EDUCATIONAL COMMENTARY – ASSAYS FOR ANTINUCLEAR ANTIBODIES

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Learning Outcomes

On completion of this exercise, the participant should be able to

- discuss autoimmunity;
- describe the nonspecific symptoms of autoimmune diseases;
- discuss the various laboratory assays and their uses in the diagnosis of autoimmune diseases; and
- compare antinuclear antibody assays.

Introduction

The role of the immune system is to defend the body against pathologic organisms such as viruses, bacteria, fungi, and parasites. In some cases, the immune system becomes dysregulated and directs itself against its own cells or organs. This condition is known as autoimmunity, and the diseases that result are designated as autoimmune diseases. When the immune system fails to recognize the body's own cells or tissues as self and mounts the same type of response that is directed against foreign antigens, the protective immunologic tolerance developed in the fetal and neonatal stages of life is compromised. A wide spectrum of autoimmune syndromes ranging from organ-specific to systemic may develop, affecting virtually any organ of the body. Autoimmune diseases are difficult to diagnose, because the symptoms are usually nonspecific with subtle onset, intermittent, highly variable from patient to patient, and often similar to those of other diseases. Symptoms may include fatigue, joint pain, swelling, abdominal pain, fever, and swollen glands. Further diagnostic confusion is created when more than one organ is affected. Often, a patient’s symptoms are considered psychosomatic or stress related. According to the American Autoimmune Related Diseases Association, it takes an average of 4.6 years and five physicians before an accurate diagnosis is determined. The many medical specialties involved in treatment, including rheumatologists, gastroenterologists, nephrologists, endocrinologists, neurologists, and hematologists, contribute to the difficulty in treating autoimmune diseases. Because not all symptoms of autoimmune diseases are treated by the same physician, similarities are often overlooked and treatment modalities are not shared.¹

The immune response is a finely balanced system. Too little activity results in disease, too much activity results in autoimmunity. Although the mechanisms are known, the cause of autoimmunity is unknown. Studies indicate that genetic and environmental factors are involved in the development of autoimmune diseases. These diseases are found to cluster in families, and often individuals diagnosed with one
syndrome develop another. Triggers of the autoimmune process include bacterial and viral infections, certain drugs, chemical and environmental irritants, and chronic inflammation related to food sensitivities. It is possible that a preceding infectious organism or substance damages cells, exposing those cells to the immune system. Inflammation plays a critical role in all autoimmune diseases. Stimulation of the immune system activates the inflammatory process. Inflammation is responsible for many of the symptoms of autoimmune diseases, which include redness, swelling, heat, and sometimes loss of function.

There are more than 100 autoimmune diseases, which can be chronic, incapacitating, and, often, life-threatening. Autoimmune disease is among the top 10 causes of death in women younger than 65 years. In most cases, the patient experiences symptoms that fluctuate between flareups and remission. Currently, there are no cures for autoimmune diseases. Autoimmune disease affects 50 million people in the United States, about 15% of the population, with about 75% of these cases occurring in women.

Laboratory Testing

Systemic autoimmune rheumatic diseases are a subset of autoimmune diseases that primarily affect the joints and muscles. These include rheumatoid arthritis, systemic lupus erythematosus, Sjögren syndrome, scleroderma, polymyositis, and dermatomyositis. The most common screening test for systemic autoimmune rheumatic diseases is the antinuclear antibody (ANA) assay. These assays detect antibodies in the patient’s serum directed against components of the cell nucleus. Antinuclear antibody testing may be performed using indirect immunofluorescence assay (IFA), enzyme immunoassay (EIA), or multiplex immunoassay.

Immunofluorescence Assay / Indirect Immunofluorescence Assay

The American College of Rheumatology issued a position statement in 2009 choosing the IFA, also known as the indirect immunofluorescence assay, as the criterion standard assay for ANAs produced in autoimmune diseases. The IFA is a screening test able to detect more than 100 autoantibodies directed against various nuclear and cytoplasmic antigens. Antinuclear antibody results report the antibody titer along with one of six patterns used by most laboratories. The patterns include homogeneous, peripheral (rim), speckled, nucleolar, centromere, and proliferating cell nuclear antigen. The IFA is a labor-intensive procedure requiring considerable expertise to properly interpret results. The procedure involves layering the patient sample on a commercial slide coated with a substrate of human epithelial (HEp-2) cells. If the patient serum contains ANAs, the ANAs will bind to the nuclei of the cells on the slide. The slide is then washed to remove all antibodies that have not combined with the substrate, and a conjugate composed of an antihuman antibody tagged with a fluorescent dye is added. A sandwich of HEp-2 cells, patient antibody if present, and conjugate is formed. Another washing removes all antibodies not incorporated
into the sandwich. The slide is then examined with a fluorescent microscope. Typically, patient specimens are screened using a 1:40 dilution. If the screen is positive, additional dilutions are performed and tested. The pattern and titer produced in positive samples is reported. Homogeneous and peripheral patterns are usually associated with systemic lupus erythematosus. Speckled patterns correspond with scleroderma, rheumatoid arthritis, and mixed connective tissue diseases. The nucleolar pattern may indicate scleroderma or Sjögren syndrome.

