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Special article

Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group☆

Diego Vergani[1,*], Fernando Alvarez[2], Francesco B. Bianchi[3], Eduardo L.R. Cançado[4], Ian R. Mackay[5], Michael P. Manns[6], Mikio Nishioka[7], Edward Penner[8]

[1]*Institute of Liver Studies, King's College Hospital, Denmark Hill, London SE5 9RS, UK*
[2]*Division of Gastroenterology, Hôpital Sainte-Justine, Montreal, Que., Canada, H3T IC5*
[3]*Department of Internal Medicine, Cardioangiology, Hepatology, Policlinico S. Orsola University of Bologna, 40138 Bologna, Italy*
[4]*Department of Gastroenterology, University of Sao Paulo School of Medicine, Brazil*
[5]*Department of Biochemistry and Molecular Biology, Monash University, Clayton 3800, Victoria, Australia*
[6]*Department of Gastroenterology, Hepatology and Endocrinology, Medizinische Hochschule Hannover, 30625 Hannover, Germany*
[7]*Ehime Rousai Hospital, Japan Labour Health and Welfare Organisation, 13-27 Minami-Komatsubara, Niihama, Ehime 792-0863, Japan*
[8]*Fourth Department of Internal Medicine, Vienna Medical University, Währinger Gürtel 18-20, A-1090 Vienna, Austria*

1. Introduction

The diagnosis of autoimmune hepatitis (AIH) has been advanced by the criteria developed by the International Autoimmune Hepatitis Group (IAIHG) [1,2]. A critical component of these criteria is detection by indirect immunofluorescence (IIF) of autoantibodies to components of the nuclei (anti-nuclear, ANA), smooth muscle (SMA) and liver kidney microsomes type 1 (anti-LKM-1). Detection not only assists in the diagnosis but also enables discrimination between two distinct subtypes of the disease [1,2], AIH-1 and AIH-2. The differing serological reactivities for the two types (ANA, SMA vs anti-LKM-1) are virtually mutually exclusive; in the rare exceptions with 'double positive' serology, the clinical expression resembles AIH-2 [3,4]. Also, consideration is needed of 'overlap' syndromes [5,6], in particular types of cholangiopathy with autoimmune serological expressions. The relatively low prevalence of autoimmune liver diseases and the operator dependency of immunofluorescence, which still remains the mainstay of liver diagnostic autoimmune serology, explain the lack of agreement between laboratories on the frequency of particular reactivities in different liver diseases, particularly the less frequent specificities such as anti-LKM-1. Perceived inaccuracy of the methodology apparently led influential authors to suggest (misleadingly) that assessment of autoantibodies be disregarded in the diagnosis of AIH because of the low sensitivity and specificity of relevant serological tests [7]. This view also may have been motivated by assignment of autoantibody testing to commercial laboratories and use of kit-based semi-automated systems often with little contact between laboratory and clinician to assist in interpretation of results. Moreover, kit-based commercial assays may have been validated only 'in-house', such that the performance characteristics would not necessarily be available to the end-user. The problems that do exist between laboratory reporting and clinical interpretation of the serological results depend in part on insufficient standardisation of the basic tests, a problem common to autoimmune diagnostic serology in general, and in part on a degree of unfamiliarity of some clinicians with disease expressions of AIH. In regard to standardisation, a lead has been taken by the diabetes community in encouraging use of standardised methods by establishing ongoing serum exchange workshops since 1986 with calibrated reference sera containing autoantibodies to

☆ The authors are the members of the committee for autoimmune serology of the International Autoimmune Hepatitis Group. DV is the chairman.

* Corresponding author. Tel.: +44-20-7346-3305/3695; fax: +44-20-7346-3700.

E-mail address: diego.vergani@kcl.ac.uk (D. Vergani).

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autoantigens relevant to type 1 diabetes mellitus [8–10]. Consequently, editors of major journals of diabetes now expect, in reports that cite reactivities of autoantibodies, that assays have been validated under international workshop conditions. Accordingly, the IAIHG established an internationally representative committee to define guidelines and develop procedures and reference standards for more reliable testing.

Although concerned with specific serological reactivity, emphasis is given to the importance for diagnosis and for monitoring treatment of a raised level of immunoglobulin (Ig) G measured by standard procedures, as noted in the IAIHG criteria.

