



Continuing medical education

Immunological techniques that support the diagnosis of the autoimmune diseases[☆]

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ABSTRACT

During the past few years technological advance have been allowed the developing of techniques that help to the diagnosis of multiple diseases. In the case of the autoimmune diseases, immunological techniques are helpful since they allow the detection of multiple autoantibodies at the same time with small volumes of sample. Together with the development of the new techniques, sensitivity and specificity in the detection of the antibodies specificities' also have been increased, in such a way that the clinicians can count with tests that allow them to make early diagnoses with greater certainty and also to follow the course of the disease based on the variation of the antibodies presents in the patient's samples. It is important to emphasize that the new techniques of laboratory that are used for the support of the diagnosis of autoimmune diseases, no longer are exclusive for research laboratories but by their facility of standardization, quality control and reproducibility they can be used in clinical laboratory of medium and small sizes. In the present paper we describe those techniques with greater application in the clinical laboratory of autoimmune diseases.

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Técnicas inmunológicas que apoyan el diagnóstico de las enfermedades autoinmunes

RESUMEN

Durante los últimos años el avance tecnológico ha permitido desarrollar técnicas que ayudan al diagnóstico de múltiples enfermedades. En el caso de las enfermedades autoinmunes, las técnicas inmunológicas son de gran ayuda ya que permiten la detección de varios autoanticuerpos simultáneamente a partir de volúmenes de muestra pequeños. Aunado al desarrollo de las nuevas técnicas, la sensibilidad y especificidad en la detección de las especificidades de los anticuerpos también han ido en aumento, de tal manera que el clínico puede contar con pruebas que le permiten hacer los diagnósticos tempranos con mayor certeza y hacer también el seguimiento del curso de la enfermedad en función de la variación de los anticuerpos presentes en las muestras de los pacientes. Cabe destacar que las nuevas técnicas de laboratorio que se utilizan para el apoyo en el diagnóstico de las enfermedades autoinmunes ya no son exclusivas de laboratorios de investigación, sino que por su control de calidad, facilidad de estandarización y reproducibilidad pueden usarse en laboratorios clínicos medianos y pequeños. En el presente trabajo se describen las técnicas de mayor aplicación en el laboratorio clínico para enfermedades autoinmunes.

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Introduction

Since the description by Hargraves of the LE cell phenomenon and its association with lupus erythematosus (SLE), the study of

the antibodies associated with autoimmune diseases assumed great importance. Currently, the support of the laboratory in the diagnosis of autoimmune diseases is due to a large number of techniques that allow both to identify and confirm, or recognize specific antigen(s). Moreover, such techniques have evolved considerably in recent decades, as tests have been developed with greater specificity and sensitivity. Among the most used techniques are: indirect immunofluorescence (IFI), immunosorbent assay (ELISA), the multiplex assay and electroimmunotransference (EIT). Each has differences, but retain the general principle of the recognition of

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antigen-antibody binding, and we will present the most important characteristics of these tests.

Indirect immunofluorescence

Indirect immunofluorescence (IFI) is one of the most common techniques used in studies of autoimmunity due to its easy handling and standardization. However, the reading and interpretation of this test requires extensive experience. The technique is based on the use of antibodies that recognize antigenic native cell structures. The interaction is demonstrated by a human anti-immunoglobulin antibody produced by a rabbit, goat or guinea pig, directed against the constant fraction (Fc) of immunoglobulins IgG, IgA and/or IgM. This human anti-immunoglobulin antibody is conjugated or coupled to a fluorophore (usually fluorescein isothiocyanate [FITC]). The results of the recognition of antigens by autoantibodies in the serum, plasma or other liquids, are assessed using a fluorescence microscope. Currently, IFI is used in studies of autoimmunity for the detection of anti-dsDNA (DNAcd) or native DNA (DNAn) using *Crithidia luciliae* as substrate. For the detection of antibodies that recognize nuclear antigens, human epithelial cell lines such as HEp-2 cells or HeLa cells are used as substrates. In the case of antibodies against components of the primary and specific granules of polymorphonuclear neutrophils or cytoplasmic antibodies (ANCA), neutrophils fixed using ethanol and formalin are employed, and in the case of antibodies that recognize organ-specific antigens, specific tissue sections are used as substrates (eg, thyroid, esophagus, stomach, adrenal glands, salivary glands, etc.).

Detection of anti-nDNA antibody using *Crithidia luciliae* as substrate

The test is based on the recognition of antibodies that react against the nDNA of giant mitochondria (kinetoplast) of the parasite *Crithidia luciliae*.¹ The test is only considered positive when the kinetoplast stains, regardless of staining in the nucleus, because there can exist reactivity against other components, leading to false positive results. The technique is highly specific but less sensitive, so it is appropriate in certain cases, to confirm the results with other more sensitive tests like ELISA.

