Antinuclear Antibody Testing: Methods, Indications, and Interpretation

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CE update [chemistry | immunology]

Antinuclear antibody (ANA) testing is commonly used in the assessment of patients who may have an autoimmune disease. This tutorial review discusses the methods currently available to perform ANA testing, the indications for ANA tests, and the interpretation of ANA results.

While ANA testing can provide helpful diagnostic information, it typically does not provide information regarding disease activity.

Given the significant rate of positive ANA results in the general population, a positive ANA test should not be over-interpreted.

Laboratory tests to assist in diagnosis and to guide therapy in autoimmune diseases have been in use for decades. One of the best-established autoimmunity tests is the assay for ANA. Although the ANA is a commonly ordered study, it is a notoriously misunderstood test. In a recent study, clinical house staff at a major teaching hospital ranged in their estimations of the sensitivity and specificity of ANA positivity in the diagnosis of lupus all the way between 0% and 100%.1 To make matters worse, new methods have been introduced within the past decade for determining ANA results, so the test performed when a clinician orders an ANA may differ entirely from laboratory to laboratory. To help reduce confusion, this article will describe the methods available to measure ANA, review the indications for ANA testing, and guide the interpretation of ANA results.

Methods of ANA Testing

The traditional method for performing an ANA test is immunofluorescence. This remains the gold standard technique. At some centers, the immunofluorescent test for antinuclear antibodies is known as the FANA. Immunofluorescence testing involves incubating dilutions of patient sera with a monolayer of fixed, permeabilized cells [F1]. Antibodies adherent to the cell monolayer are visualized with an anti-human immunoglobulin reagent that has been conjugated to a fluorescent tag. Trained technicians identify the presence or absence of nuclear staining and the pattern of nuclear staining by fluorescence microscopy.

When performed in a proficient laboratory, immunofluorescence is a highly sensitive assay for the presence of antinuclear...
antibodies. Since cells are being used as the test substrate, a wide variety of characterized and uncharacterized targets of autoantibody binding can lead to a positive result. In comparison to other ANA testing methods, advantages of immunofluorescence include the high sensitivity of the test, and the ability to interpret the extensive body of published data on the performance of FANA testing in a wide variety of clinical scenarios. Also, the FANA test provides information that may be relevant to the ordering clinician that other testing techniques may not provide. This information includes the pattern of nuclear staining, and the presence or absence of cytoplasmic staining. Disadvantages of immunofluorescence testing relate to the complexity of the procedure. It is more time consuming, and more dependent on highly trained laboratory personnel than other test modalities. The performance of the test also depends critically on having quality reagents. This can be a challenge, since different laboratories use different cell substrates, and even standard cells from a single cell line (such as the frequently used HEp-2 cell line) prepared by a single manufacturer can show significant variation from lot to lot. For modern levels of test sensitivity, it is necessary to use a human rather than an animal-derived cell line. Given the inherent variability in FANA testing, laboratories performing FANA testing reliably must perform a high volume of tests and have an active program of quality control supervised by experienced personnel to ensure valid results.

The other common method for ANA testing currently in use is the enzyme-linked immunosorbent assay (ELISA). In this technique, the test manufacturer coats multi-well plates with a homogenate of antigens. While the antigen sources are proprietary, manufacturers generally use either preparations derived from cell nuclei, mixtures of purified characterized nuclear autoantigen proteins, or combinations of both. Dilutions of patient sera are incubated in antigen-coated wells, followed by incubation with an antibody human immunoglobulin reagent linked to an enzyme tag. Antibody binding is quantitated by colorimetry, adding a substrate to the wells that changes color in the presence of the enzyme tag.

