

Antinuclear antibodies (ANA) as a criterion for classification and diagnosis of systemic autoimmune diseases

Luis Eduardo C. Andrade^{a,b,*}, Jan Damoiseaux^c, Diego Vergani^{d,e}, Marvin J. Fritzler^f

^a Rheumatology Division, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, Brazil

^b Immunology Division, Fleury Medicine and Health Laboratories, São Paulo, Brazil

^c Central Diagnostic Laboratory, Maastricht University Medical Center, Maastricht, the Netherlands

^d King's College London Faculty of Life Sciences & Medicine at King's College Hospital, London, United Kingdom

^e Institute of Liver Studies, MowatLabs, King's College Hospital, London, United Kingdom

^f Department of Medicine, Cumming School of Medicine, University of Calgary, Calgary, Canada

ARTICLE INFO

Keywords:

Autoantibodies
Antinuclear antibodies
Autoimmune diseases
Classification criteria
Systemic lupus erythematosus
Juvenile idiopathic arthritis
Autoimmune hepatitis
Primary biliary cholangitis

ABSTRACT

The classification and diagnosis of systemic autoimmune diseases are frequently based on a collection of criteria composed of clinical, laboratory, imaging, and pathology elements that are strongly associated with the respective disease. Autoantibodies are a distinctive hallmark and have a prominent position in the classification criteria of many autoimmune diseases. The indirect immunofluorescence assay on HEp-2 cells (HEp-2 IFA), historically known as the antinuclear antibody test, is a method capable of detecting a wide spectrum of autoantibodies. A positive HEp-2 IFA test is part of the classification criteria for systemic lupus erythematosus (SLE) and juvenile idiopathic arthritis (JIA), as well as the diagnostic criteria for autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC). A positive HEp-2 IFA test can appear as different morphological patterns that are indicative of the most probable autoantibody specificities in the sample. Only some of the HEp-2 IFA patterns are associated with the specific autoantibodies relevant to SLE, JIA, AIH, and PBC, whereas some other patterns occur mainly in non-related conditions and even in apparently healthy individuals. This paper provides a critical review on the subject and proposes that the classification and diagnostic criteria for SLE, JIA, AIH, and PBC could be improved by a modification on the HEp-2 IFA (ANA) criterion in that the staining patterns accepted for each of these diseases should be restricted according to the respective relevant autoantibody specificities.

1. Classification and diagnostic criteria

Accurate classification and diagnosis of diseases is a long-term historical goal of medicine and an ideal classification system should ensure that two distinct disease types are not ascribed to the same disease category [1]. An accurate classification system is important to understand the causes of diseases as well as several aspects relevant to disease management, such as the early diagnosis, prognosis, predictive outcomes, and response to evidence-based therapeutic approaches. Some diseases are classified successfully according to an identified causal agent (e.g., infectious diseases), and some are classified according to individual constitutional predisposition or risk (e.g., monogenic diseases). In many systemic autoimmune/inflammatory diseases (SAID), no single causal agent, environmental exposure, or gene abnormality is typically identified. Therefore, the classification of SAID usually relies

on a set of clinical, laboratory, imaging, and pathology elements that optimally segregate patients into a particular SAID category. Appropriate classification criteria are particularly important to ensure a level of consistency of the pathological status of the individuals under consideration for clinical studies and trials. Classification criteria are not intended as the approach in achieving the diagnosis of individual patients, because a partial and initial expression of a SAID in each patient may not fulfill the classification criteria, which are meant to represent mature or definite disease. As expected, classification criteria need occasional re-evaluation and revision according to progress in the knowledge of the diseases. Autoantibodies are prevalent in and considered a hallmark of SAID, and some are an integral part of the formal classification and diagnostic criteria for some SAID. The indirect immunofluorescence assay on HEp-2 cell substrates (HEp-2 IFA), historically known as the antinuclear antibody test, is widely used as a

* Corresponding author. Rua Luis de França Jr 201, casa 8. São Paulo, SP, CEP 04648-070, Brazil.

E-mail address: luis.andrade@unifesp.br (L.E.C. Andrade).

<https://doi.org/10.1016/j.jtauto.2022.100145>

Received 14 January 2022; Accepted 17 January 2022

Available online 19 January 2022

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screening test for a wide spectrum of autoantibodies and has been accorded a key part of the classification criteria of some SAID. This review will address potential opportunities for improving the utilization of the HEp-2 IFA test in this context.

