboratories from Australia, New Zealand, and several South East Asian countries together with manufacturers and purveyors of kits participate in this programme. The Tissue Antibodies module includes AMA, SMA and anti-LKM1 antibodies. Feedback to the laboratories is by a report to all participants in which any single laboratory can identify its own performance versus that of all other participants. The RCPA issues certification of participation in this QAP. The reports sent back to laboratories are inspected by the National Association of Testing Authorities (NATA) during laboratory assessment visits. In order to be accredited, laboratories must participate and perform satisfactorily in the relevant proficiency testing programmes. There is a 'regulatory' element here in that NATA certification is required for access to fees under the Medicare rebate scheme.

As expected, quality assurance programmes have highlighted difficulties encountered by peripheral laboratories. In mid-2007, UK NEQAS distributed a serum with a typical anti-LKM1 antibody staining; a substantial proportion (53 out of 356, 15%) of the laboratories reported negativity for anti-LKM1 antibody test and, among these 53 laboratories, 43 incorrectly reported positivity for AMA instead (Peter White, UK NEQAS, personal communication). Also, rather worryingly, several additional laboratories did not return reports on anti-LKM1 either because they themselves do not offer this test or because they ignore its significance (Peter White, UK NEQAS, personal communication).

It is clear that exchange of calibrated reference sera and rigorous standardization programmes on liver-related autoantibody serology are urgently needed. Such initiatives will need to involve initially researchers and laboratories with a special interest in the respective antibody specificities and subsequently clinical laboratories performing routine screening tests. To this end, efforts have been made recently by the IAIHG to arrange an exchange of sera at international level but whether such an initiative will take off depends on securing financial support. Administrative sponsorship should initially come from the International Association for the Study of Liver (IASL), the American Association for the Study of Liver Diseases (AASLD) or the European Association for the Study of Liver (EASL) or from Clinical Immunology Societies of developed countries.

In conclusion, practice guidelines on liver autoimmune serology based on consensus of experts in the field have been issued and need to be steadily updated[55]. The more the clinician is aware of these guidelines, the greater the chance of correct and clinically relevant autoantibody diagnosis. It is in the best interest of the patient to obtain eventually the highest possible commitment and coordination of all organizations, agencies, industrial partners and networks working in the field.

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