The advantages of ELISA testing include the speed and simplicity of the assay. Also, there is the hope that mass-produced coating antigen preparations may be more consistent from lot to lot than immunofluorescence cell substrates. Based on published data, ELISA assays vary in quality, but some approach immunofluorescence in their sensitivity and specificity for the identification of antinuclear antibodies. Disadvantages of ELISA testing include reduced antigen diversity leading to decreased sensitivity for the identification of antinuclear antibodies, and reduced ability to assess the quality of the antigen preparation by direct inspection of results of the assay compared to immunofluorescence. Since antigen quality deteriorates over time as ELISA plates are stored, even if the original preparations are uniform, close quality control is needed for this assay as well. Indeed, since the assay itself is less likely to reflect qualitative changes in antigen condition, quality control procedures for ELISA-based ANA determinations must be even more rigorous than those for immunofluorescence testing. A strategy of performing immunofluorescence testing to confirm positive results on ELISA testing is used by some laboratories and clinicians, but this approach is inadequate to prevent or identify potential false negative results on initial ELISA testing. Thus, although in theory, performing an ELISA-based ANA test requires less training and experience, running an accurate ELISA-based ANA testing laboratory requires at least as high a level of expertise and scrutiny as a fluorescence-based laboratory. Both

**Characteristics of ANA Testing Methods**

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tests should only be performed at high volume centers dedicated to immunopathology assays. Since some ELISA kits include supplementary tests to test for antigens that correlate with the nuclear pattern on immunofluorescence, the clinician should not assume that immunofluorescence testing was performed if a staining pattern is reported.

Most laboratories offering ANA tests offer additional autoantibody tests as well. Often, these tests are bundled together into an “ANA profile” that clinicians order at the same time as the antinuclear antibody test. While the precise make-up of the “ANA profile” varies from laboratory to laboratory, typical components in such panels include tests for antibodies recognizing single-stranded and double-stranded DNA, ribonucleoprotein antigens, and protein autoantigens including Ro, La, Sm, and topoisomerase I (Scl-70).

The “ANA profile” emerged to satisfy 2 interests on the part of clinicians. First, an “ANA profile” has higher sensitivity for identifying patients with autoimmune disease than an ANA test alone, even when using immunofluorescence. As an example, at our center, we find that 95% of all lupus patients have positive FANA test results, but 98% all lupus patients had positive ANA profiles.11 Second, the ANA profile can provide additional diagnostic and prognostic information about the nature of the autoimmune process based on the targeted antigens. For example, antibodies to DNA are highly specific for lupus,12 and antibodies to topoisomerase I are specific for scleroderma.13 A variety of assay techniques can be used to perform the tests making up the ANA panel. A full discussion of these methods is beyond the scope of this article. Some ANA ELISA kits offer antigen-specific ELISA tests for “ANA profile” antigens. The published performance of such tests has been inconsistent.14,15 While ELISA assays for some tests in the ANA profile may be equal to more traditional assay methods, performance of total kits has not yet matched established gold standard techniques.

Even as ELISA assays continue to improve, researchers are developing nanotechnology arrays of defined autoantigens in the hope that these will supplant traditional ANA testing in the future.17 These arrays would combine the homogeneity of antigen preparations and simplicity of assay procedures of ELISAs with the potential to provide even more detailed information about the specific antigens targeted by the immune response than immunofluorescence. As immune responses toward specific antigens become correlated with clinical diagnoses (or even clinical expressions of disease within the same diagnosis), this information may be a major advance in testing for laboratory manifestations of autoimmune disease. To date, nanotechnology is limited by technical and knowledge hurdles. Technically, investigators have only been able to make detectable antigen nanospots with an estimated 50% of known autoantigens,18 limiting the sensitivity of these assays to less than that available by immunofluorescence. From a knowledge standpoint, few correlations between clinical manifestations of disease and specific autoantibodies have been rigorously made. In the cases where such correlations have been made, fewer still are known to convey clinically useful information.19

