Screening and Laboratory Diagnosis of Autoimmune Diseases Using Antinuclear Antibody Immunofluorescence Assay and Specific Autoantibody Testing

Paul P. Doghramji, MD

Educational support provided by Quest Diagnostics, Inc.

Introduction

Autoimmune diseases occur when the immune system attacks the normal tissue within joints, vasculature, and other organ systems, causing inflammation, pain, diminished mobility, fatigue, and other non-specific symptoms.[1] The strong overlap of signs and symptoms among the autoimmune diseases can lead to delays in diagnosis and appropriate treatment. According to a survey by the Autoimmune Diseases Association, it takes up to 4.6 years and nearly 5 doctor visits to receive a proper autoimmune disease diagnosis.[2]

Laboratory testing, in addition to clinical assessment, is necessary for differential diagnosis and disease classification of autoimmune diseases. However, research shows that primary care physicians tend to overuse common autoantibody tests for autoimmune conditions, which can limit the positive predictive value and clinical utility of such testing.[3] To facilitate appropriate referral to specialists, if necessary, laboratory testing should be reserved for patients who have signs and symptoms consistent with one or more autoimmune disease (Table 1).

The antinuclear antibody (ANA) immunofluorescence assay (IFA) is a first-line screening test for patients with a suspected autoimmune disease. This test is the gold standard because of its high sensitivity compared to other assays.[4,5] Positive results should prompt clinicians to continue investigating the cause of a positive ANA IFA and narrow the field of potential culprits. The following describes how ANA IFA in combination with specific autoantibody testing can be used in the differential diagnosis of a suspected autoimmune disease.

Laboratory screening and diagnostic testing for disease classification

The recommended ANA screen approach uses HEp-2 human tissue culture cells in an IFA. In this assay, the patient's blood sample is mixed with HEp-2 cells fixed to a slide. ANAs present in the sample react with the cells and treatment with a fluorescent anti-human IgG antibody allows visualization of antibody binding under fluorescence microscopy. This test screens for a large number of known autoantibodies, approximately 150, directed against nuclear antigens and cell cytoplasm. A positive screen result is followed by evaluation of antibody titer and pattern (consult side bar below).

