ANA and Antibody Series

Scleroderma ANA and Antibody Testing Basics

ANA Testing

The long-standing way of doing ANA testing is a method called indirect immunofluorescence (IFA). It is a time consuming, manual process subject to individual interpretation and is therefore fairly expensive. An ANA by IFA can detect the presence of up to 150 different types of antibodies (antigens) but does not determine specifically which antibody or antibodies are present. The main result of an ANA by IFA is called a titer (it is actually a dilution factor), but a staining pattern is also reported.

The titer reported with a positive ANA test is a measure of antibody levels, which is quite significant in some autoimmune diseases but not generally in systemic scleroderma. It should be reported as a dilution factor in a format such as 1:320, but sometimes you will only see the second number. The second number will always be a multiple of 40, so titer results might look like 1:40, 1:80, 1:160, 1:320, 1:640, etc. Depending on the lab, a titer of either 1:40 or 1:80 will be considered positive. However, an ANA titer of 1:80 or less is not generally considered meaningful since a significant percentage of people in the general population will have a positive ANA at these levels with no clinical symptoms. When you get to a titer of 1:160 or higher, then this is much more likely to be an indication of an underlying autoimmune condition. Note that if you see an ANA test result that looks like a single low number (e.g., 3.8),this indicates that the ANA test was done using an alternative testing method (ELISA or Multiplex). These alternative testing methods are discussed in some detail below.

The staining pattern can be somewhat subjective but overall it gives a rough idea of what type of antibody is detected. Some specific staining patterns are fairly specific to certain diseases (for example, a centromere pattern is highly correlated with the presence of centromere antibodies and limited systemic scleroderma). Other patterns are specific to lupus or can occur with more than one autoimmune disease. In modern clinical practice, staining patterns are not considered to be definitive for specific diseases since pattern interpretation is a bit of an "art form" and different labs train their technicians differently on interpretation of staining patterns. For this reason, detailed follow-on antibody testing is a much more accurate way of determining a likely diagnosis.

Scleroderma Antibody Testing

If the titer is high enough to suggest an autoimmune disease, then the next step is to run a separate diagnostic panel to try to identify the specific antibody that was detected by ANA/IFA (fewer than 2% of patients with systemic scleroderma have more than one scleroderma specific antibody). Note that some of these screening panels are specific for an autoimmune disease, e.g., lupus, scleroderma, or rheumatoid arthritis. There is also often a general rheumatic disease screen panel offered by each testing lab that includes some of the most common antibodies for several autoimmune diseases.

Diagnostic testing panels use a variety of different testing methods, depending on the reference lab. However (and this is VERY important), an antibody screening panel can *only* detect the specific antibodies that are included in the panel. While this sounds obvious, in fact the significance of this cannot be over stressed, as will be discussed below.

In recent years, the standard way of doing ANA testing has started to change. As was mentioned above, doing an ANA test by IFA is somewhat expensive since it requires human analysis and to some extent the results are subject to individual interpretation. Two alternative ways of doing ANA testing are now commonplace: solid phase immunoassays (ELISA or EIA) or a related technique known as a Multiplex platform. These new methods are faster, cheaper, and eliminate the subjective interpretation problem and are generally very accurate. Unfortunately, they also introduce a significant major problem of false negative results – especially for patients with scleroderma.

ANA testing by IFA tests for the presence of up to 150 different antibodies at one time. In contrast, typical ELISA testing includes only 8 to 10 antibodies and typical Multiplex testing may include up to 13 different antibodies. These testing systems can be set up for different antibody panels. For example, an ELISA test for lupus-related antibodies would test for a different set of 8 to 10 antibodies than an ELISA test for scleroderma-related antibodies. Research shows that these two methods (ELISA and Multiplex) are generally very reliable in detecting the antibodies they are designed to test for (with one important exception – see below).

Falsely Reported Negative ANA Result and Diagnostic Risk

If a clinician suspects that her patient might have some form of systemic scleroderma based on the patient's symptom profile, she would typically order an ANA test with reflex to a scleroderma-specific antibody panel if the ANA is positive. In the past, what the lab would have done is to first run an ANA test by IFA. If that result was positive, then a scleroderma-specific antibody panel would be run to try to identify which (if any) scleroderma-related antibodies the patient has.

Now, however, when the clinician orders ANA testing with a reflex antibody panel, the initial ANA testing is usually performed by either ELISA or Multiplex instead of IFA. If the ELISA/Multiplex test detects the presence of one of the 8 to 13 antibodies included in the panel, then in most cases a separate ANA by IFA will then be done to confirm the result

and also measure the titer level and staining pattern. However, if none of these 8 to 13 antibodies are detected, then the doctor receives a report that the ANA is negative.

