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The Past, Present and Future of Antinuclear Antibody (ANA) Testing

Marvin J. Fritzler, MD, PhD

This article is in memory of Dr. Eng M. Tan (Emeritus: The Scripps Research Institute) and acknowledges the remarkable mentorship and tremendous contributions to our understanding of anti-nuclear antibodies (ANA). Dr. Tan passed away in 2024 at the age of 97.

Introduction

More than 70 years have passed since the discovery of the lupus erythematosus (LE) cell and the development of the LE cell test, which led to the 'tipping point' for the discovery of antinuclear antibody (ANA), or what should more correctly be referred to as anti-cellular antibodies (ACA).[1] Paralleling the evolution of ANA testing based on the indirect immunofluorescence assay (IFA) on cryopreserved organ sections in the 1960s and through the early 1970s was an 'explosion' in the spectrum of ANA and a remarkable transition in technologies used to detect ANA. This included the transition to IFA on HEp-2 cell substrates beginning in the late 1970s.[2] While some of the 'octogenarian' immunoassays such as double immunodiffusion, hemagglutination, complement fixation, radioimmunoassay, and counterimmunoelectrophoresis are fading into oblivion, the ANA IFA has prevailed because of its world-wide use as a screening test for systemic autoimmune rheumatic diseases (SARD), diagnostic criteria for autoimmune hepatitis, a risk factor for the development of uveitis in juvenile idiopathic arthritis, and the entry criterion for classification of systemic lupus erythematosus (SLE).[1,3] ANA testing, once regarded the domain of rheumatologists and clinical immunologists, has witnessed a widening spectrum of clinicians using these tests because of its links to a growing spectrum of autoimmune and autoinflammatory conditions.[2] All of this is set against the background of remarkable advances in autoantibody detection, especially the emergence of newer high-throughput (i.e., faster turn-around-time for results), multi-analyte array technologies (MAAT). These technologies use comparatively small serum or plasma volumes and provide higher specificity while detecting a broad range of SARD autoantibodies.[4]

Over 180 autoantibodies have been described in SLE, more than 30 in systemic sclerosis, and greater than 20 in autoimmune inflammatory myopathies (AIM). The ongoing discovery and expanding spectrum of autoantibodies in SARD