2. Autoantibody testing by indirect immunofluorescence

The basic technique for the routine testing of autoantibodies relevant to AIH is IIF on a freshly removed rodent (usually rat) multi-organ substrate panel that should include kidney, liver and stomach. This allows for detection of ANA, SMA, anti-LKM-1 as well as anti-mitochondrial antibody (AMA), and also antibodies to liver cytosol type 1 (anti-LC1). For sera positive at the screening stage, further examination is required to assess the pattern of nuclear staining, by use of HEp2 cells, or to define the nature of the SMA reaction (see below) (Table 1).

2.1. Preparation of the substrate

Liver should be cut in cubes of 3–4 mm diameter, but orientation of the sectioning is of minor importance since the liver is morphologically homogeneous. Kidney requires careful orientation, and should first be cut sagitally and then in 3–4 mm cubes that contain both medulla and cortex. This is because both AMA and anti-LKM-1 stain renal tubules, but with different patterns distinguishable only in the presence of both proximal and distal tubules. Thus AMA stains preferentially the distal tubules which are smaller in size, whereas anti-LKM-1 stains characteristically the third portion of the proximal tubules (P3). Stomach should be carefully washed and cut in longitudinal strips. Liver and kidney cubes are juxtaposed to each other, and to a rolled up gastric strip, to form a homogeneous block. The block is then gradually frozen in a vapour of liquid nitrogen on a cryostat chuck, or in pre-cooled isopentane; both the relatively small size of the block and gradual freezing prevent crystal formation. Liver, kidney and gastric tissues not immediately used can be sealed in foil and stored in liquid nitrogen for use up to several years or at −70 °C (storage 3–6 months). The cryostat chuck is mounted in the cryostat and the first few sections are discarded until the surface of the block is homogeneous. The block is then cut in sections 4–5 μm thick, which are mounted on standard or multi-well microscope slides. The sections are dried in air and used without further fixation or can be stored in air-tight histological boxes at −20 °C, and will last 4–8 weeks. Equivalent sections that are commercially available are of variable quality because, to lengthen shelf-life, they are treated with fixatives (acetone, ethanol or methanol), which readily result in enhanced background staining that limits interpretation of fluorescence patterns.

2.2. Application of test sera

25–30 μl of test and control sera diluted in phosphate buffered saline (PBS) pH 7.2 are applied to the slide to cover the entire tissue section. The sections are left for

Table 1
Methods, associations and reactants for autoantibodies in liver diseases

Autoantibody[a]	Conventional method of detection[b]	Molecularly based assays	Disease association	Molecular target(s)
ANA	IIF	N/A	AIH-1; overlap syndromes	Multiple targets, particularly chromatin
SMA	IIF	N/A	AIH-1; overlap syndromes	Microfilaments (actin?), Intermediate filaments (vimentin etc)
Anti-LKM-1	IIF[c]	ELISA, IB, RIA	AIH-2; HCV infection (5%)	Cytochrome P450 2D6
Anti-LC1	IIF, DID, CIE	ELISA, RIA	AIH-2	Formiminotransferase cyclodeaminase
SLA/LP	Inhibition ELISA	ELISA, IB, RIA	AIH-1; AIH-2 and AIH negative for other reactivities	tRNP(Ser)Sec (see text)
Atypical pANCA pANNA	IIF	N/A	AIH-1; sclerosing cholangitis	Unidentified antigen(s) at nuclear periphery
AMA	IIF	ELISA, IB, RIA	Primary biliary cirrhosis	E2 subunits of 2-oxo-acid dehydrogenase complexes, particularly PDC-E2

[a] ANA, anti-nuclear antibody; SMA, anti-smooth muscle antibody; LKM-1, anti-liver/kidney microsomal antibody type 1; LC1, anti-liver cytosol type 1 antibody; SLA/LP, anti-soluble liver antigen/liver pancreas antibody; pANCA, perinuclear anti-neutrophil cytoplasmic antibody; pANNA, perinuclear anti-neutrophil antibody; AMA, anti-mitochondrial antibody.