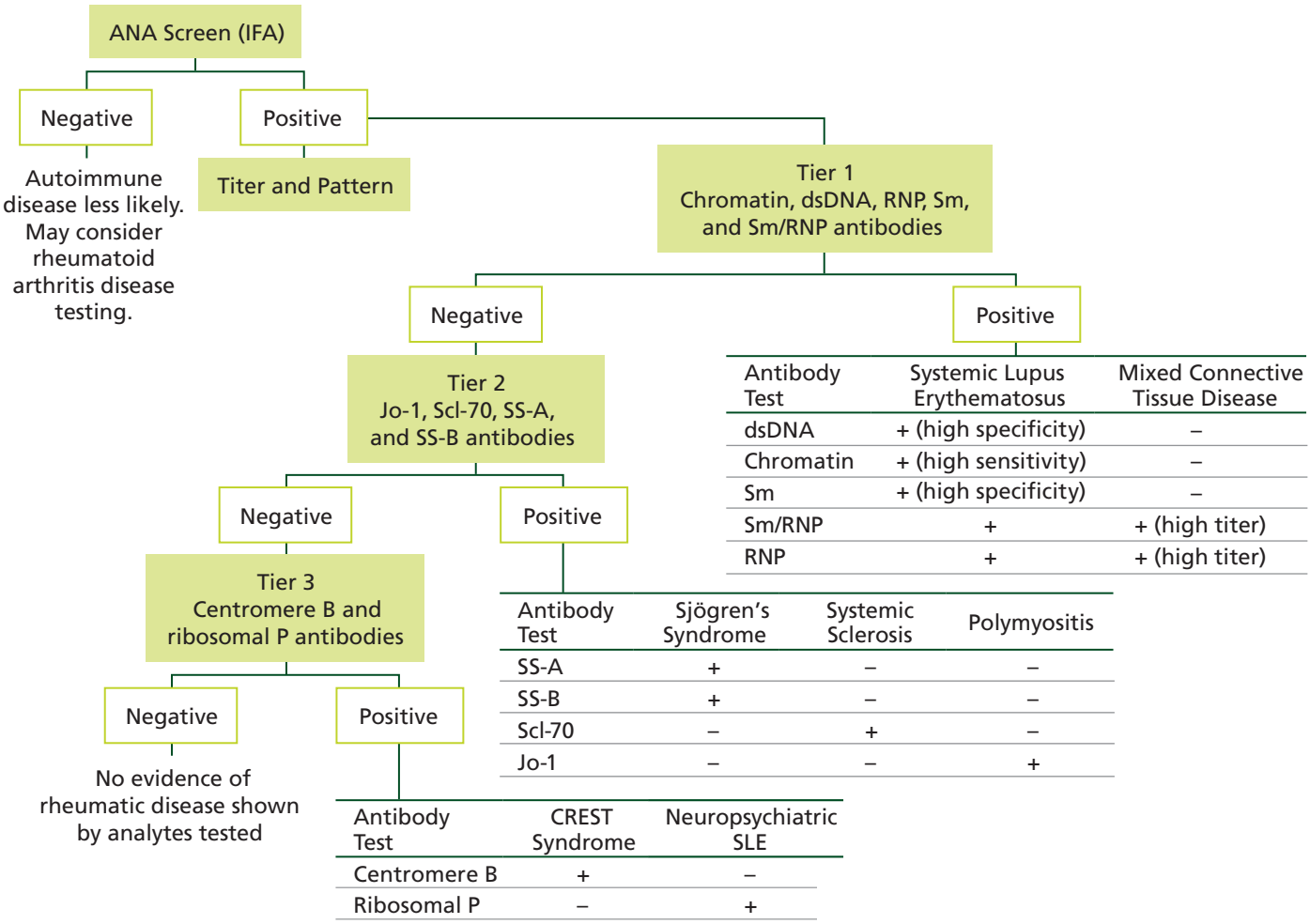
With a positive ANA IFA screen and titer, the clinician needs to determine the root cause of the positivity. This can be accomplished through a reflex to an algorithm of specific antibody tests to help identify autoantibodies associated with specific autoimmune diseases.

An ANA reflex algorithm tests for specific antibodies in a clinically logical sequence. With a combination of ANA IFA plus a reflex algorithm of specific antibody testing, positive results automatically reflex to a tier of disease-specific autoantibodies. Testing begins with the most prevalent autoimmune diseases and continues until a positive result is found, or all tests in the algorithm have returned a negative result. This algorithm/reflex approach provides the clinician with a rational approach to confirming a diagnosis in a patient with a suspected autoimmune disease, with a single blood draw.

Titer and Pattern

If the ANA IFA screen is positive, testing for antibody titer and pattern can help evaluate the presence and type of autoantibody disease. ANA titers are determined by diluting the liquid portion of the blood sample in saline at a ratio of 1:40 to 1:1280. The titer is thus the highest dilution that yields a positive ANA result. Any titer above 1:40 is considered positive, and titers above 1:80 are consistent with an autoimmune disease. To assess the nuclear and cytoplasmic staining patterns of samples with positive results, patient antibodies react with indicator cells and the localization of patient antibodies is visualized by a second fluorescein antihuman IgG antibody evaluated under a fluorescence microscope. These patterns may provide additional information to rule out or further implicate a suspected condition and can guide the selection of additional testing for specific autoantibodies.

Figure 1.
Use of ANA (IFA) and Specific Antibody Testing Cascade (Test Code 16814) for the Diagnosis of Rheumatic Disease[6-10]



Antibody Test	Systemic Lupus Erythematosus	Mixed Connective Tissue Disease
dsDNA	+ (high specificity)	–
Chromatin	+ (high sensitivity)	–
Sm	+ (high specificity)	–
Sm/RNP	+	+ (high titer)
RNP	+	+ (high titer)

Antibody Test	Sjögren's Syndrome	Systemic Sclerosis	Polymyositis
SS-A	+	–	–
SS-B	+	–	–
Scl-70	–	+	–
Jo-1	–	–	+

Antibody Test	CREST Syndrome	Neuropsychiatric SLE
Centromere B	+	–
Ribosomal P	–	+

The acronym CREST refers to a syndrome defined by presence of calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia. dsDNA indicates double-stranded DNA; Sm/RNP antibody, Smith/ribonucleoprotein antibody; SS-A and -B antibodies, Sjögren's Syndrome A and B antibodies; Scl-70 antibody, scleroderma (topoisomerase I) antibody; Jo-1 antibody, histidyl-tRNA synthetase antibody; and SLE, systemic lupus erythematosus.