Antinuclear antibodies

The identification and quantification of antinuclear antibodies (ANA) not only includes antigens that are located in the nucleus of HEp-2 cells, but also antigens of the cytoplasm. The importance of using HEp-2 cells as substrate is based mainly on them having a bigger nucleus than normal due to its large number of nuclear antigens (eg more than 46 chromosomes, more than three nucleoli, abundant ribonucleoprotein, etc.) and cytoplasm (eg mitochondria, lysosomes, ribosomes, etc.) which allows for easy detection and identification of antigens recognized by autoantibodies present in the sera of patients with autoimmune diseases. Also, by being a very active cell line, all phases of the cell cycle may be observed in culture, thereby facilitating the identification of antigens present only in certain phases of division, such as centromeres or *proliferating cell nuclear antigens* (PCNA). ANA detection with IIF using HEp-2 as a substrate is useful as an initial screening test, because the most common staining patterns are related to a variety of antigens (Table), but it is necessary to perform more sensitive and specific tests than ELISA for the identification of the antigen or antigens recognized by autoantibodies. There may also be for specific staining patterns antigens such as nuclear lamina proteins, centrioles, Jo-1 and Scl-70, in which cases the observed pattern is useful to specifically identify the recognized antigen, which may be confirmed by ELISA or EIT.

Table 1
Antigens recognized by autoantibodies that give ANA staining patterns

Pattern	Antigen	
Nuclear	Homogeneous	dsDNA, histones, Ku
	Speckled	RNP, Sm, Scl-70, SSB, RNA
	Perinuclear	dsDNA, ssDNA
Cell cycle	Nucleolar	RNA, RNP
	Cytoplasmic	SSA, clathrin
Cytoplasmic	Mitochondrial	Mitochondrials, mitochondria phospholipids
	Intermediate filaments	Vimentin, actin, desmin, etc.

dsDNA indicates double stranded; RNP, ribonucleoproteins; ssDNA, single strand DNA.

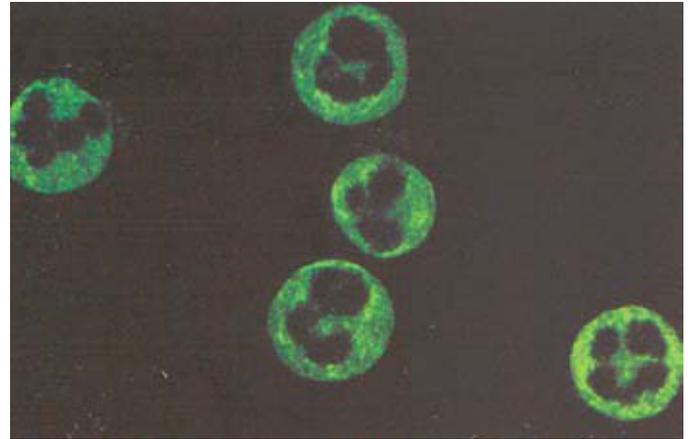


Figure 1. cANCA pattern. Antibodies recognizing weakly cationic proteins such as proteinase-3 (PR-3) and the 57kDa (CAP-57) cationic protein lead to a cytoplasmic pattern on alcohol or acetone fixed neutrophils.

Neutrophil cytoplasmic antibodies

For the detection of ANCA, IFI is also used as initial the screening test because it can detect the three staining patterns that are associated with clinical manifestations of autoimmunity. The patterns are: cytoplasmic or cANCA, perinuclear or pANCA and atypical or xANCA. The cANCA pattern is characterized by cytoplasmic staining on ethanol or acetone fixed neutrophils. Antibodies associated to the cANCA pattern are weakly cationic or neutral proteins such as proteinase-3 (PR-3), and the 57kDa cationic protein (CAP-57), which are released from specific granules by treating cells with alcohol or acetone and uniformly distributed in the cytoplasm of neutrophils (Figure 1).

The pANCA pattern is observed in neutrophils fixed with ethanol or acetone and is perinuclear homogeneous. The pattern is given by antibodies that recognize highly cationic proteins such as myeloperoxidase (MPO), elastase and azurocidine which, when released from primary neutrophil-specific granules and rearrange at the periphery of the nucleus, which is negatively charged by DNA (Figure 2A). The pANCA pattern should be confirmed in formalin-fixed neutrophils. Formalin is an organic solvent that reduces the effect of attraction of the cationic proteins (MPO, elastase, and azurocidine) to DNA, with which they are distributed in the cytoplasm. The antibodies that recognize it are associated to a cytoplasmic IFI pattern (Figure 2B).