Quality control for ANA testing is essential and involves multiple steps. As with any successful quality assurance program, maintaining quality is the responsibility of the entire laboratory, not just a quality control officer. All measurements must be accurate, all reagents fresh and pure, and all substrates without evidence of blemishes or spoilage. Careful inventory control is necessary. New lots of substrate should be tested extensively against older established lots before being brought into clinical use. All assays should be run, including well-characterized positive and negative control sera, and repeated if controls fail to perform to established standards. All subjective readings (such as immunofluorescence titers and staining patterns) should be validated by at least 2 trained readers. All measurement equipment (such as ELISA plate readers) should receive frequent routine maintenance and calibration. The laboratory should routinely and frequently process blinded positive and negative test samples to validate laboratory performance. Laboratory results should be correlated with the clinical context and prior laboratory studies. If incongruities are identified, laboratory results should be re-checked. Periodic monitoring of the statistical performance of the laboratory may also be performed. Variations between similar patient populations in test results between a baseline time period and a new time period may identify divergences in test quality related to changes in substrate, reagent, or personnel.

**Indications for ANA Testing**

The major use of ANA testing is as a diagnostic tool. If a diagnosis has already been confidently established without ANA testing, the test generally does not need to be performed. It is notable that 57% of ANA tests ordered by clinicians in one prospective study were in patients whose diagnoses were already established.1

In general, laboratory tests can help clinicians in 3 ways. They can provide insight regarding diagnosis, prognosis, or disease activity. Weaknesses in the ability of ANA testing to provide information particularly regarding disease activity limit the number of times when ANA testing is indicated. Studies suggest that the results of ANA tests provide no information regarding disease activity. Changes in ANA titers have not been associated with autoimmune disease activity.20 Thus, in a patient who has been previously found to be ANA positive, there is seldom a reason to reassess their ANA status.

While assays when performed at a competent lab can identify antinuclear antibodies with a high degree of accuracy, ANA testing is also limited as a diagnostic test. The problem relates to the frequency with which people have antinuclear antibodies even when they have no autoimmune disease. Using an ANA titer of 1:40 as a cut point between positive and negative, approximately 10% of normal individuals are ANA positive.21 Increasing the cut point of the test to titers of 1:320 or greater leaves 3% of normal people with positive values, indicating a specificity of ANA testing of 97%. While a test with 97% specificity may sound like it should be able to give useful diagnostic information, remember that autoimmune diseases are relatively uncommon—lupus, for example, has an estimated population
prevalence of approximately 10 cases per 10,000 people. Thus, the positive predictive value of ANA testing is often low. In lupus, 95% of patients are ANA positive at a titer of 1:40 or higher and 75% are ANA positive at a titer of 1:320 or higher. However, given the rate of positive ANA tests in the general population, an ANA result of 1:40 or higher in isolation has a positive predictive value for the diagnosis of lupus of only 0.9%. At a titer of 1:320 or higher, a positive ANA test in isolation has a positive predictive value of only 2.4% for a diagnosis of lupus. As these examples illustrate, a positive ANA result by itself is not sufficient to lead to the diagnosis of an autoimmune disease.

Rather than being used to diagnose autoimmune disease, one of the most effective uses of ANA testing is to exclude the diagnosis of ANA-associated autoimmune diseases. In the case of lupus, for example, the absence of ANA positivity at a titer of 1:320 or higher has a negative predictive value of over 99% in an unselected population. In patients with manifestations of disease that are potentially consistent with lupus, a more aggressive screening strategy is available to help exclude lupus. To maximize the negative predictive value of ANA testing in such patients, an “ANA profile” can be performed. To be most useful, such autoantibody panels should be optimized for high sensitivity of detection of autoantibodies. Under these conditions, a completely negative ANA profile dramatically reduces the likelihood that the patient has an ANA-associated disease, allowing the clinician to focus on alternative potential diagnoses.

**Interpretation of ANA Tests**

As described above, the most definitive result from ANA testing is a negative test. This result, especially when coupled with negative tests on an ANA profile, suggests that lupus or other strongly ANA-associated diseases are unlikely to be present. On the other hand, in the absence of other clinical or laboratory data supporting a diagnosis, a positive ANA test is seldom useful. The most likely cause of a positive ANA test is the presence of no disease at all. Even in subjects who are clinically ill, a positive ANA test must be interpreted with caution. While antinuclear antibodies are common in relatively rare conditions such as lupus, scleroderma, mixed connective tissue disease, and Sjögren’s syndrome,11,24-26 they also can be seen at moderate frequency in more common disorders including thyroid disease, rheumatoid arthritis, HIV disease, and hepatitis C infection.27 The presence of ANA positivity and related findings can provide significant additional information in specific clinical situations, though. In these circumstances, ANA titers at or above 1:320 are considered positive while lower titers are considered indeterminate.