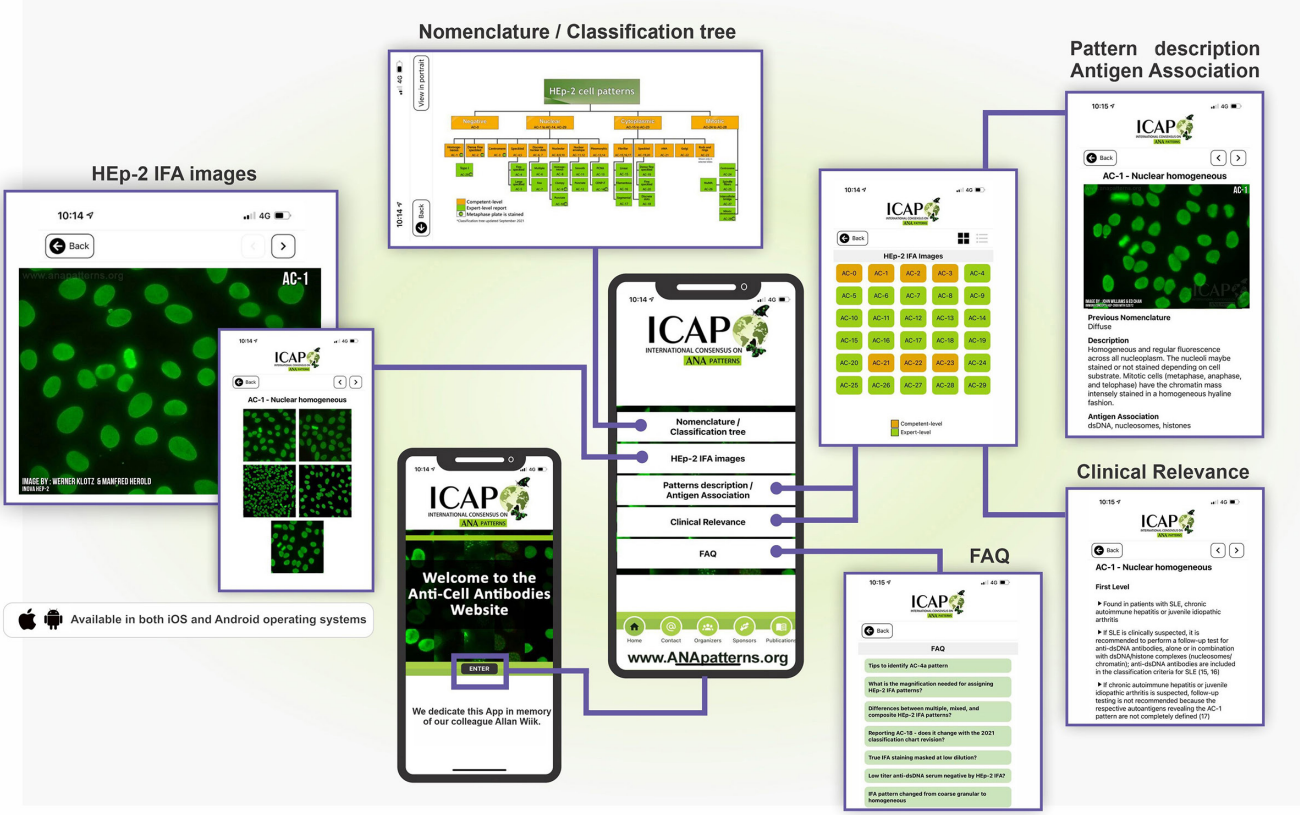

Figure 1. A wealth of information on the nomenclature and related clinical features associated with a wide spectrum of anti-nuclear antibodies (ANA) is available on the **Consensus on Autoantibody Patterns (ICAP) website** and can be easily accessed through this ICAP app available free of charge; *courtesy of Marvin J. Fritzler, MD, PhD.*

might be considered as unnecessary but a primary rationale for these efforts is to identify new and clinically actionable ANAs that close the 'seronegative gap' in SARD.[5]

Despite over half a century of 'progress', one of the major challenges continues to be the standardization and harmonization of ANA testing.[6] The history of this problem is extensive and still plagued by significant limitations despite the concerted efforts of various global committees representing world-wide input. These include the Serology Sub-Committee of the International Union of Immunology Societies, which has provided easily-accessed reference sera containing the major autoantibody specificities, and an extension of those efforts by the International Consensus on Autoantibody Patterns (ICAP) to standardize the nomenclature of the main ANA patterns and ANA test reports.[3,7] Clinicians should take advantage of the wealth of ANA information on the **ICAP website** that can be easily accessed with the ICAP "app" (**Figure 1**).

Another major challenge to the clinical use and interpretation of ANA testing is evidence showing that the prevalence of positive ANAs in the general population is increasing, with some studies reporting rates higher than 30%.[8] This increase has been attributed to several factors including environmental agents and exposure to xenobiotics, climate change, high sensitivity and low specificity of many ANA methodologies, and pandemics including COVID-19.[9,10]

It is important to understand that for an accurate interpretation of the ANA test results, both the titers and IFA patterns are very important.[7] Although there are geographic variations in ANA IFA pattern reporting with some regions restricting reports to 'nuclear' staining patterns, many laboratories also report IFA staining of cytoplasmic and mitotic components cells.[1,7] Many sera also demonstrate more than one AC pattern (e.g., mixed patterns) or have an IFA pattern not currently characterized by ICAP that then receives an AC-XX designation followed by a descriptor.[7]

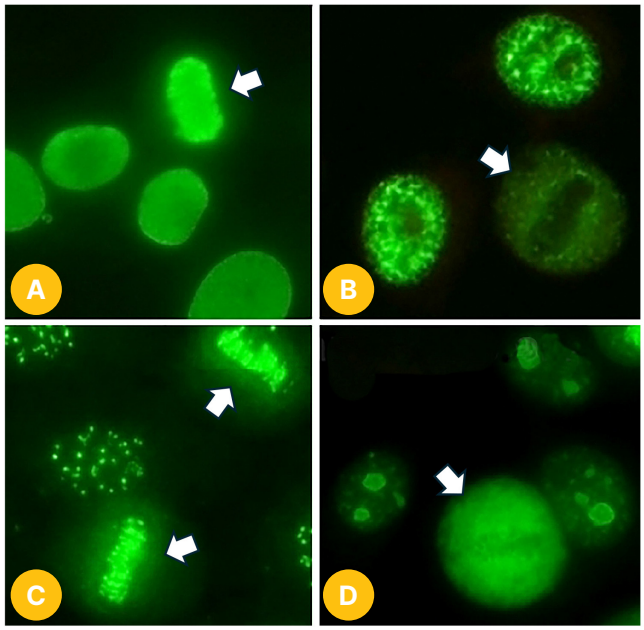

Figure 2. Common HEp-2 indirect immunofluorescence assay (IFA) patterns observed in systemic autoimmune rheumatic diseases (SARD) sera: **a)** homogeneous/diffuse nuclear staining (AC-1) associated with antibodies to dsDNA and nucleosomes; **b)** speckled nuclear staining (AC-4, AC-5) associated with antibodies to Sm and other nuclear ribonucleoproteins (RNP); **c)** discrete speckled nuclear staining (AC-3) associated with anti-centromere antibodies and limited cutaneous systemic sclerosis; **d)** anti-nucleolar antibodies (AC-8, AC-9, AC-10) associated with diffuse cutaneous systemic sclerosis (SSc); *courtesy of Marvin J. Fritzler, MD, PhD.*