2. HEp-2 IFA and the International Consensus on ANA patterns (ICAP)

The IFA method for the detection of autoantibodies was introduced several decades ago with the use of cryopreserved rodent tissue sections as substrates [2]. This was a significant advance and predominantly allowed the detection of antibodies to the cell nucleus and some tissues (e.g., smooth muscle) or extracellular matrix (e.g., glomerular basement membrane, skin dermal/epidermal junction). Nonetheless, its application to SAID focused on the cell nucleus and thus the test became known as the antinuclear antibody (ANA) test. In the late 1970s, the human laryngeal epidermoid carcinoma HEp-2 cell line was introduced as an alternative substrate for cryopreserved rodent tissue sections [3]. The use of HEp-2 cell substrate had several important consequences, as it provided the opportunity to detect some autoantibodies not readily recognized with the use of rodent tissue substrates because the cells were larger and allowed more detailed visualization of the intracellular cell structures bound by the autoantibodies. In addition, it allowed the recognition of autoantibodies reacting with the cytoplasm and cell-cycle specific targets including the mitotic apparatus. Within a few years, HEp-2 cells became the universal substrate of choice for the ANA test and today the most popular test for screening for autoantibodies worldwide.

The use of the HEp-2 as the antigenic substrate in IFA increased the sensitivity of the ANA test and contributed to its use to evaluate a widening array of diseases [4]. The progressive popularization of the HEp-2 IFA test and its use by an expanding spectrum of medical specialties, frequently in a low pre-test probability scenario, contributed to a decrease in the clinical specificity of a positive HEp-2 IFA test. This is a problem since there is an increasing number of reports in various clinical situations that are difficult to interpret and tend to provide more challenges than solutions. Accordingly, in contrast to low serum dilutions (1:10, 1:20) used on rodent section substrates, the screening serum dilution when HEp-2 substrates are used was increased to 1:80 or 1:160. Another consequence is that the former "anti-NUCLEAR antibody (ANA)" test could now detect antibodies against antigens in the cytoplasm and the mitotic apparatus. Therefore, in the last few years, it has been proposed that the name of the test be changed to the anti-cell antibody (ACA) test or the HEp-2 IFA test [5,6].

One of the important pieces of information provided by the HEp-2 IFA test is the IFA staining pattern. Several studies have reported on the HEp-2 IFA patterns associated with specific autoantibodies (Table 1). Some IFA patterns are strongly associated with specific targets to the point that the patterns are named after the respective autoantibody specificity, such as the centromere pattern, the nuclear mitotic apparatus (NuMA)-like pattern, the proliferating cell nuclear antigen (PCNA)-like pattern, the centromere protein F (CENP-F)-like pattern, and the Topoisomerase I-like pattern. The recognition of the clinical relevance of a widening spectrum of HEp-2 IFA patterns prompted different groups to develop guidelines for the classification of these patterns [9–11]. The clinical interest in the HEp-2 IFA staining patterns has been further stimulated by a seminal study highlighting the role of the HEp-2 IFA pattern in discriminating positive HEp-2 IFA results from SAID and non-SAID patients. This study showed that some patterns (e.g., the homogeneous nuclear pattern and the coarse speckled nuclear pattern) were observed exclusively in SAID patients, whereas some other patterns (e.g., the dense fine speckled nuclear pattern) were observed most predominantly in healthy subjects [12]. Starting at the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA-2014) held in São Paulo, Brazil, and followed by a sequence of annual workshops, an international panel of specialists started the

Table 1

Associations of some HEp-2 IFA patterns with autoantibody antigenic specificities (reviewed in [7] and [8]).

Pattern	Autoantibodies to
Nuclear homogeneous (AC-1)	dsDNA, nucleosome, histones
Nuclear dense fine speckled (AC-2)	DFS70/LEDGF-p75
Centromere (AC-3)	CENP-A, CENP-B, CENP-C
Nuclear coarse speckled (AC-5)	Sm, U1-RNP
Multiple discrete nuclear dots (AC-6)	Sp100, PML, MJ/NXP-2
Few discrete nuclear dots (AC-7)	p80-coilin
Nucleolar clumpy (AC-9)	Fibrillarin (U3-RNP)
PCNA-like (AC-13)	PCNA/cofactor of DNA polymerase delta
CENP-F-like (AC-14)	CENP-F/mitosin
Cytoplasmic reticular (AC-21)	Anti-mitochondria/E2-pyruvate dehydrogenase complex
NuMA-like (AC-26)	NuMA protein
Topo I-like (AC-29)	DNA topoisomerase I

HEp-2 IFA: indirect immunofluorescence assay on HEp-2 cells; dsDNA; double-stranded DNA; DFS70: dense fine speckled 70 kDa protein; LEDGF-p75: lens epithelium-derived growth factor p75; Sm: Smith antigen; CENP: centromere protein; U1-RNP: U1-ribonucleoprotein; Sp100: speckled 100 kDa; PML: promyelocytic leukemia nuclear bodies; NXP-2: nuclear matrix protein 2; U3-RNP: U3-ribonucleoprotein; PCNA: proliferating cell nuclear antigen; NuMA: nuclear mitotic apparatus protein.

