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Review

ANA as an entry criterion for the classification of SLE

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ABSTRACT

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease with highly variable clinical and immunological manifestations. In the classification of patients with this condition, the presence of an antinuclear antibody (ANA) is an important element, with new criteria from the American College of Rheumatology and European League against Rheumatism positioning ANA positivity by an immunofluorescence assay on HEp2-cells (HEp2-IFA) or by an equivalent solid phase assay as the entry criterion. This positioning is based on assumptions about the frequency of ANA positivity in SLE as well as the reliability of the assays. Studies indicate that these assumptions are still a matter of uncertainty since both types of assay show considerable variability and patients with SLE may display negative results in ANA testing. These findings suggest caution in positioning ANA positivity as an entry criterion for classification and point to the value of alternative serological approaches for ANA determinations.

1. Introduction

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease which displays highly variable clinical and immunological manifestations [1,2]. Among these manifestations, the production of antibodies to components of the cell nucleus (antinuclear antibodies or ANA) is a prominent serological finding. These antibodies target DNA, RNA, proteins and protein-nucleic acid complexes, with antibodies to DNA and Sm, a complex of proteins and uridine-rich RNA molecules, highly associated with SLE. Other ANA responses, such as anti-Ro, are frequent in SLE but also occur in other autoantibody-associated rheumatic diseases (AARD). The use of serological testing for classification of patients with SLE is now a topic of great interest and controversy and is the subject of this review.

2. ANA testing in SLE

2.1. ANA assays

For the measurement of ANA, the indirect immunofluorescence assay on HEp2 cells (HEp2-IFA) has long been viewed as a gold standard and therefore widely used [3–5]. This assay, which uses the HEp2 cell line as a source of cells, allows detection of antibodies to a wide variety of nuclear molecules; antibodies to antigens located in the cytoplasm or expressed by mitotic cells can also be detected by this assay. Results are reported in terms of a titer and pattern of cellular staining [3,6]. While the pattern of staining can provide some insight into the molecule targeted by an ANA (e.g., anti-centromere antibodies), in general, the antigens recognized by individual sera are unknown and many lupus sera contain multiple specificities that can lead to highly complex patterns of fluorescence. At present, many alternative approaches allow ANA detection. These assays include ELISA, fluoroenzyme immunoassays (FEIA), chemiluminescence assays (CLIA) and laser addressable bead multiplex assays (ALBIA) that utilize purified or cloned antigens since the specificity of many target antigens has been defined molecularly.

2.2. Criteria for SLE classification

Because ANA were originally discovered in patients with SLE, these antibodies have been considered a key if not invariable immunological finding. As such, the presence of ANA has been considered a criterion in the classification of patients with SLE in either the American College of Rheumatology (ACR) or the Systemic Lupus International Collaborating Clinics (SLICC) criteria set [7,8]. For the ACR criteria, a positive antinuclear antibody is defined as “An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs.” The SLICC criteria designate “ANA

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above laboratory reference range" as an immunological criterion. These criteria sets also include the expression of antibodies to DNA or the Sm antigen as a separate criterion. While both markers for SLE, anti-DNA and anti-Sm are independent responses and show distinct patterns of expression over time [9].

An important consideration in the use of ANA positivity as a classification criterion concerns the high frequency of ANA expression in the general population. Depending on the assay and on the titer used as the cutoff for the HEp2-IFA, up to 20% of otherwise healthy individuals are ANA positive, with the frequency higher in women than in men [10–12]. The specificity of antibodies leading to ANA positivity among healthy individuals is, in general, not known although ANA positivity can result from antibodies to an antigen known as DFS70 [13–15]. These antibodies produce an IFA pattern known as dense fine speckled. The high frequency of false positivity can complicate serological testing in the routine clinical setting, especially when the pre-test probability of lupus or another AARD is low. On the other hand, positivity for specific autoantibodies can be clinically more significant since, in asymptomatic individuals, it raises the possibility of pre-autoimmunity for an AARD [16,17].