In general, SARD are characterized by high titer (>1:320) ANA, with the most specific HEp-2 IFA patterns (**Figure 2**) including AC-1 (anti-dsDNA, anti-nucleosomes), AC-3 (anti-centromere in limited cutaneous systemic sclerosis [SSc]), AC-4 and AC-5 (anti-Sm and U RNPs in SLE and mixed connective tissue disease), AC-8, AC-9, AC-10 (nucleolar autoantibodies characteristic of diffuse cutaneous SSc [dcSSc]), AC-29 (associated with anti-topoisomerase I/Scl-70 in dcSSC), and AC-30 (anti-Ro60 and anti-nucleosomes observed in Sjögren disease [SjD] and SLE). From this overview, it appears that SARD autoantibodies typically stain and target the HEp-2 nuclei. Nevertheless, other IFA patterns are occasionally observed in SARD, and often point to overlapping conditions, inflammatory and infectious diseases, malignancy, or even the absence of overt disease.

For example, the AC-2 HEp-2 IFA pattern, when confirmed as monospecific (e.g., no other known autoantibodies detected) anti-DFS70 antibodies by an antigen-specific immunoassay, rules out the diagnosis of SARD in >95% of cases,[11] while the AC-1, AAC-4, and AC-30 IFA patterns, which are similar to AC-2, -3 tends to "rule in" a SARD diagnosis. It needs to be appreciated that despite the high sensitivity of the HEp-2 IFA test for SARD, the approximate frequency of a negative ANA is 5% in SLE, 3% in SSc, 1% in MCTD, 25% in SjD and 40 % in the broad spectrum of autoimmune inflammatory myopathies (AIM). In addition, many of the HEp-2 IFA patterns observed in AIM are not closely correlated with the specific routinely-detected autoantibodies (e.g., anti-Jo1, anti-MDA5, anti-HMGCR). To summarize, the clinician should not rely on HEp-2 IFA screening when a diagnosis of SjD or AIM is being considered.

As evidenced by the Choosing Wisely recommendations endorsed by the Canadian Rheumatology Association in 2015, and 'consensus' statements of the American College of Rheumatologists and the American College of Pathologists, the use and abuse of ANA testing is the subject of considerable criticism.[12] There is general agreement that once a SARD diagnosis has been established the ANA should not be repeated. However, there are exceptions in which a repeat ANA may be helpful, such as in SARD patients who develop features of another or overlapping condition, with an example being limited cutaneous SSc patients who develop anti-mitochondrial antibodies suggesting the presence or onset of primary biliary cholangitis. Recently, the Effective Health Care (EHC) Program stated that there is a "broad clinical consensus that ANA testing (including ANA sub-serologies) should not be used to screen for SARDs in primary care," Therefore, "there is no clinical uncertainty that a new systematic review could potentially address."[13] In other words, despite evidence to the contrary[12], this evidence indicates that ANA testing must be curtailed (particularly in primary care) because of its "poor positive and negative predictive values (positive predictive value [PPV] 29%, negative predictive value [NPV] 77%), leading to increased health care costs with unclear clinical benefit." With these issues in mind, my perspectives on the future of ANA testing are summarized in four questions.

First, what should be done with well-known evidence that some ANA and related autoantibodies antedate the diagnosis of SARD by up to 20 years?[14] Unfortunately, the proclamations from Choosing Wisely and the EHC arise from a rather myopic perspective that ANA testing should be limited to patients with a high PPV/low NPV for SARD. Clearly, because the frequency of a positive ANA test approaches 30% in the population, ANA testing should not be done on patients without any clear evidence of a SARD.