International Consensus on ANA Patterns (ICAP) dedicated to building a consensus on the nomenclature and classification of the most prevalent and relevant HEp-2 IFA patterns [6,13–15]. Aiming to achieve the harmonization of the nomenclature, the designation of each HEp-2 IFA pattern was established by consensus and an alpha-numeric code (AC-#, for anti-cell) was assigned for each pattern. For example, the homogeneous nuclear staining pattern was designated AC-1 and the centromere pattern AC-3. The ICAP classification system and relevant information on all patterns, which contribute to the national and international harmonization of AC nomenclature, are now available in 14 languages on the ICAP website (www.anapatterns.org).

3. HEp-2 IFA as a criterion for classification of systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a multi-system chronic inflammatory disease with a variety of immunologic abnormalities and a plethora of circulating autoantibodies [16]. The diagnosis of SLE relies on the presence of well recognized and validated clinical and laboratory elements, most of which are not specific to SLE. Therefore, the diagnosis of SLE may be challenging and demands expertise and critical consideration of alternative diagnoses. Accordingly, dating to the 1970s, classification criteria have been established aiming to assure a minimal homogeneity in series of patients participating in clinical studies. All editions of the classification criteria for SLE have included the ANA and SLE-specific autoantibodies [17–22]. In the formulation of the classification criteria, individual variables potentially contributing to the definition of patients with definite SLE were carefully analyzed and parsed by experts. In general, the analysis includes a comparison of the performance of each variable in a large cohort of patients and appropriately matched controls. As shown in Table 2, the first set of classification criteria for SLE, elaborated by a specially designated committee by the American Rheumatology Association in 1971, contained two independent criteria related to autoantibodies: the LE cell test and a false-positive serological test (VDRL) for syphilis. These can be considered as indirect evidence for the presence of autoantibodies to deoxy-ribonucleoproteins and phospholipids, respectively. The first revision of the SLE classification criteria in 1982 promoted several modifications in the immunologic criteria. The LE cell test and the false-positive syphilis test were merged into a single criterion and anti-double-stranded DNA (dsDNA) and anti-Sm (Smith: U2–U6 ribonucleoprotein) were added to

Table 2
Historical perspective on the classification criteria for systemic lupus erythematosus.

Autoantibodies	1971	1982	1997	2012	2019
LE cell test	Criterion #8				
Anti-dsDNA		Shared		IC #2	Shared IC #3
Anti-Sm		criterion #10	Shared	IC#3	
False-positive VDRL	Criterion #9		criterion #10	Shared IC #4	
Anti-phospholipid*					IC #1
ANA IFA		Criterion #11	Criterion #11	IC#1	Entry criterion
Direct Coombs test				IC#6	

Shaded slots mean that the item was not part of the classification criteria. IC: immunologic criterion; LE: lupus erythematosus; Sm: Smith antigen; dsDNA: double-stranded DNA; VDRL: venereal disease research laboratories; ANA IFA: antinuclear antibody indirect immunofluorescence test.

* Starting 2019, only the anti-phospholipid syndrome criteria autoantibodies (anti-cardiolipin, anti- β 2 glycoprotein I, and lupus anticoagulant) are accepted as SLE criteria.

this criterion. Therefore, the presence of any of these four serologic variables would count as a single criterion. In addition, the ANA IFA test was included as a separate immunological criterion. The 1997 revision of the SLE classification criteria removed the LE cell test (ostensibly due to lack of specificity) and included three tests as evidence of anti-phospholipid antibodies, i.e., the false-positive serologic test for syphilis, the lupus anticoagulant test, and the anti-cardiolipin antibody test. Together with anti-dsDNA and anti-Sm, these were regarded as a single immunologic criterion while the ANA test persisted as an independent immunologic criterion. In the criteria elaborated by the Systemic Lupus International Collaborating Clinics (SLICC) in 2012, anti-dsDNA and anti-Sm antibodies were considered as two independent criteria, and a positive direct Coombs test was included as an additional immunological criterion. Again, the ANA test remained an independent immunologic criterion. Finally, the most recent classification criteria established by the European League Against Rheumatism (EULAR) and the ACR in 2019 promoted the following changes: removal of the direct Coombs test and the false-positive syphilis serologic test, merging of anti-dsDNA and anti-Sm as a single immunologic criterion, and establishment of a positive ANA test at a dilution of 1:80 or greater as a required entry criterion.