2.3. The position of ANA in the new classification criteria

While the high frequency of false positivity of ANA has long been established, nevertheless, a new set of classification criteria by the ACR and the European League Against Rheumatism (EULAR) have repositioned the importance of ANA [18–21]. Thus, in the new criteria, a positive ANA is required for further consideration for classification. In the original version of these criteria, ANA positivity was defined as positivity of HEp2-IFA at a titer ≥1:80; because of limited availability of IFA testing in certain settings, the final version of the criteria allowed ANA positivity by a solid phase assay with at least equivalent performance. Other clinical and laboratory features can be judged as evidence for SLE using a scoring system. A number of assumptions on the nature of ANA expression in SLE as the gold standard entry classification criterion underlie these criteria. In view of the impact of repositioning ANA testing for classification, it is important to review recent information on serological testing to determine the utility of the ANA as a starting point for classification.

2.3.1. Ideas underpinning the new criteria

In considering the role of serological testing for disease classification, three ideas have long dominated thinking about the nature of ANA expression in SLE; these ideas underpin the new ACR-EULAR criteria using either the IFA-HEp2 or an equivalent solid phase assay. The first idea is that ANA expression is essentially invariable in SLE and, to the extent that it may occur, ANA negative lupus is rare [22]. The second idea is that SLE expression persists over time; as such, ANA testing can be used for disease classification at any point in the disease course. The third idea is that the HEp2-IFA or more recent equivalent solid phase assays are the gold standard for ANA detection, with different assays or kits providing similar results [12].

The evidence regarding these ideas can be reviewed to help answer the question of whether the ANA should be used as the entry criterion for patient classification.

2.3.2. The frequency of ANA expression in SLE

While many studies have shown a high frequency of ANA positivity in SLE, few studies have ever reported a frequency of 100%; a somewhat lower number is more common (i.e., 95–99%) although the demonstration of a lower frequency of positivity is not unusual in cross-sectional studies [18,22–24]. One reason that these findings do not generate more interest or scrutiny relates to the nature of studies to validate immunoassays. Since both HEp2-IFA and solid phase assays for ANA testing are useful for many AARD, including Sjogren's syndrome, myositis and progressive systemic sclerosis, the patient cohorts used for assay validation can include many different AARD. As long as the frequency of false positive results is low, an assay can be considered useful and reliable and gain regulatory approval. On the other hand, the impact of ANA negative SLE on efforts at classification should nevertheless be recognized even if the frequency of seronegative disease is reasonably low as indicated in more recent literature [22].

In many respects, the actual frequency of ANA positivity in SLE is not known since it depends on the population studied, disease duration, prior therapy and, as will be discussed, the assay used for ANA determinations. Thus, the idea that ANA expression is invariable in SLE remains somewhat of an assumption, with existing data suggesting caution in positing that demonstration of ANA positivity is required for classification. In this regard, studies on murine models of SLE are not really helpful since the designation of a strain or experimental model as "murine lupus" almost always requires the presence of serological abnormalities (ANA or anti-DNA) and usually renal disease. Without these immunological and clinical findings, a murine model would not attain the status as lupus.

2.3.3. The persistence of ANA expression over time

In the clinical context, ANA testing is usually performed at the time of initial patient evaluation for consideration of SLE or another AARD. If this testing is done close to the time of the development of signs, symptoms or laboratory findings of SLE (e.g., proteinuria, increased creatinine), then the serology should reflect events indicative of ongoing inflammation or autoimmunity. Once ANA positivity is established and the criterion met, there is little reason in the routine clinical setting to repeat this test especially as levels of ANA are not useful markers for activity; the same is true for antibodies to ENA (extractable nuclear antigen) such as anti-Sm, anti-RNP or anti-Ro. In contrast, testing for anti-DNA or complement is commonly performed over time because of the utility of these markers for assessing disease activity [25].