In patients with established diagnoses of autoimmune diseases, ANA positivity can subdivide patients with regard to prognosis and response to therapy. Examples include juvenile chronic arthritis, where ANA positivity is associated with an increased risk of uveitis,31 and autoimmune hepatitis where ANA positivity defines a disease subtype.32

In patients with scleroderma, the presence of a centromere pattern of staining may suggest the CREST syndrome, while a diffuse or nucleolar pattern of staining would be more consistent with diffuse cutaneous scleroderma.33 This distinction may be important, since the incidence of major end organ complications such as interstitial lung disease is lower in CREST syndrome.34 In contrast, scleroderma patients with anti-topoisomerase I (Scl-70) antibodies (one of the causes of a diffuse pattern of ANA staining) have increased scleroderma lung disease.35

In some situations, the pattern of nuclear staining on ANA may be nonspecific, but able to support the findings on more specific autoantibody testing. Antibodies to DNA, for example, are typically associated with homogenous or rim pattern nuclear staining. Antibodies to Sm often are associated with a speckled pattern of nuclear staining. Confirmation of the presence of DNA or Sm antibodies is strongly suggestive of a diagnosis of lupus.12 Recent data suggest that the 3 major assays for anti-DNA antibodies—immunofluorescence against *Crithidia luciliae*, the Farr radioimmunoassay, and ELISA tests—all have similar performance characteristics in clinical practice.36

If inflammatory myositis is suspected, attention also should be paid to the presence of anti-cytoplasmic antibodies. While nonspecific, these can be seen in patients with the anti-γRNA syntheseshisitis syndromes.37

One way that specific antigen testing may constitute an advance over traditional ANA testing could be the “false” positive rate. As described above, at least 3% of normal individuals have positive results on traditional ANA testing. These are “false” results not in the sense of being inaccurate—these subjects do have antinuclear antibodies. They are “false” in the sense that they could mislead the test interpreter into thinking that the subject has an autoimmune disease or is at risk for developing one. In contrast to ANA tests, the likelihood of “false” positive results for individual autoantigen antibodies may be lower.12,38 As nanotechnology

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### Conditions Associated With Antinuclear Antibodies

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<th>Condition</th>
<th>Frequency of ANA +</th>
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<td><strong>Uncommon conditions with a high frequency of antinuclear antibodies</strong></td>
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<tr>
<td>Lupus</td>
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allows laboratories to screen for large arrays of specific autoantigens, clinicians may have additional information available to help classify the clinical significance of specific patterns of autoimmune reactivity.

Summary
Tests for autoantibodies are entering a phase of evolution in medical practice. New methodologies are emerging for old tests, new understandings are developing of current tests, and entirely new kinds of tests are appearing on the horizon. However, the old established approaches to laboratory medicine still apply to tests for autoantibodies: tests must be performed under rigorously controlled conditions to ensure valid results, and tests should only be ordered when clinically indicated in order to yield relevant results. The immunofluorescence test for antinuclear antibodies remains a gold standard test for the assessment of humoral autoimmunity. The test can be most valuable diagnostically when a negative result is obtained. The title of an ANA result has not been shown to correlate with disease activity. Although new test methods make ANA testing seem more accessible to smaller laboratories, the quality control oversight requirements for these newer tests are even more exacting than for older tests. Use of high-volume laboratories with extensive experience performing ANA assays may be the best way to ensure reliable results.

Acknowledgment
This work is dedicated to a pioneer in the field of anatomic antibody testing, Dr Gordon C. Sharp on the occasion of his 70th birthday. This work was supported by the Medical Research Service of the Department of Veterans Affairs and by NIH grant AR 43308, AR 48055 and AI 101842.