Unfortunately, there is a big difference between testing for up to 150 antibodies (as IFA testing does) and testing for up to 13 antibodies. A recent (2011) study looked at a typical Multiplex scleroderma screening panel and determined that the antibodies included in the test missed *up to 43%* of scleroderma patients that in fact tested positive by IFA because the panel simply did not test for antibodies that are now known to occur in a significant percentage of systemic scleroderma patients. And, note that this was a scleroderma-specific screening panel. If instead, the clinician was concerned that the patient might have *some* type of autoimmune condition, potentially including lupus, scleroderma, and mixed connective tissue disorder / MCTD), he would start by ordering a general rheumatic screening panel. This time, however, even fewer scleroderma-specific specific antibodies would be included in the test panel and the likelihood of a false negative ANA report would be much higher.

To get a better idea of how much of a problem this is in the real world, I recently reviewed several national testing labs to see what types of ANA / autoimmune screening panels were offered by these labs and which specific antibodies were included in the scleroderma screening panels. Unfortunately, in this quick survey I found only two national reference labs (RDL and ARUP) where their scleroderma panel included all but one of the testable antibodies that are now known to be associated with systemic scleroderma. (The remaining scleroderma related antibody – Ku – is associated with a scleroderma/myositis overlap syndrome and can be added to the scleroderma panel upon request at these two labs.) Perhaps, not surprisingly, these two labs also routinely do initial ANA testing by IFA rather than Multiplex or ELISA. In all the other labs that I reviewed, the scleroderma screening panel excluded antibodies that represent more than 50% of the antibodies that are present in the overall population of scleroderma patients. In fact, the general rheumatic disease screening panels often include only one scleroderma-specific antibody – Scl-70, representing only about 20% of the total systemic scleroderma patient population.

Discussion and Recommendations

The *American College of Rheumatology*, in a 2011 Position Statement, recommends that testing by IFA "should remain the gold standard for ANA testing". While that may be ideal, for cost reasons it is expected that initial ANA screening will increasingly be performed using new lower-cost methods such as ELISA or Multiplex. However, because of the very real potential of a false negative ANA result that can potentially cause major delays in accurate diagnosis and early treatment, if the ordering clinician suspects that an underlying autoimmune condition is the root of the patient's presenting symptoms, then it is very important that any negative result of an ANA test done by ELISA or Multiplex be verified by re-testing for ANA using the IFA method.

It is important to note that a negative ANA done by the IFA method does not completely eliminate the possibility of a diagnosis of systemic scleroderma since about 6% of patients with a clear diagnosis of systemic scleroderma are ANA negative by IFA according to recent research. In these cases, diagnosis is made entirely based on the patient's clinical profile. It is also important to note that if an ANA is negative when done by the IFA method, then there is no need to run individual antibody tests since they should be negative. ANA

testing by IFA detects the presences of all known scleroderma specific antibodies, so if an individual antibody tests positive with a negative ANA by IFA, it is almost certainly a false positive result.*

Based on current research, initial antibody screening following a positive ANA by IFA should at a minimum include the three most common scleroderma-specific antibodies: Scl-70, centromere, and RNA Polymerase III. These three antibodies are found in the majority of all patients diagnosed with systemic scleroderma. Clinicians should not assume that ordering a "scleroderma antibody panel" will include these three antibodies and needs to verify that they are included in the panel or are ordered individually if needed.

If these three antibodies are negative, then additional antibody testing is needed for the rarer antibodies: Th/To, U1-RNP (MCTD), U3-RNP, PM-Scl, and Ku. In some cases, the patient's symptom profile can be a guide for additional testing. For example, if the patient has a clinical profile consistent with limited systemic scleroderma, then testing for Th/To and U1-RNP antibodies would be appropriate next testing steps. If the clinical profile suggests diffuse disease cutaneous disease, then U3-RNP antibody testing would make sense. PM-Scl and Ku antibodies are associated with scleroderma overlap syndromes that include myositis. (There are two additional recently identified scleroderma-specific antibodies: U11/U12-RNP and RuvBL1/2. Unfortunately, commercial testing is not yet available for these two rare antibodies.)

The ultimate problem in ANA and antibody testing is that many clinicians have no training in ANA testing methods and are not aware of the testing issues discussed in this article. As a result, when clinicians get a report of a negative ANA result in a patient with suspected autoimmune disease, they will conclude that the patient does not have an autoimmune condition and as a result will start looking for alternative explanations for their patient's often confusing symptom profile. This can lead to significant delays in getting a correct diagnosis. Ironically, 20 years ago this would not have been as much of a problem since all ANA testing was done by IFA.

* There is a known problem with false-positive Scl-70 antibody results when done by ELISA. These will usually be low positive results. If the ANA is negative by IFA then any positive individual scleroderma-specific antibody result is almost certainly a false positive.

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