Available data on the persistence of ANA experiments are limited, although a few studies have assessed the expression of certain specific ANA during the course of disease. These studies have shown that, over a timeframe of a few years (e.g., 2–3 years), levels of antibodies such as anti-Sm, anti-RNP and anti-La are relatively constant. Such a pattern would be considered consistent with a role of plasma cells, although autoantibody responses may differ from that of conventional antibodies in the emergence of antibody secretory cells from naïve B cells over time [26,27]. In contrast, studies have provided some informative examples for both the acquisition and loss of autoantibody expression [28–33]. It is unclear whether the loss occurs as a result of treatment or natural history of disease and whether the generation of a new antibody can occur in the face of treatment. While these issues require further investigation because of their implications for disease mechanisms, they nevertheless indicate that autoantibody expression may not be durable and persistent. Data clearly show that anti-DNA production is variable, accounting for the value for repeat testing [25].

Repeat ANA testing may occur in at least two circumstances. The first is re-evaluation of a patient with established disease, often when the patient is evaluated by a new provider. In face of a historical diagnosis of SLE, many providers would "restage" the patient to ensure the diagnosis and help assess disease activity. The second circumstance is a clinical trial. Most trials of new agents or treatment approaches for SLE involve patients with established disease with persistent disease activity. In the trial setting, disease duration can be many years, with 10 years not unusual. This setting clearly differs from that of a new patient evaluation, with serology possibly changing as a consequence of prior treatment involving agents that affect B cells (e.g., rituximab or cyclophosphamide)

Among studies that have awakened interest in ANA expression in patients with established (and active) SLE, the trials with belimumab have been very informative [34,35]. These studies demonstrated that, in the Phase 2 trials, the frequency of ANA negativity was higher

(approximately 30%) than that reported by more recent literature, including data used in the development of the new criteria. While the reason for this high frequency of ANA negativity in the Phase 2 population is not known, treatment response was much more likely in patients who were ANA positive. As a result, the Phase 3 studies required evidence of serological activity in terms of a positive ANA and/or positive anti-DNA and/or low complement levels. As these trials were successful and led to regulatory approval, other sponsors have followed this approach to enrich patients whose condition is likely to respond to immune modulation. An alternative approach allows entry of patients with historical ANA positivity but negativity at the time of screening as long as the diagnosis is confirmed by experts in an adjudication process. This approach can lead to the inclusion of a significant percentage of ANA negative patients into trials [36].

2.3.4. ANA detection

The discordancy between a clinical diagnosis (or classification) of SLE and the serological status is at the heart of any question about the use of the HEp2-IFA or solid phase assays as the gold standard tool for ANA detection. At least two factors can lead to a high frequency of ANA negativity in patients with established SLE: biological and technical. Thus, it is possible that serology varies over time in a way not previously appreciated, whether from the natural history of disease or the effects of treatment. The second factor is technical and relates to the performance characteristics of existing assays.

2.3.5. ANA assay variability

Two recent studies have addressed the issue of assay variability. The first involved a cross-sectional study of 103 patients with SLE followed at an academic center [37]. The cohort included patients with and without renal disease as well as varying disease duration. Sera were assayed with three different HEp2-IFA kits that are widely available. The sera were also tested with an ANA ELISA as well as a multiplex assay for ANA detection. As these results showed, the frequency of ANA negativity with HEp2-IFA kits ranged from 4.9 to 22.3%. The frequency of ANA negativity was 11.7% by ELISA and the frequency of negativity by multiplex was 13.6%. Thus, no assay format produced 100% positivity although assay combinations could approach that number.

A second study included a large population of patients who had participated in a Phase 2 trial for an anti-IL-6 agent to treat active lupus [38]. For this study, entry of ANA negative patients by the laboratory at screening was possible if an adjudication committee considered that the diagnosis of active lupus was correct. By the screening laboratory, 23.8% were ANA negative. Sera of these patients were then tested with five different commercially available HEp2-IFA kits. The results were similar to the cross-sectional study since the frequency of ANA negativity ranged from 0.6 to 27.6%. Only one patient was ANA negative by all five kits. Interestingly, the presence of the interferon signature was more common in the ANA positive than the ANA negative population.