This figure was developed by Quest Diagnostics based on references 6-10. It is provided for informational purposes only and is not intended as medical advice. A physician's test selection and interpretation, diagnosis, and patient management decisions should be based on his/her education, clinical expertise, and assessment of the patient.

Figure 1 above illustrates an example of an algorithm approach with a set of reflexing tiers for suspected rheumatic disease, a more common group of autoimmune diseases.[6-10] With this algorithm, samples with a positive IFA result are tested for five autoantibodies associated with systemic lupus erythematosus (SLE) and mixed connective tissue disease: dsDNA, chromatin (nucleosomal), Smith (Sm), ribonuclear protein (RNP), and Sm/RNP antiodies. (In the cell, Sm and RMP proteins form a complex.) If the first tier yields a positive result, the results are reported and the testing stops. If the first tier of antibody testing is negative, the testing reflexes to the 2nd tier, which includes four autoantibodies associated with Sjögren's Syndrome, systemic sclerosis, and polymyositis: SS-A, SS-B, Scl-70, and Jo-I antibodies. If a positive result is determined for any of these autoantibodies, the results

Table 1. Common Signs and Symptoms of Autoimmune Rheumatic and Related Diseases[a,b]

Sign or Symptom	Gout	JIA	MCTD	PM/DM	Pseudogout	RA	Sarcoidosis
Joint-/muscle-related							
Joint pain, stiffness, or inflammation	●	●	●	●	●	●	●
Muscle weakness			●	●			
Myalgia							
Skin-/hair-related							
Alopecia							
Rash		●	●	●			●
Raynaud's phenomenon			●	●		◐	
Skin lesions		●		◐[c]			
General							
Anorexia						●	●
Cough							●
Ear involvement	◐[d]						
Eye involvement		◐					●
Fatigue				●		●	●
Fever	●	●	●	●		●	●
GI involvement							
Malaise	●					●	●
Nasal symptoms							◐
Nervous system involvement							◐
Respiratory involvement		●					●
Weight loss				●		●	●
Other							
Adenopathy		●					
Anemia						◐	
Dysphagia				●			
Swelling of hands			●	◐			◐

(Continued)

Table 1. Common Signs and Symptoms of Autoimmune Rheumatic and Related Diseases[a] *(Continued)*

Sign or Symptom	SjS	SLE	SpA				SSc	Systemic Vasculitis		
			AS	ReA	PsA	EA		GPA	EGPA	MPA
Joint-/muscle-related										
Joint pain, stiffness, or inflammation	●	●	●	●	●	●	●	●	●	●
Muscle weakness		○								
Myalgia		○						●	●	●
Skin-/hair-related										
Alopecia	○	●					○			
Rash	○	●							●	●
Raynaud's phenomenon	●	●					●			
Skin lesions		○		○	●		●	○		○
General										
Anorexia			●							
Cough								●	●	
Ear involvement								●[d]		
Eye involvement	●	●	●	●				●		●
Fatigue		●	●	●					●	●
Fever		●	●	●				●	●	●
GI involvement						●	●		●	●
Malaise		●							●	●
Nasal symptoms								●	●	
Nervous system involvement		●						●	●	●
Respiratory involvement								●	●	
Weight loss			●	●					●	●
Other										
Adenopathy	●	●								
Anemia		●	●							
Dysphagia	○						●			
Swelling of hands					●		●			

● indicates common; ○ indicates less common but not rare.