XANCA or atypical pattern is the result of recognition of the following proteins: cathepsin G, lysozyme and lactoferrin. These proteins are released from specific granules of neutrophils and

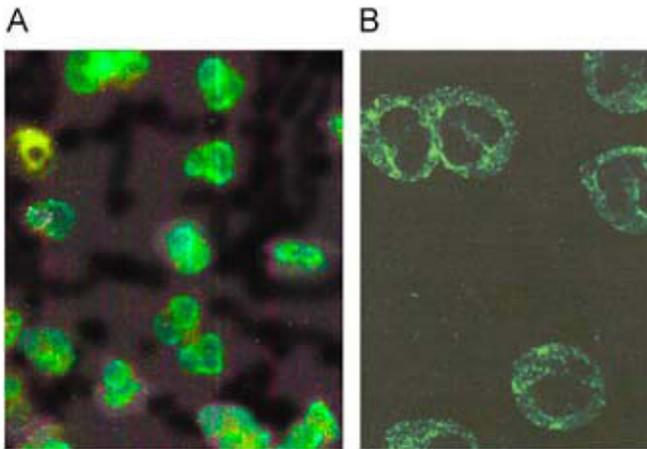


Figure 2. pANCA pattern. A) Antibodies recognizing strongly cationic proteins such as myeloperoxidase (MPO), elastase and azuridine giving a perinuclear pattern in alcohol or acetone fixed neutrophils. B) These antibodies give a cytoplasmic pattern when tested in formalin fixed neutrophils.



Figure 3. xANCA or atypical pattern. The antibodies recognizing cathepsin G, lysozyme and lactoferrin give an xANCA or atypical pattern in alcohol, acetone or formalin fixed neutrophils.

redistributed at the periphery of the nucleus when treated with ethanol, acetone or formalin. It is important to emphasize that the effect of perinuclear rearrangement of the above mentioned proteins is not modified in formalin-fixed neutrophils (Figure 3). Because a perinuclear pattern can be confused with a pANCA pattern, it is important to study samples of patients with this pattern in alcohol or acetone-fixed neutrophils and formalin-fixed neutrophils.

Antibodies specific organ

The reactivity of autoantibodies in samples from patients with autoimmune diseases is identified by IFI, which recognizes antigens specific to particular organs. To characterize the specific antigens or those recognized by antibodies, ELISA that contains the specific antigens should be used. For the detection of organ specific antibodies, slides containing tissue sections of organs, mostly from primates, are used. These include: adrenal glands, heart muscle, esophagus (to identify anti-endomysium antibodies), kidney (to identify anti-glomerular basement membrane antibodies) and pancreas (to identify anti pancreatic islets cells antibodies).

Immunosorbent assay

As mentioned above, ELISA is one of the techniques used to identify or confirm the specificity of antibodies present in samples from patients with autoimmune diseases. Furthermore, due to its easy standardization, management and the variety of antigens available, the test has significantly displaced other techniques such as radioimmunoassay for the detection of anti-dsDNA (also known as the Farr test), it does not use radionuclides, which makes it accessible and with a low technical risk. While there are different types of ELISA, the most commonly employed in autoimmunity diagnosis laboratory is the indirect ELISA, which is based on the recognition of specific antibodies present in patient samples, using an antibody directed against the human Fc region of any isotype (IgG, IgA or IgM) and including any subclass (IgG1, IgG2, IgG3, IgG4, IgA1 and IgA2). Anti-Fc antibodies are bound to enzymes such as peroxidase or alkaline phosphatase. The antigens used in ELISA plates can be native, recombinant (full-or epitope-specific antigen) or synthetic (specific epitope). After allowing for the interaction of antibodies from the samples of patients with antigen attached to the ELISA plate and washing out to remove nonspecific antibodies, human anti-immunoglobulin antibody is added together with an enzyme, allowing the interaction for a fixed period of time. Then, the solution containing the chromogenic substrate-specific enzyme (3, 3', 5, 5'-tetramethylbenzidine [TMB] to peroxidase or p-nitrophenylphosphate for alkaline phosphatase) is added, which will change color based the amount of enzyme conjugated antibodies and whose amount depends on patient's antibodies recognizing the antigen attached to the plate. In other words, the color intensity is directly proportional to the amount of patient antibody bound to antigen³ (Figure 4). The technique can be qualitative if it is only required to know whether or not reactive antibodies to specific antigens exist, or quantitative if it is required to know the amount of antibodies present in samples, for which the test should include a specific reactivity pattern curve. ELISA has the advantage that no sophisticated equipment is needed for its interpretation.