In contrast to these results, determination of ANA status in a large international SLE inception cohort (n = 1137) indicated that 6.2% of SLE patients were negative for anti-cellular antibodies by HEp2-IFA [22]. Other studies have demonstrated similar sensitivities for HEp2-IFA and solid phase assays [12,39–41]. In this regard, although several studies have reported a high sensitivity of HEp2-IFA for SLE, the variance in results is higher for HEp2-IFA compared to FEIA, as recently reported in a meta-analysis [42]. The lower variance for FEIA is related to the fact that the assay is automated and comes from a single manufacturer.

Results obtained by ELISA from different manufacturers can also show significant variance. De Almeida Brito et al. studied 72 SLE patients with three different ELISA systems and found a sensitivity ranging between 83% and 97% [43]. These studies clearly indicate that ANA methods show considerable variability which may result from a variety of technical factors. For IFA-HEp2, these factors include the culture conditions of cells, fixative, adherence to slides, the conditions of the incubation steps, the nature of the anti-immunoglobulin reagents for detection, and the performance of the microscope [44]. For ELISA and other solid phase assays, the nature of the antigens used for the assay, either purified or recombinant, can affect assay performance. Overall, assay variability appears lower for automated solid phase assays, especially when kits come from the same manufacturer; substantial variability, however, can occur with ELISAs from different manufacturers.

2.3.6. ANA assay specificity

The amount of autoantibody measured is also an important consideration for the use of ANA assays for classification. For the HEp2-IFA, a cutoff of 1:80 is generally considered to have a low specificity. In general, the specificity of FEIA, CLIA and ALBIA is higher than the specificity of HEp2-IFA at a cutoff of 1:80 [10,12,39,40,42,45–49]. In contrast, the specificity of ELISA varies depending on the assay used [43]. In the majority of the studies, the sensitivity of FEIA, CLIA and ALBIA has been lower than the sensitivity of HEp2-IFA [43].

The low specificity of HEp2-IFA relates to the significance of low levels of ANA expression. In general, studies indicate that higher levels of antibodies are associated with a higher specificity and likelihood ratio for disease. For example, in a large study including 9851 unique consecutive patients tested for ANA (including 83 SLE patients), the likelihood ratio of a HEp2-IFA titer 1:80 for SLE was estimated to be 1.1, which indicates no difference in pre-test to post-test probability [49]. In this study, the estimated titer-specific likelihood ratio increased to 4.1, 7.0, 10.8, 14.7 and 21.8, for a titer of 1:160, 1:320, 1:640 and ≥ 1:1280, and reactivity to overexpressed SS-A on the HEp-2000 substrate respectively [49].

This variability is significant and, if single tests are used to determine eligibility, the screening process could limit entry of many patients. Correspondingly, with single tests, patient classification could also be affected especially for patients with longstanding disease.

Other studies have shown that HEp2-IFA can detect antibodies in SLE patients (even at high antibody titer) that are not detected by ALBIA [45], FEIA [10], or CLIA [50]; of note, FEIA, CLIA or ALBIA can detect antibodies that are not detected by IFA [10,45,50]. Thus, increasing evidence supports the idea that the combination of an IFA with solid phase assay adds value and can increase overall diagnostic accuracy [12,41,46–54]. This approach has mainly been documented for combining HEp2-IFA with FEIA or CLIA. A recent meta-analysis has confirmed that combining HEp2-IFA with FEIA adds clinical value compared with single test results alone [42]. A combination approach is important since double positivity has a higher likelihood ratio than single positivity and either assay can detect antibodies that are missed by the other assay.