[a]AS=ankylosing spondylitis, EA=enteropathic (inflammatory bowel disease-associated) arthritis, EGPA=eosinophilic granulomatosis with polyangiitis, GPA=granulomatosis with polyangiitis, JIA=juvenile idiopathic arthritis, MCTD=mixed connective tissue disease, MPA=microscopic polyangiitis, PM/DM=polymyositis/dermatomyositis; RA=rheumatoid arthritis, PsA=psoriatic arthritis, ReA=reactive arthritis, SjS=Sjögren's Syndrome, SLE=systemic lupus erythematosus, SpA=spondyloarthropathies, SSc=systemic sclerosis.

[b]This is not a complete list of signs and symptoms; some conditions have more signs and symptoms than could be presented here.

[c]In dermatomyositis.

[d]External ear in gout; middle ear in GPA.

are reported and the testing stops. If 2nd tier antibodies are negative, the testing continues with a reflex to 3rd tier. The 3rd tier includes two autoantibodies associated with CREST syndrome (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) and neuropsychiatric SLE: centromere B and ribosomal P antibodies.

Although a positive result in the 1st or 2nd tier will stop the reflex testing to the next tier, it does not rule out the presence of additional clinically relevant autoantibodies, including those in the next tier or other autoantibodies outside the algorithm. Additionally, negative results for all three tiers do not rule out an autoimmune disease, as not all autoimmune diseases are represented in this algorithm. Note that the clinician can request full reporting through all three tiers, especially if a rheumatic autoimmune disease is strongly suspected.[11]

Clinical suspicion and correlation are generally necessary to proceed with additional testing for other specific antibodies beyond the algorithm of the most common rheumatic diseases. For example, a positive ANA IFA is a diagnostic criterion for drug-induced lupus, polymyositis/dermatomyositis, other forms of idiopathic inflammatory myopathy, and oligoarticular juvenile chronic arthritis. A positive ANA result is also consistent with organ-specific autoimmune diseases, including thyroid diseases (eg, Hashimoto's thyroiditis, Grave's disease), gastrointestinal diseases (eg, autoimmune hepatitis, primary biliary cholangitis, inflammatory bowel disease), and pulmonary disease (eg, idiopathic pulmonary fibrosis).

A positive ANA result alone is not diagnostic of an autoimmune disease. The prevalence of ANAs in healthy individuals is about 3-15%.[11] The production of these autoantibodies is strongly age-dependent, and increases to 10-37% in healthy persons over 65. Even healthy people with viral infections can have a positive ANA, albeit for a short time. Patients with infectious diseases may also test positive for ANA. These include viral infections (hepatitis C, parvovirus), bacterial infections (tuberculosis), and parasitic infections (schistosomiasis). Certain medications and some lymphomas may also cause a positive ANA.

Case Studies

1 SLE

A 32-year-old Caucasian woman presented for an initial visit because of soreness in her hands. This soreness began six weeks previously, with no history of injury or prior pain and soreness. The middle fingers of both hands (fingers 3 and 4) were swollen and tender, and she had trouble making a fist and opening jars. Other joints were stiffer than usual, especially her knees, ankles, and feet. She had difficulty getting out of bed in the morning, with joint stiffness and painful walking lasting many hours. The patient denied fevers, chills, and any recent febrile illnesses, yet reported feeling especially tired since this issue began. She had been taking over-the-counter naproxen for "some" relief. She denied rash, dry eyes/mouth, sun sensitivity, trouble swallowing, and cardiovascular symptoms. The patient's medical history was unremarkable. Her medication list included birth control pills and she denied any medication allergies. Her family history was significant for thyroid disease (mother and older sister) and type 1 diabetes (father); social history included smoking ½ pack per day for the past 18 years. She had no surgical history, and a review of systems was unremarkable (Table 2).