This series of criteria editions for the classification of SLE emphasizes the importance of autoantibodies as a hallmark of the disease and indicates that the ANA test has a prominent role across several versions of the classification criteria, as it remained an independent criterion and ultimately it was established as an entry criterion. In this context, some aspects of the ANA test deserve further comment. The ANA test is extremely sensitive for the diagnosis of SLE, as most studies have reported that 92–99% of the patients will present a positive test at some time during the disease [23,24]. This may explain why it has been suggested as an entry criterion in the 1982 publication [18] and ultimately established as an entry criterion in the 2019 EULAR/ACR version [21,22]. However, in cross-sectional studies, the frequency of a positive ANA in SLE can be lower [25–27]. Although the HEp-2 IFA is the predominant assay used worldwide for ANA determination, the recent EULAR/ACR version of the classification criteria mentions that an equivalent solid-phase immunoassay is also acceptable, but the precise performance features of this alternative are vague. The methodology used in the establishment of the five editions of the classification criteria for SLE most likely did not address ANA assays other than the HEp-2 IFA systematically, and most clinical studies refer to the HEp-2 IFA method as the assay used to fulfill the SLE classification criteria.

As mentioned above, the HEp-2 IFA method provides key information, so that positive tests from two different samples may have distinct

clinical and immunological relevance. The HEp-2 IFA titer provides a semi-quantitative estimate of the concentration of the autoantibodies in the sample. This is relevant because SLE patients typically have moderate to high titer ANA, whereas patients with non-autoimmune conditions and healthy individuals with a positive HEp-2 IFA test usually have lower titers [12,28,29]. Most importantly, the IFA staining pattern provides indirect information on the antigenic specificity of the autoantibodies in the sample. Therefore, different HEp-2 IFA patterns have distinct clinical relevance. For example, the homogeneous nuclear (AC-1) and the coarse speckled nuclear (AC-5) are frequently observed in SLE patients, as these patterns are related to the SLE-associated autoantibodies directed against dsDNA/nucleosomes and ribonucleoproteins/U1-RNP/Sm, respectively. In contrast, the dense fine speckled nuclear pattern (AC-2) is very rarely observed in SLE patients and more frequently observed in patients with non-autoimmune diseases and healthy individuals with a positive HEp-2 IFA [12, 29–31]. In a pivotal study on the relevance of HEp-2 IFA patterns, Mariz et al. systematically studied samples from 918 healthy individuals and 153 patients with SAID and found that the AC-1 and AC-5 patterns were exclusively observed in the SAID patients whereas the AC-2 pattern was only observed in healthy individuals. The AC-2 pattern occurred in one-third of the healthy individuals with a positive HEp-2 IFA test [12]. The AC-2 pattern is also quite prevalent in patients with non-autoimmune diseases, as demonstrated by Agustinelli et al. who reported that the AC-2 pattern occurred in 16.7% of non-autoimmune patients with a positive HEp-2 IFA test. In contrast, only 1 out of 197 SLE patients (0.5%) presented the AC-2 pattern [29]. As shown by several groups, the AC-2 pattern is strongly associated with antibody to the transcription coactivator DFS70 [32–34] and anti-DFS70 is observed in 2–5% of the general population as well as in SAID patients [30,32]. However, the bona fide AC-2 pattern is observed when the sample contains only anti-DFS70 antibody, as the presence of additional antinuclear antibodies will mask the distinctive characteristics of the AC-2 pattern [31]. This is the basis for the fact that the AC-2 pattern is seldom present in SLE and frequently observed in the general population and non-autoimmune patients [12,29–31]. Considering the much higher frequency of the AC-2 pattern in non-SLE patients with a positive HEp-2 IFA test (20%–30%) than in SLE patients (<1%), the chances that a HEp-2 IFA test with the AC-2 pattern supports the diagnosis or classification of SLE are very low. In contrast, the AC-1, AC-4, and AC-5 patterns occur in the majority of SLE patients. In a recent cross-sectional survey on HEp-2 IFA staining patterns in SLE, we observed the AC-1, AC-4, AC-5, and multiple overlapping patterns in 94% of 259 sequential SLE patients. In contrast, the AC-2 and other patterns were rare

Table 3

Frequency of HEp-2 IFA patterns in 259 sequential patients with systemic lupus erythematosus (23).

Pattern	Frequency	Pattern	Frequency
Nuclear homogeneous (AC-1)	29.3%	Nuclear envelope (AC-11/AC-12)	0.4%
Nuclear dense fine speckled (AC-2)	0.4%	CENP-F-like (AC-14)	0.4%
Centromere (AC-3)	1.2%	Cytoplasmic reticular (AC-21)	1.2%
Nuclear fine speckled (AC-4)	28.6%	Mitotic spindle fibers (AC-25)	1.2%
Nuclear coarse speckled (AC-5)	14.7%	Cytoplasmic Golgi-like (AC-22)	0.4%
Nucleolar (AC-8/AC-9/AC-10)	0.8%	Cytoplasmic dense fine speckled (AC-20)	0.4%
Multiple patterns	21.2%		

HEp-2 IFA: indirect immunofluorescence on HEp-2 cells; CENP-F: centromere protein F.