In 2002, to address the problems incurred in the interpretation of ANAs, automation was introduced to ANA IFA. Digital image analysis and recognition algorithms are now used to discriminate between negative and positive test results and to determine the pattern produced. Light intensity correlates with titer. Digital images can be viewed by the technologist and verified or revised. Problems with the automated system occur when the patient sample has mixed patterns or presents with one of the less common patterns.4

*Enzyme-Linked Immunosorbent Assay / Enzyme Immunoassay*

Enzyme-linked immunosorbent assay (ELISA) is a solid-phase immunologic assay. Monoclonal antibodies coated on microtiter plates act as antigens and are used to measure antibodies, other proteins, hormones, and peptides in patient specimens. This procedure is also known as *enzyme immunoassay*. A monoclonal antibody is adsorbed on a solid-phase microtiter plate. The patient serum is then added and incubated. If the serum contains the antibody sought (in this case, ANA), the antibody will attach to the antigen on the solid-phase. The microtiter plate is washed to remove any antibodies in the patient serum that are not bound to the antigen-antibody complex attached to the solid phase. A conjugate is added, composed of a secondary antibody with an enzyme tag that will attach to the patient antibody in a different location. The primary enzymes used as tags in this procedure are alkaline phosphatase and horseradish peroxidase. This step is followed by an incubation period. The plate is washed again to remove any excess conjugate that has not attached to the patient antibody. A substrate for the enzyme tag is added, and a color is produced. After a specified period of time, a stop solution is added. The resultant color is read on a spectrophotometer. The more color produced, the higher the concentration of antibody present in the patient serum. Improper washing, failure to incubate properly, and poor pipetting may result in aberrant results.

*Multiplex Immunoassay*

Multiplex immunoassay is another solid-phase immunoassay. Multiplex immunoassay for ANAs involves microspheres (usually 8-µm beads) coated with clinically significant nuclear antigens. The number of antigens involved varies by manufacturer but usually involves 11 to 13 beads. The beads, when detected by a laser, will produce a specific color and can be identified. Each of the different colored beads is
coated with a unique nuclear antigen. One of each of the colored beads coated with antigen is added to a tube. Patient serum is added to the tube and can react with the antigens on the beads. If the patient serum contains antibody(ies) to the antigen(s) on the various beads, the antibodies will attach to the antigens. Washing removes nonspecific antibodies. A fluorescent tag attached to an antihuman antibody is added. A second washing step is performed. The tube is then placed in a flow cytometer that has two laser detectors. One laser detects differences in color, thereby identifying the bead, and one detects the fluorescent particle attached to the patient antibody attached to the bead. Three quality control beads are also added to each tube: a serum-verification bead to confirm the addition of the patient serum, an internal control bead to standardize the detector, and a reagent blank bead. False-negative results may occur as the system may fail to recognize autoantibodies produced in autoimmune liver disease and autoantibodies directed against nucleolar antigens.

Comparison of ANA Assays

The IFA has higher sensitivity (> 95%) than solid-phase assays, but lower specificity. The use of HEp-2 cells increases the sensitivity of IFA, because many antigens capable of participating in autoimmunity are present in the substrate. Solid-phase assays (EIA and multiplex immunoassay) use a limited number of the most common autoantigens. Patients with rarer types of autoimmune disease may not test positive in solid-phase immunoassays because of the small number of autoantigens involved in the assay.4

Enzyme immunoassays and multiplex immunoassays have several advantages when compared with IFA. These assays are more cost-effective, less labor-intensive, easier to interpret, require less sample volume, and have faster turnaround times. On the other hand, multiplex assays are often not validated with the criterion standard assay or standardized between multiplex assays that use different technologies and different antigens.5 As stated earlier, they also use fewer antigens than IFA. Multiplex testing allows for the simultaneous detection of several autoantibodies, whereas EIA testing detects a single antigen per assay. This allows results for common ANAs and antibodies to extractable nuclear antigens to be available without reflex testing.

Usefulness of Assay Results

Results from ANA testing alone are not diagnostic. The assays’ lack of specificity leads to the production of many false-positive results. Positive test results are appropriately used as aids in the diagnosis of autoimmune diseases. The patient must present with the clinical signs and symptoms of an autoimmune disease. Antinuclear antibody testing is used to support the physician’s suspicion of an autoimmune disease and to assist in establishing a definitive diagnosis. The presence of antibodies to particular ANAs and antibodies directed against extractable nuclear antigens may lead to a specific diagnosis of systemic
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lupus erythematosus, Sjögren syndrome, scleroderma, rheumatoid arthritis, or others. Diagnosis of a specific autoimmune disease is important, as treatment differs for each disease.

Conclusion

Autoimmune disease affects a large portion of the US population, especially women. The diagnosis of a specific syndrome is difficult and treatment is costly. Antinuclear antibody testing is one of several tools used in the diagnosis of autoimmune disease. False-positive results may confuse the diagnostic process and lead to unnecessary follow-up testing. The physician must carefully consider all aspects of the patient’s condition and test results before making a definitive diagnosis.

References


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