Table 2.
SLE Patient Vital Signs and Physical Examination

Height	5'6"
Weight	129 lb
BMI	21
HEENT	Normal
Neck Adenopathy	None
Heart/Lung Auscultation	Normal
Abdomen	Benign
Hands	Boggy, tender, warm 3rd and 4th metacarpophalangeal joints on the right, minimally same on the left; rest of joints normal
Skin	No rash

The patient underwent laboratory testing using with the ANA IFA with 3rd tier specific autoantibody reflex cascade (Figure 1). The results were positive for ANA IFA at a titer of

1:160 with a mixed speckled and homogeneous pattern. The 1st tier of testing was positive for dsDNA, chromatin, and Sm antibodies, which pointed to a diagnosis of SLE. The patient was then referred to a rheumatologist who confirmed the diagnosis of SLE, and started her on hydroxychloroquine. The primary care physician continues to work collaboratively with the rheumatologist to manage this patient.

2 Sjögren's Syndrome

A 58-year-old Japanese woman presented to the clinic complaining of a very dry mouth and thirst that had been going on for months and was now worsening. She was worried about a diagnosis of diabetes mellitus (T2DM) due to her family history (ie, parents had T2DM). The patient had trouble speaking normally without constantly drinking water and found it difficult to chew and swallow food. Upon further questioning, she reported that she also had dry eyes but no polyuria or weight loss, no swollen salivary glands, no joint pain, and no skin changes (Table 3). Her medical history was significant for the deep vein thrombosis and for major depressive disorder, which had been treated and was now in remission. She had no medications or allergies to such. In addition to T2DM, her family history was positive for hypertension (parents), hyperlipidemia (parents), and rheumatoid arthritis (brother). The patient had never smoked cigarettes and the review of systems was unremarkable. On examination, the patient appeared comfortable and in no pain; vital signs were normal.

Table 3.
Sjögren's Patient Vital Signs and Physical Examination

Height	5'1"
Weight	121 lb
BMI	23
HEENT	Dry mouth and eyes; leathery tonge; no partoid swelling
Neck Adenopathy	None
Heart/Lung Auscultation	Normal
Abdomen	Benign
Hands	Normal
Skin	Normal

The results of routine laboratory testing (complete blood cell count, sedimentation rate, and comprehensive metabolic panel) were normal. The ANA IFA was positive at a titer of 1:320 with speckled pattern. First tier cascade testing (Figure 1) was negative, yet the 2nd tier was positive for SS-A and SS-B. Rheumatoid factor was ordered and was positive (≥14 IU/mL). The labs indicated a diagnosis of Sjögren's Syndrome.

After the diagnosis of Sjögren's Syndrome, the patient was referred to a rheumatologist for further evaluation and treatment. Consultation confirmed the diagnosis and she was started on prednisone 10 mg daily and hydroxychloroquine 200 mg daily. She was to follow-up with the rheumatologist for further definitive treatment. The primary care physician continues to work collaboratively with the rheumatologist to manage this patient.

3 Autoimmune Hepatitis

A 43-year-old woman presented with a 5-month history of unintentional weight loss (13.2 lb), anorexia, irritability, malaise and generalized pruritus. On physical examination, she was mildly icteric with numerous scratch marks, palmar erythema, and hepatosplenomegaly; vital signs and physical exam were unremarkable (Table 4). Laboratory results showed a low hemoglobin level (8 g/dL) with a normal white-cell count and an elevated erythrocyte sedimentation rate (140 mm/h; normal ≤ 20 mm/h). The prothrombin time was prolonged, yet urea and electrolytes, calcium and phosphate concentrations were normal. Although the serum albumin was normal (4.1 g/dL), the following were elevated: total serum proteins (9.3 g/dL; normal range 6.1-8.1 g/dL), serum bilirubin (1.8 mg/dL; normal range 0.2-1.2 mg/dL), alanine transaminase (152 IU/L; normal range 7-55 IU/L), and aspartate transaminase (164 IU/L; normal range 8-48 IU/L). The alkaline phosphatase level was normal (83 IU/L; normal range 45-115 IU/L). Her serum immunoglobulins showed an increased IgG level (44 g/L; normal range 7.2-19.0 g/L) with normal IgA and IgM levels.