(Table 3) [23]. Patterns referring to the staining of the mitotic apparatus and cytoplasm, including the anti-ribosomal P-related cytoplasmic dense fine speckled pattern (AC-20), were also rare. Therefore, we conclude that a positive HEp-2 IFA result with the AC-2 pattern (or some of the other rare patterns) may not represent the “positive ANA test” observed in the series of patients originally used for the establishment of the SLE classification criteria [18–22.] and AC-2 should not represent a “positive ANA test” as required by the recent EULAR/ACR recommendation. The same consideration probably applies to other patterns, such as the centromere (AC-3), discrete nuclear dots (AC-6 and AC-7), nucleolar (AC-8, AC-9, and AC-10), CENP-F-like (AC-14), and several of the cytoplasmic and mitotic apparatus patterns. For example, anti-centromere antibodies are reported in less than 2% [35] and anti-nucleolar antibodies are reported in less than 10% of SLE sera, but when they are found they tend to be associated with overlap syndromes (i.e., the presence of anti-PM/Scl antibodies and myositis, Raynaud’s phenomenon, or malignancy [36]. The NuMA-like pattern (AC-26) is a pattern reported in some SLE patients, but it is more frequently observed in patients with Sjögren syndrome [37,38]. HEp-2 IFA patterns represent a vast array of autoantibodies and their specific molecular targets, but only a minority of them are related to SLE. Thus, the generalization of any HEp-2 IFA pattern to be used as a criterion for classifying a patient as suffering from SLE may be misleading, as SLE-associated patterns (e.g., AC-1 nuclear homogeneous or AC-5 nuclear coarse speckled) could be equally valued as patterns not associated with SLE (e.g. nuclear dense fine speckled). A specific study dedicated to establishing the frequency of the HEp-2 IFA patterns in a large cohort of SLE patients and appropriate controls should contribute to the definition of which patterns should be considered “criterion-grade” for SLE classification.

There are several precedents in the acknowledgment of particular features of laboratory and imaging parameters in the context of classification criteria. For example, in the classification criteria for systemic sclerosis, interstitial lung disease is defined as pulmonary fibrosis most pronounced in the basilar portions of the lungs on high-resolution chest tomography [39]. In the most recent classification criteria for Sjögren’s syndrome, there are explicit restrictions applied to the definition of the criteria on salivary gland histopathology, ocular staining score, Schirmer test, and unstimulated whole saliva flow rate [40]. In the classification criteria for rheumatoid arthritis, there is increasing stratification in the semi-quantitative weight of criteria according to the number and size of involved joints, duration of symptoms, and serum levels of rheumatoid factor and anti-citrullinated peptide antibodies [41]. We propose that the HEp-2 IFA criterion used in the SLE classification system be evaluated according to specific inclusions and exclusions of AC patterns and titer, and not merely as a dichotomic positive/negative parameter. Thus, we propose the organization of an international task force of experts to work with interested members of

the previous SLE criteria committees to evaluate the specific aspects of the HEp-2 IFA test regarding its use as a classification criterion for SLE. By delimiting the patterns and incremental titer thresholds to be considered, the HEp-2 IFA criterion may provide a better contribution to the classification of SLE patients.

Although this critical review focuses on the HEp-2 IFA method, it is important to mention that the current ACR/EULAR criterion “ANA (antinuclear antibodies)” includes the option of results obtained with solid-phase ANA screening immunoassays with at least equivalent performance. There is no strict quantitative specification of what constitutes this ‘equivalence’ and apparently this is left to each laboratory’s discretion. However, it has been shown that the likelihood ratio of ANA screening solid-phase immunoassays is strongly dependent on the intensity of reactivity [28]. This aspect should be addressed as another special task for the definition of the ANA criterion for classification of SLE.

4. HEp-2 IFA as a criterion for classification of autoimmune liver diseases

The three main autoimmune liver diseases are autoimmune hepatitis (AIH), primary biliary cholangitis (PBC), and primary sclerosing cholangitis (PSC), which also utilize autoantibodies as relevant biomarkers in their diagnosis and management. This review will focus on AIH and PBC, because these two diseases have autoantibodies as elements in their diagnostic criteria.