[b] IIF, indirect immunofluorescence (recommended cut-off titre for positivity is 1/40 except in children—see text); DID, double dimension immunodiffusion; CIE, counter-immuno-electrophoresis; ELISA, enzyme linked immunosorbent assay; IB, immunoblot; RIA, radio-immunoprecipitation assay.

[c] Anti-LKM-1 and AMA both stain renal tubules and are frequently confused (see text).

30 min at room temperature (RT) in a humidified box and then washed in an excess of PBS with mild shaking. The sections are then exposed to fluorochrome-labelled anti-human IgG, IgA and IgM antiserum, or fluorochrome-labelled antiserum to human IgG only, for an additional 30 min at RT in the humidified box and then re-washed as above. The slides are then mounted in 9/1 glycerol/PBS mounting medium and examined by epifluorescence.

2.3. Dilution of sera and fluorochrome-labelled reagents

Conventionally, the starting serum dilution is 1/10, but at this dilution sera from some healthy adults can be reactive, so that a clinically significant level of positivity would start at the arbitrary dilution of 1/40. In contrast, for subjects up to the age of 18 years, any level of autoantibody reactivity in serum is infrequent, so that positivity at dilutions of 1/20 for ANA and SMA and even 1/10 for anti-LKM-1 is clinically relevant. Hence, the laboratory should report any level of positivity from 1/10, with the result interpreted within the clinical context and the age of the patient; for example, an ANA of 1/20 in a 16-year-old girl with abnormal liver function tests, including a high level of immunoglobulin G and a liver biopsy showing interface hepatitis, would be indicative of AIH-1 with prompt requirement for immunosuppression. The optimal dilution of the revealing fluorochrome-labelled antiserum depends on the original strength of the commercially available antiserum used; this usually varies between 1/50–1/100.

2.4. Controls

Controls must include sera with known negative and positive reactivity for autoantibodies associated with autoimmune liver disease, (ANA, SMA, anti-LKM-1 and AMA). The future availability from the IAIHG Serology Committee of positive reference antisera with calibrated reactivities for each of the major specificities is anticipated, but meanwhile the laboratory should develop in-house reference sera and a reference range based on the local population.

2.5. Immunofluorescence reactivities

The three tissues should be identified and examined sequentially starting at the lowest microscope magnification, usually with $\times 10$ objective.

ANA is readily detectable as a nuclear staining in each of the three tissues. On liver particularly, the ANA pattern may be discerned as homogeneous, or coarsely or finely speckled. However, a much clearer definition of ANA pattern is obtained using HEp2 cells that have prominent nuclei. The HEp2 preparation should contain sufficient mitoses to allow ready identification of anti-centromere. HEp2 cells should not be used at the screening stage because of the high positivity rate for ANA at low serum dilution, 1/40, in normal subjects [11]. The usual ANA pattern in active phases of AIH-1 is homogeneous, but speckled patterns are not infrequent, particularly after a concurrent homogeneous pattern has faded on remission, but more definitive data on this are needed. For ANA, the likely molecular targets include nuclear chromatin and histones, as in SLE, and there are probably others [12]. In the future more refined techniques using recombinant nuclear antigens and immunoassay formats may enable the identification of reactants, and assessment of their specificity for diagnosis and possible role in pathogenesis of AIH-1.

SMA stains the walls of the arterial vessels in all three tissues, the muscular layer of the stomach and, in addition, it stains the vascular axis of the lamina propria of the gastric mucosa. The examination of the kidney is of importance since this allows for visualization of the V, G and T patterns; V refers to vessels, G to glomeruli, and T to tubules [13]. The V pattern (attributable to vimentin) is present also in non-autoimmune inflammatory liver disease, as well as in autoimmune diseases not affecting the liver and in viral infections, but VG and VGT patterns are more reliably associated with AIH. The VGT pattern, as detected on composite rodent tissue, corresponds to what is known as the ‘F actin’ or microfilament (MF) pattern which is optimally observed using cultured fibroblasts and serum heat-inactivated at 56 °C for 30 min [14,15]. However, neither the VGT pattern nor anti-microfilament positivity as observed on cultured fibroblasts are per se entirely specific for the diagnosis of AIH-1.The VGT-MF pattern in AIH may represent a separate population of autoantibodies or simply reflect high titre SMA; the former view is favoured by the V (vascular) pattern seen as the sole reactivity in various viral infections, and the latter view by the observation that the G and T components, supposedly AIH specific, recede when AIH-1 is successfully treated. The molecular target of the microfilament reactivity that is observed in AIH-1 remains to be identified. A recent retrospective study shows that SMA-T and anti-MF were present in 80% of patients with AIH-1 with the two reactivities being strongly correlated [16]. These data confirm the strong association (sensitivity 80%) between ‘anti-actin’ reactivity and AIH-1, but also show that some 20% of SMA positive patients with AIH-1 lack the ‘actin’-SMA pattern, such that the absence of anti-actin SMA does not exclude the diagnosis.