Antinuclear antibodies by IFA were strongly positive (titer 1:320) in a homogeneous pattern, and 1st tier autoantibody

Table 4. Hepatitis Patient Vital Signs and Physical Examination	
Height	5'7"
Weight	111 lb
BMI	17.4
HEENT	Normal
Neck Adenopathy	None
Heart/Lung Auscultation	Normal
Abdomen	Distended; liver palpable below costal margin
Hands	Normal
Skin	Mildly icteric; pruritic with scratch marks on various locations including forearms, abdomen, and thighs; palmar erythema on both hands

testing was revealed positive for only dsDNA antibodies. The ANA IFA also showed cytoplasmic staining of actin elements. Testing was positive for antibodies to smooth muscle consistent with autoimmune hepatitis. Hepatitis B surface antigen and hepatitis C antibody was absent and alpha fetoprotein was not detected. The immunological picture and absence of hepatitis B and C infection strongly favored a diagnosis of autoimmune hepatitis. The ANA titer 1:320 was a definitive diagnosis. The patient was therefore started on prednisolone (30 mg/day) and vitamin K, and showed dramatic improvement. Her serum bilirubin, transaminases, and prothrombin time returned to normal over the next two weeks. A diagnostic liver biopsy showed chronic active hepatitis with cirrhosis. She was continued on prednisolone (15 mg/day) and is fully reassessed every six months, including repeat liver biopsy as appropriate.

Conclusion

An ANA IFA cascade with reflex to specific testing has clinical significance in the proper setting. A positive ANA IFA by itself can show pre-clinical autoimmune disease, yet utilizing the ANA IFA cascade with reflex to specific testing can lead to early diagnosis and early treatment of potentially devastating diseases, putting some patients in remission. Test results should be interpreted in a clinical context that includes a history and physical, basic chemistry panel, imaging studies, and assessment of signs and symptoms.

References

1. Hajj-ali RA. Introduction to rheumatic diseases. https://www.merckmanuals.com/professional/musculoskeletal-and-connective-tissue-disorders/autoimmune-rheumatic-disorders/introduction-to-autoimmune-rheumatic-disorders. Accessed August 29, 2016.
2. Autoimmune disease in women. American Autoimmune Related Diseases Association, Inc. http://www.aarda.org/autoimmune-information/autoimmune-disease-in-women/. Accessed August 29, 2016.
3. Suarez-Almazor M, Gonzalez-Lopez L, Gamez-Nava I, et al. Utilization and predictive value of laboratory tests in patients referred to rheumatologists by primary care physicians. *J Rheumatol.* 1998; 25:1980-1985.
4. American College of Rheumatology Position Statement: Methodology of testing for antinuclear antibodies. Published January 2009. Updated August 2015. http://www.rheumatology.org/Portals/0/Files/Methodology%20of%20Testing%20Antinuclear%20Antibodies%20Position%20Statement.pdf. Accessed August 29, 2016.
5. Salzman BE, Nevin JE, Newman JH. A primary care approach to the use and interpretation of common rheumatologic tests. *Clinics in Family Practice.* 2005; 7(2):335-358.
6. Kavanaugh A, Tomar R, Reveille J, et al. Guidelines for the clinical use of the antinuclear antibody test and tests for specific autoantibodies to nuclear antigens. *Arch Pathol Lab Med.* 2000; 124:71-81.
7. Satoh M, Chan EKL, Sobel ES, et al. Clinical implication of autoantibodies in patients with systemic rheumatic diseases. *Expert Rev Clin Immunol.* 2007; 3:721-738.
8. Stinton LM, Fritzler MJ. A clinical approach to autoantibody testing in systemic autoimmune rheumatic disorders. *Autoimmun Rev.* 2007; 7:77-84.
9. Petri M, Orbai AM, Alarcón GS, et al. Derivation and validation of systemic lupus international collaborating clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum.* 2012; 64:2677-2686.
10. Cappelli S, Randone SB. "To be or not to be," ten years after: Evidence for mixed connective tissue disease as a distinct entity. *Semin Arthritis Rheum.* 2012; 41:589-98.
11. Gutierrez-Adrianzen OA, Koutouzov S, Mota RM, et al. Diagnostic value of anti-nucleosome antibodies in the assessment of disease activity of systemic lupus erythematosus: a prospective study comparing anti-nucleosome with anti-dsDNA antibodies. *J Rheumatol.* 2006; 33:1538-1544.

MI6203 12/2016