AIH is a chronic inflammatory liver disease with unknown etiology and characterized by high serum levels of immunoglobulin G (IgG) and gamma globulin, circulating autoantibodies, and plasma cell hepatic infiltrates, as specified by the International Autoimmune Hepatitis Group (IAIHG) [42,43]. Currently, serum IgG levels are preferred over serum total gamma globulin, as this represents the main component associated with AIH. Autoantibodies are useful biomarkers and help in the differentiation of the two types of autoimmune hepatitis. Type I AIH affects mainly adults and is the most prevalent form of AIH, being associated with ANA, anti-smooth muscle antibodies (SMA), and anti-F-actin antibodies [44,45]. Actually, anti-F-actin antibodies represent the most AIH-specific autoantibodies in the SMA system (see ahead). Type 2 autoimmune hepatitis occurs in younger individuals, frequently in children, has a more aggressive course, and is associated with antibodies to liver/kidney microsome 1 (LKM-1) and liver/cytosol 1 (LC-1) [46,47]. Other autoantibodies specifically associated with AIH are those against the soluble liver antigen (SLA), also called the liver/pancreas antigen [48], and against the hepatocyte membrane asialoglycoprotein receptor (ASGPR) [49]. Some AIH patients, especially children, may present autoantibodies traditionally associated with SLE, including anti-dsDNA, anti-Sm, anti-SS-B/La, and anti-P ribosomal antibodies [50–52].

In the diagnostic criteria for AIH, a positive ANA test using IFA on rat tissue or a positive SMA test score 1 or 2 points at titer $\geq 1:40$ and $1:80$, respectively [45,53,54]. Because of the higher sensitivity of the HEp-2 IFA test [55], the IAIHG has suggested titers of $\geq 1:80$ and $\geq 1:160$, respectively, as being above the normal reference range, when using the HEp-2 IFA test to address the diagnostic criteria of AIH [53]. In addition, the consensus statement from the IAIHG committee for autoimmune serology recommended that positive samples at the screening stage using IFA on rat tissue should be examined by the HEp-2 IFA test in order to assess the IFA nuclear staining pattern [56]. However, not all HEp-2 IFA staining patterns are equally associated with AIH. Nowadays, many laboratories perform the ANA test only by IFA on HEp-2 cells and the most frequently reported HEp-2 IFA patterns in AIH patients are the homogeneous nuclear (AC-1) or a combination of the homogeneous and the fine speckled nuclear (AC-4), with the homogeneous component prevailing [56]. In addition, the fibrillar cytoplasmic pattern (AC-15) may occur in patients with anti-F actin antibodies, although this association is not absolute [57]. In contrast, other HEp-2 IFA patterns, such

as the Golgi apparatus (AC-22), the cytoplasmic “rods and rings” (AC-23), and the mitotic apparatus (AC-24-28) are not commonly seen in patients with autoimmune hepatitis [7,37,58–60]. Of special interest, the centromere (AC-3), the nuclear multiple dots (AC-6), and the punctate nuclear envelope patterns (AC-12) are preferentially observed in another autoimmune liver condition, namely PBC. The cytoplasmic reticular pattern (AC-21) is strongly associated with anti-mitochondria antibodies, which represent robust evidence for PBC and are taken as evidence against the diagnosis of AIH. However, the AIH diagnostic criteria recommendations established no restriction specifying which patterns should be considered as a criterion [53,54,56]. Thus, as stated in the SLE section above, the ANA diagnostic criterion for AIH could be improved and achieve better performance by restricting the eligible HEP-2 IFA staining patterns to be considered ‘criterion-grade’ patterns. And this is especially relevant because there are specific HEP-2 IFA patterns associated with PBC, an autoimmune liver disease that should be differentiated from AIH.

A few comments are relevant concerning the interpretation of autoantibodies in the IFA test on HEP-2 and rodent tissue. SMA can be found, generally at low titer, in a variety of inflammatory liver diseases, ranging from viral hepatitis to non-alcoholic fatty liver disease (NAFLD). SMA at higher titers and especially those giving the vessel (V)–glomerulus (G), and/or tubule (T) (e.g., VG or VGT) patterns are strongly associated with AIH (Fig. 1). This pattern is frequently referred to as the anti-F-actin pattern even though the titer of this IFA pattern and the levels of anti-F-actin antibodies measured by ELISA do not always coincide. In addition, some samples may yield a VGT pattern on IFA and a negative result in anti-F-actin ELISA, and vice-versa. ELISA anti-F-actin is considered specific for AIH only at high levels. IFA on especially prepared human fibroblasts is considered a very specific method for detecting anti-F-actin antibodies in AIH. SMA is as important as ANA (homogeneous or speckled) in the diagnosis of type 1 AIH. SMA cannot be detected on HEP-2 cells, hence the importance of using the triple rodent tissue IFA in the diagnostic approach to AIH. As mentioned before, use of the triple tissue substrate allows the detection of LKM-1, LC-1, and anti-mitochondrial antibodies, which are important in the diagnosis of autoimmune liver diseases. IFA on HEP-2 cannot replace the IFA on triple rodent tissue, though it may be complementary. ANA are not detected in all patients with type 1 AIH who can have a strongly positive test for SMA.