Anti-LKM-1 stains brightly the liver cell cytoplasm and the P3 portion of the renal tubules strongly and diffusely, but spares cells of the gastric glands. In contrast, AMA, which can be confused with anti-LKM-1 [17], gives a fainter staining of the liver cell cytoplasm but a strong staining of all renal tubules especially the smaller distal ones; the difference from

anti-LKM-1 is best appreciated at low magnification (×10 objective). AMA also stains the gastric parietal cells, with a bright granular pattern. Examination of the three tissue substrates should allow a ready distinction between AMA and anti-LKM-1, but the inadvisable use of renal tissue in isolation (as often done), or faulty orientation of the tissue, can result in erroneous interpretations. HEp2 cells are being increasingly used and recommended for the diagnosis of AMA but, since positivity on HEp2 cells may not correspond to AMA detectable on a composite 3-tissue substrate [18], or to anti-M2 reactivity observed by Western blot, the exclusive use of HEp2 cells to detect AMA is inadvisable. In the context of AIH, there can be positivity for AMA in a small subset of patients (3–5%) in whom there are overlapping features with PBC [19,20].

2.6. Antibody testing based on recombinant/purified antigens

The identification of the molecular targets of certain of the specificities described has led to the establishment of various immuno-assays based on use of recombinant or purified antigens, as cytochrome P4502D6 (CYP2D6) (anti-LKM-1) and enzymes of the 2-oxo-acid dehydrogenase complexes (AMA) [21–23]. While radioligand assays for these autoantibodies remain confined to research laboratories [24,25] ELISA formats are available commercially. Some of these ELISAs are accurate for detection of anti-LKM-1, at least in the context of AIH-2 [26], and reasonably accurate for detection of AMA, but they await external validation. Importantly, possible confusion between the anti-LKM-1 and AMA reactivities by immunofluorescence can be resolved by immunoassays that detect antibodies to purified/recombinant enzyme antigens.

2.7. Antibodies not tested for routinely

2.7.1. Anti-LC1

This was originally described either in association with anti-LKM-1, or in isolation, and in both instances it defined a clinical entity resembling AIH-2 [27,28]. Later, anti-LC1 has been also found occasionally in association with the serological markers of AIH-1 [29] and in patients with HCV infection [28]. Anti-LC1 can be detected by IIF using the standard tissue panel. It stains the cytoplasm of liver cells with relative sparing of the centrilobular area, but is usually obscured by concurrent presence of anti-LKM-1, which stains hepatocyte cytoplasm throughout the lobule. In the presence of anti-LKM-1, anti-LC1 can be detected by use of liver cytosol in double-dimension immunodiffusion, or counterimmunoelectrophoresis, using a positive reference serum [30], by formation of lines of identity between the test serum and the positive reference serum. Anti-LC1 can also be detected by Western blot as a reactivity with a a 58–60 kD protein using a human liver cytosolic fraction as substrate [29]. The clinical relevance of anti-LC1 is still being assessed, but should soon become clearer since the molecular target has been identified as formimino-transferase cyclodeaminase (FTCD), and commercial kits have become available [31,32]. The presence of anti-LC1 in isolation scores positively towards a diagnosis of AIH-2, allowing prompt initiation of treatment.