Second, the circular logic is difficult to rationalize because, if the suspected SARD patient has a high PPV, why should the ANA test be performed at all? Some argue that this is necessary for suspected SLE individuals to fulfill the ACR/EULAR classification criteria. However, it is important to remember that these are classification criteria, not diagnostic criteria. An important aspect that seems to be overlooked is that when conventional diagnostic and an 'intent to treat' approach to ANA testing is used, the diagnosis of SARDs is delayed. As a result, a considerable proportion of patients have active disease and end organ damage at the time of or shortly after the diagnosis is made.[12] This delay in diagnosis is associated with remarkably high direct and indirect health care costs.[15] Conditions such as renal disease, pulmonary fibrosis, hypertension, and irreversible joint damage, to name a few, require much more intensive and expensive care. This leads to a decreased health-related quality of life and additional increased indirect costs. These observations are prompting many clinicians to reconsider their approach to SARDs, making concerted efforts to achieve much earlier diagnoses, as exemplified by studies of undifferentiated connective tissue disease[16] and the Very Early Diagnosis of Systemic Sclerosis (VEDOSS) cohort.[17] It needs to be appreciated that achieving an earlier diagnosis is currently primarily in the domain of primary health care providers, who serve as the SARD 'case finders'.[12] Screening tests such as ANA for SARD are used as part of 'case finding'. Then, based on clinical acumen, patients are referred to subspecialists for evaluation and appropriate management. In my view, it is quite unfortunate that some rheumatologists are unhappy when they receive a referral for an ANA-positive individual who has "nothing". I think that this situation is a win-win for the patient, the physician, and the health care system. In my view, a much clearer and proactive approach is needed for assuring these apparently 'healthy' individuals that a positive ANA is not a diagnostic of a disease. It is beyond the scope of this brief overview to cite the growing literature that other biomarkers can be used as predictors of disease in ANA-positive individuals that might have a low pre-test probability of a SARD. This approach will be more realistic and actionable when artificial intelligence (AI) (discussed briefly below) is used to weigh and sort various aspects of an individual's health to predict an emerging SARD or other condition.

Third, if primary care physicians and 'nurse' practitioners are not the early SARD case finders in the real world when there is a severe shortage of tertiary care rheumatologists, who is?

Fourth, given the documented and perceived limitations of the ANA IFA test as a screen for SARD,[18] what should replace it? As a succinct reply to this question, some modern laboratories are migrating to ANA immunoassay platforms that are highly automated and digital,[2] as well as to MAAT, which offer higher throughput and faster turn-around-times.[4] Recent evidence indicates that the best approach for ANA testing is to screen with the relatively inexpensive HEp-2 IFA ANA and then reflex to a MAAT.[2,19] Some laboratories use solid phase ANA tests, which, despite earlier limitations, now have performance that is comparable to, if not better than, the HEp-2 IFA when used in a reflex test setting.[19] Similarly, the digital automated ANA test systems referred to above have superior performance characteristics compared to 'manual' systems, offering hope that this is an important step toward harmonization of the ANA test.

Many clinicians often find the wealth of laboratory investigation and imaging results overwhelming and confusing (e.g., low titer ANA, obscure ANA IFA patterns, and MAAT results), making it unclear how they are clinically actionable. There is considerable optimism that AI and machine learning approaches will help clarify this by combining testing data into likely diagnoses and subsets of SARD, while also recommending actionable approaches and prognostic considerations for managing patients.[20]

Key Considerations in Ordering and Interpreting ANA tests
The ANA test is not standardized or harmonized; hence, there are differences in results generated by different labs.
Up to 30% of the general population can have a positive ANA.
Useful and clinically actionable ANA tests should be performed on individuals with a moderate-to-high pre-test probability of a SARD.
Both the titer and immunofluorescence patterns should be reported because they are important in interpreting the results.
It is recommended that a positive ANA be followed by testing for specific targets using multi-analyte arrays.
Unless there is a change in the clinical features of the patient, the ANA should not be repeated once the diagnosis of SARD is established.
In the future, ANA results will only be one input considered as artificial intelligence will analyze and 'interpret' multiple inputs to more accurately inform the clinician's diagnostics.

Conclusion

In summary, despite its long history, there is a strong need for evidence-based approaches to ANA testing. Future laboratory testing needs to consider the importance of disease prevention fostered by 'case finding' and the attenuation of significant morbidity and health care expenditures.[12,18] As MAATs improve and decrease in price, it is possible that the ANA test will no longer be the SARD screening assay of choice. In the meantime, the judicious use of the ANA test should focus on making an early and accurate diagnosis of SARD, with the best 'value' of the ANA test being in individuals with a moderate pre-test probability of the disease. Given the high frequency of ANA in the general population, individuals with either a high or low pre-test probability are unlikely to benefit from the test. Individuals with a high pre-test probability will likely gain more benefit from proceeding directly to MAAT analysis of autoantibodies.

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