PBC is a chronic granulomatous inflammation affecting the small intrahepatic bile ducts and predominates in women over the age of 45 years [61]. Because of its very insidious onset and progression, the disease is frequently diagnosed only at advanced stages when liver cirrhosis is already established. PBC should be investigated in patients with fatigue, chronic pruritus, and elevated serum canalicular liver enzymes (mainly alkaline phosphatase and gamma-glutamyl transferase). Elevated serum IgM and cholesterol are additional hints for suspicion of the disease. Circulating autoantibodies are present in virtually all

patients and over 95% of the patients have anti-mitochondria antibodies (AMA). AMA bind enzymes in the outer mitochondria membrane with E2 pyruvate dehydrogenase complex (PDC-E2) being the primary target. AMA are highly specific for PBC and one of the three criteria for the diagnosis of the disease, the two others being cholestatic enzyme biochemistry and compatible liver histopathology [62]. Other relevant autoantibodies strongly associated with PBC include anti-gp210 [63–65] and anti-sp100 [66]. In addition, up to 30% of PBC patients have anti-centromere antibodies [63,67]. It is relevant to note that most autoantibodies associated with PBC produce specific staining patterns on the HEP-2 cell substrate. AMA produce a distinctive cytoplasmic reticular pattern (AC-21), anti-gp210 produces a characteristic punctate nuclear envelope pattern (AC-12), anti-sp100 produces a typical multiple nuclear dot pattern (AC-6), and anti-centromere antibodies produce the unique centromere pattern (AC-3) [68]. Not infrequently, PBC patients have more than one PBC-associated autoantibody, eliciting the

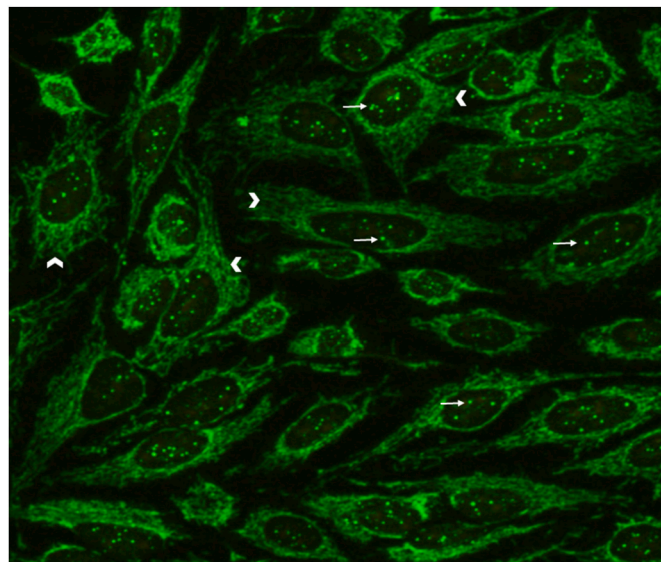


Fig. 2. Indirect immunofluorescence assay on HEP-2 cells (HEP-2 IFA) showing multiple patterns relevant to Primary Biliary Cholangitis (PBC). The image shows two independent HEP-2 IFA patterns staining the cytoplasm and the nucleus, respectively. The coarse granular reticular staining extending throughout the cytoplasm (arrowheads) is highly suggestive of anti-mitochondria antibodies and is designated AC-21 by ICAP. The multiple discrete nuclear dots pattern designated AC-6 (arrows) is suggestive of anti-Sp100 antibodies. Anti-mitochondria and anti-Sp100 antibodies are associated with PBC and, therefore, this combination of patterns should elicit the suspicion of this disease. Serum from a patient with PBC diluted 1:160. Original magnification: x400.

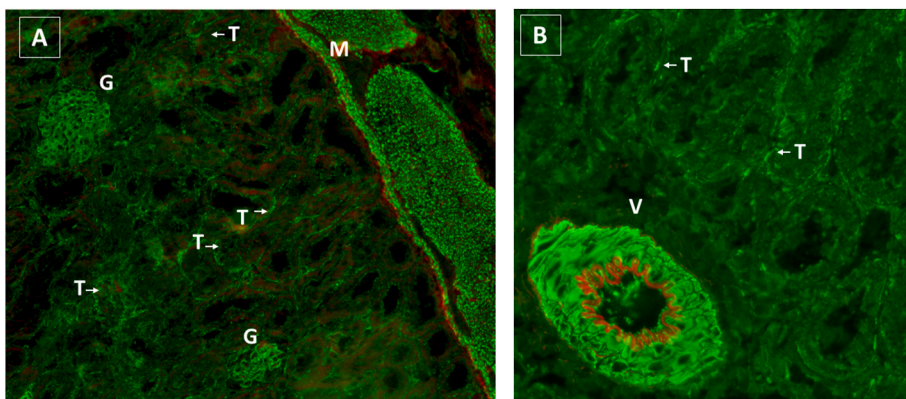


Fig. 1. Indirect immunofluorescence assay on cryopreserved rodent tissue sections showing the patterns relevant to anti-smooth muscle antibodies. Indirect immunofluorescence on composite kidney and stomach sections, using human serum diluted 1:80. (A) Reactivity with the glomeruli (G), extracellular fibrils of the renal tubules (T), and the muscle layer of the stomach (M). (B) Reactivity with the muscle layer of small arteries (V) and extracellular fibrils of the renal tubules (T). The VGT pattern has higher specificity regarding the associations with anti-F-actin antibodies. The elastic layer of arterioles appears in red due to the characteristic autofluorescence of elastin. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