2.7.2. Variant liver microsomal antibodies: anti-LM, anti-LKM2 and 3

These are mostly directed against P450 cytochrome isoforms other than 2D6 (LKM-1) and occur particularly in drug-induced hepatitis. Anti LM antibodies stain only the liver cytoplasm and not the kidney or other organs, react with liver specific cytochrome P4501A2 [33,34] and occur in dihydralazine induced hepatitis [33] and in hepatitis associated with the autoimmune polyendocrine syndrome type 1 (APS-1) [35]. An anti-LKM-1 like pattern of immunofluorescence is given by autoantibodies to P4502A6, as judged by recombinant cytochrome P4502A6 based immunoassays [36,37]; these antibodies occur in APS-1 and occasionally in hepatitis C, but in contrast to anti-LM they are not specifically associated with liver disease in APS-1.The term anti-LKM-2 was originally applied to LKM-1-like microsomal antibodies produced during hepatitis induced by tienilic acid [38] (no longer marketed) and are directed against cytochrome P4502C9. Anti LKM-3 also gives an immunofluorescent pattern resembling that of anti-LKM-1, but these antibodies occur in hepatitis D (delta), and rarely in autoimmune hepatitis type 2 [39]; they react with members of the family 1 UDP glucuronosyltransferases (UGT) and target an epitope common to all members of this family [40–42].

2.7.3. Anti-soluble liver antigen/liver–pancreas antigen (anti-SLA/LP)

Two earlier described autoantibodies in AIH, anti-SLA [43] and anti-liver–pancreas [44], target the same antigen [45]. Anti-SLA was thought to identify a third type of AIH in which there were negative tests for the conventional autoantibodies [43], but the early report predated the publication of IAIHG recommendations [1,2] and used a cut-off point for autoantibody levels higher than that currently recommended for the diagnosis of AIH. The SLA antigen was first described in 1992 [46] as marker for particularly severe forms of autoimmune hepatitis. Screening of cDNA expression libraries by various groups using high titre anti-SLA serum identified the target antigen at the molecular level as UGA tRNA suppressor associated antigenic protein (tRNP(Ser)Sec) [45,47,48]. Molecularly based diagnostic assay kits are available [49] but their full evaluation is pending. Though anti-SLA/LP is found occasionally in patients with AIH who are negative for ANA, SMA or anti-LKM-1, it is also frequently present in typical cases of AIH-1 and AIH-2, and also in AIH/sclerosing cholangitis overlap syndrome [50,51]; in

fact anti-SLA appears highly specific for the diagnosis of AIH, and its detection at the time of diagnosis identifies patients with more severe disease and a likely adverse outcome [46,49,50,52].

2.7.4. Antineutrophil cytoplasmic antibodies (ANCA)

These are autoantibodies directed at cytoplasmic components of neutrophils [53,54] and give either a perinuclear (p-ANCA) or a cytoplasmic (c-ANCA) pattern. c-ANCA mainly react with proteinase 3 and p-ANCA with myeloperoxidase. Many patients with AIH-1 (50–96%) are p-ANCA seropositive while, interestingly, most with AIH-2 are negative [55–58]. The classical p-ANCA staining pattern is an artefact of the ethanol fixation of the neutrophils, caused by migration of strongly cationic cytoplasmic proteins (such as myeloperoxidase) to the negatively charged nuclear membrane; the cytoplasmic pattern is retained if neutrophils are fixed with cross-linking agents such as formaldehyde. However, p-ANCA in AIH ('atypical' p-ANCA) often differ from classical p-ANCA by retention of perinuclear staining on formaldehyde-fixed cells; such antibodies which are also found in primary sclerosing cholangitis (60–92%), ulcerative colitis (60–87%) and Crohn's disease (5–25%) may react with nuclear membrane components (perinuclear anti-neutrophil antibodies, p-ANNA) [59,60]. Detection of atypical p-ANCA can act as an additional pointer towards the diagnosis of AIH, particularly in the absence of other autoantibodies.

3. Concluding remarks

Detection of autoantibodies plays a pivotal role in the diagnosis of liver disorders with an autoimmune pathogenesis and may be a useful tool in monitoring disease activity. However, both the laboratory personnel and the clinician need to become more familiar with the interpretation of the liver autoimmune serology to derive maximum benefit for the patient. Akin to what has already been achieved for other autoimmune diseases, especially type 1 diabetes, this can only be obtained through standardization of the methodology employed and exchange of sera with known and calibrated reactivities. This should enable meaningful data on sensitivity and specificity of the assay systems to become available. The IAIHG Autoimmune Serology Committee is working towards this goal.

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