2.3.7. Alternative approaches for ANA detection for classification

Assuming that ANA may be an entry criterion for SLE classification and that all patients with SLE are ANA positive at some point in their illness, current assays, especially when used alone, limit this determination. As discussed above, the HEp2-IFA is subject to considerable variability although some kits appear capable of detecting 95% or greater of patients even in those patients with established disease [10,12,39–41]. Together, these studies suggest that the combination of the HEp2 and another approach (e.g., ELISA, FEIA, CLIA, LINE assay, ALBIA) provides complementary and confirmatory evidence to establish serological status.

Every assay for the autoantibody detection in AARD has limitations, including those directed for detection of specific autoantibodies. For example, among anti-DNA assays, which also can be used for classification, the frequency of positivity can vary depending on the type of DNA used and the measurement strategy (i.e., a fluid phase Farr assay vs an ELISA) [25,55,56]. For antibodies to proteins, variability may occur because of the use of cloned proteins vs purified proteins vs peptides as the test antigens. Furthermore, some antibodies bind to

Table 1
Strengths and limitations of HEp2-IFA (A) and solid phase assays (SPA) (B) for ANA detection in SLE classification.

A. HEp-2 IFA

Strengths	Limitations
Variety of different target autoantigens (> 100)	High frequency of ANA positivity in the general population (low specificity)
Discovery tool for novel autoantibodies	Subjectivity of reading; need for training and expertise; difficulty in distinguishing patterns
High antibody levels (≥1:640) have a high specificity for SLE and AARD	Low sensitivity for detection of certain clinically relevant autoantibodies to anti-ribosomal P, SS-A/Ro60, Ro52/TRIM21, Ku
Detects (high titer) antibodies that are undetected by SPA	Assay variability and poor standardization across manufacturers
Pattern can give an indication of the underlying antigen specificity	Technical issues in assay performance
Automated systems with quantification of light intensity units allow for improved quality control and harmonization across laboratories	Variable expression of ANAs over time (?)
	Uncertainty concerning whether ANA expression is essential to SLE

B. Solid phase assays

Strengths	Limitations
Higher reproducibility than HEp2-IFA	Marked differences in performance between different assays and technologies of manufacturers
Allow automation	A limited number of antigens included, limiting sensitivity
May detect antibodies that are not detected by HEp2-IFA (e.g., anti-SS-A, anti-ribosomal P antibodies)	

Table 2
Strengths of combining HEp2-IFA with SPA.

Combined positivity has a higher likelihood ratio for disease than single positivity
Combined negativity has the lowest likelihood ratio for disease
HEp2-IFA might detect reactivities that are missed by SPA and SPA can detect reactivities that are missed by HEp2-IFA (e.g., anti-SS-A, anti-ribosomal P).

conformational determinants that may result from interactions among molecules (e.g., DNA and histones) or a multicomponent complex [25]. Table 1 presents strengths and limitations of the HEp2-IFA and solid phase assays for ANA testing for SLE classification.

3. Conclusions

As this discussion highlights, the main question in serological testing for determining ANA status is not whether HEp2-IFA is better than solid phase assays or vice versa. Rather, data from many studies indicate that each approach has advantages and disadvantages that may affect the use of a single method for ANA detection as an entry criterion for SLE classification. Furthermore, emerging evidence indicates that the combination of the two techniques provides the best results regarding sensitivity and specificity (Table 2). In addition to classification, assay combinations may be informative in the setting of routine diagnosis in the clinic.

Disease classification and, by extension disease diagnosis, of a clinically and immunological heterogeneous disease is often difficult. In the face of data on assay variability and the unknown (and unknowable) frequency of ANA positivity in SLE, requiring ANA positivity as the preeminent criterion for classification has uncertain aspects that should be taken into account. While future research will be needed to resolve these issues, recent studies clearly show that combinations of the available IFA and the solid phase ANA assays may offer a practical and sensible solution at the present. Improvements of testing in the future will likely be provided by assays based on profiles of autoantigens specifically recognized by autoantibodies in SLE; these assays are being tested now and hopefully will be available for general use soon [57].

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