appearance of HEp-2 IFA multiple patterns that are very typical for PBC (e.g. AC-6/AC-21, AC-12/AC-3, AC-3/AC-12/AC-21) (Fig. 2). This observation highlights the relevance of the HEp-2 IFA pattern in the investigation of patients suspected of having PBC.

The classic clinical presentations of AIH and PBC are so characteristic that there is usually no difficulty in discriminating the two diseases in many patients. However, there is a considerable proportion of patients in whom the clinical presentation is not clear-cut. In such cases, the application of the current version of the AIH diagnostic criteria may be troublesome concerning the HEp-2 IFA criterion. A positive HEp-2 IFA test can score up to 2 positive points for the diagnosis of AIH [53,54], however, a positive HEp-2 IFA test with AC-3, AC-6, AC-12, or AC-21 patterns reflects autoantibodies associated with PBC and not AIH. This represents a genuine opportunity for improving the diagnostic criteria for AIH, by restricting the HEp-2 IFA patterns to be considered as a positive score. The homogeneous nuclear pattern (AC-1), the fine speckled nuclear pattern (AC-4), and the cytoplasmic fibrillar pattern (AC-15) should be considered as relevant results for this criterion. In contrast, the cytoplasmic reticular pattern (AC-21), the punctate nuclear envelope pattern (AC-12), the multiple nuclear dot pattern (AC-6), and the centromere pattern (AC-3) might be considered as a negative scoring parameter for the diagnosis of AIH, as these suggest the diagnosis of PBC or AIH/PBC overlap syndrome [68].

5. Juvenile idiopathic arthritis

Juvenile idiopathic arthritis (JIA) encompasses several forms of chronic arthritis of unknown etiology with onset before 16 years of age. There are specific sets of classification criteria for each JIA subtype. The classification criteria for the early-onset oligoarticular ANA-positive JIA subtype includes three elements, one of which is the presence of two positive ANA tests with a titer $\geq 1:160$ (tested by IFA) at least three months apart [69]. In addition to representing a disease classification criterion, the positive ANA is used by clinicians to predict the risk of uveitis in JIA [70,71]. Although the HEp-2 IFA pattern is not mentioned in most publications on JIA, some studies report that the prevalent patterns are the nuclear homogeneous (AC-1) or nuclear fine speckled pattern (AC-4) [72]. As observed in the above sections for SLE and autoimmune liver diseases, it is likely that a restricted set of HEp-2 IFA patterns yet to be clearly defined are relevant for the classification and prognosis of early-onset oligoarticular ANA-positive JIA.

6. Conclusions

The classification criteria for SLE and certain JIA subtypes as well as the diagnostic criteria for AIH include the HEp-2 IFA (or equivalent solid-phase immunoassay) test result. A positive HEp-2 IFA test is represented by different patterns and each pattern is associated with a limited subset of autoantibodies. Considering that a positive HEp-2 IFA test represents a vast array of autoantibodies and many of these are not directly relevant to these diseases, specification of the HEp-2 IFA patterns to be considered as a classification criterion offers an opportunity to restrict the array to relevant 'criterion grade' autoantibodies associated with these diseases. It is worthwhile considering whether and how specifying which HEp-2 IFA patterns qualify as a valid criterion would impact the reliability and accuracy of classification/diagnostic criteria for SLE, AIH, and JIA. This may marginally decrease the sensitivity of the HEp-2 IFA test for SLE (and AIH and JIA-uveitis) and accordingly limit the utility of the ANA as an entrance classification criterion for SLE. On the other hand, this approach would likely increase the specificity of the ANA criterion in each of these conditions. It must be emphasized that the HEp-2 IFA patterns are very useful indicators of the potential autoantibody specificities present, however, in all cases the suspected autoantibodies should be determined using antigen-specific immunoassays.

Funding

This review did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. Luis Andrade receives grant PQ-1D 310,334/2019-5 from the Brazilian National funding agency CNPq.

Credit author statement

LECA: preparation of the first draft version of the manuscript and coordination of the exchange of contributions from the other authors. Editing and finishing the final version. Obtaining approval of other authors for the final version. **JD:** edition of the first and successive versions of the manuscript and contribution of appropriate content. **DV:** edition of the first and successive versions of the manuscript and contribution of appropriate content. **MJF:** edition of the first and successive versions of the manuscript and contribution of appropriate content.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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