Multiple luminometric assay

In recent years, the multiple luminometric test (MLT) has become popular because it allows for the detection of up to 100 different antibodies that recognize the same number of antigens in a single determination and with a small volume of sample (about 50 µl) These are the most important advantages compared to the ELISA test, since the same number of specific determinations by ELISA would require 100 plates and 5 ml of sample. The technique is a variant of indirect ELISA, but detection is different: ELISA uses a chromogenic compound, while MLA detection is through fluorescence, making assay sensitivity higher. MLA is based on the detection of antibodies that react against specific antigens, which coat spheres containing different proportions of a fluorophore (combinations of red and infrared fluoride). The determination of the amount of antibody bound to antigen is revealed by the interaction of an anti-human immunoglobulin antibody conjugated to biotin. Subsequently, addition of a streptavidin-phycoerythrin conjugate (PE) is added. The test is read in a luminometer, an instrument that has the same principle as a flow cytometer. In simple terms, when passed through a fluorescence detector, the microspheres containing the antigen-antibody-streptavidin-PE, the dye of the microspheres and PE are excited by beams of light with wavelengths of 635 and 488 nm respectively. Laser light passing through the 635 nm wavelength filter excites the fluorophore of the microsphere, allowing the identification of antigens, and the laser that passes through the 488 nm filter excites the PE-conjugated streptavidin, allowing the detection of the amount of specific antibody in the sample of the patient (Figure 5).^{2,4}

Electroimmunotransference

EIT, along with ELISA and the MLA, it is one of the techniques with greater sensitivity and specificity for the detection of antibodies against specific antigens, however, EIT has the drawback of being qualitative or semiquantitative. It is based on the recognition of antibodies that have specificity for antigens that are absorbed by

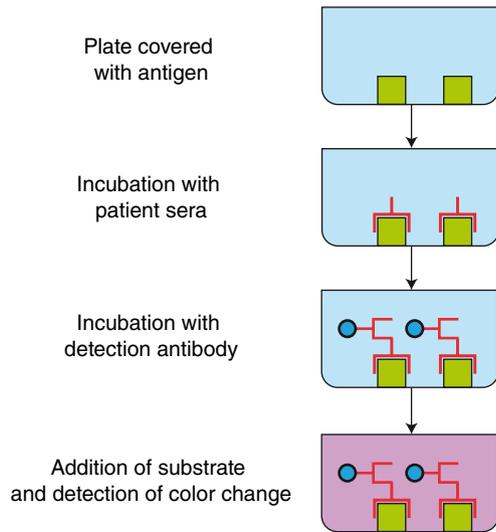


Figure 4. ELISA. A 96 well plate is covered with specific antigens, which are recognized by the antibodies in the patient's sera. Binding is revealed when an anti-human Fc enzyme-conjugated antibody is added. By adding the substrate, the enzyme changes the color of the medium.

a membrane (nitrocellulose or nylon). The antigens adsorbed on the membrane were previously separated in polyacrylamide gel sodium dodecyl sulfate (SDS-PAGE) and transferred to membranes. The binding of antibodies to specific antigens detected by the addition of an antibody that recognizes the Fc region of human immunoglobulin, which is coupled to an enzyme (alkaline phosphatase or peroxidase). This binding is revealed after the addition of a soluble chromogenic substrate (alpha-naftol/Fast network or NBT / BCIP [tetrasodium chloride blue/toluidine salt 5-bromo-4-chloro-3-indolfosphate] respectively, for each enzyme) which precipitates on the site where the antigen-antibody complex is located by the action of the enzyme. EIT is an accessible and easily interpreted technique, as it does not require any tool for reading and is performed visually.⁶

Multiple luminometric nanoassay

The multiple luminometric nanoassay (NLA) is one of the recently developed multiple detection methods. It can identify simultaneous reactivity against 10 autoantigens. It is a technique derived from EIT and is based on the recognition of autoantigens absorbed in small quantities, in 96-well plates for ELISA with a nitrocellulose membrane background. The antigen-antibody complex is detected by a second antibody against the Fc region of human immunoglobulin, which is conjugated a fluorophore. The reactivity is measured by an image analyzer.⁵ The technique is qualitative and requires a reading instrument and a computer program for its interpretation (Figure 6).

Conclusions

- IFI is an initial screening technique that, according to the substrates used (HEp-2 cells, neutrophils or tissue), can identify potential

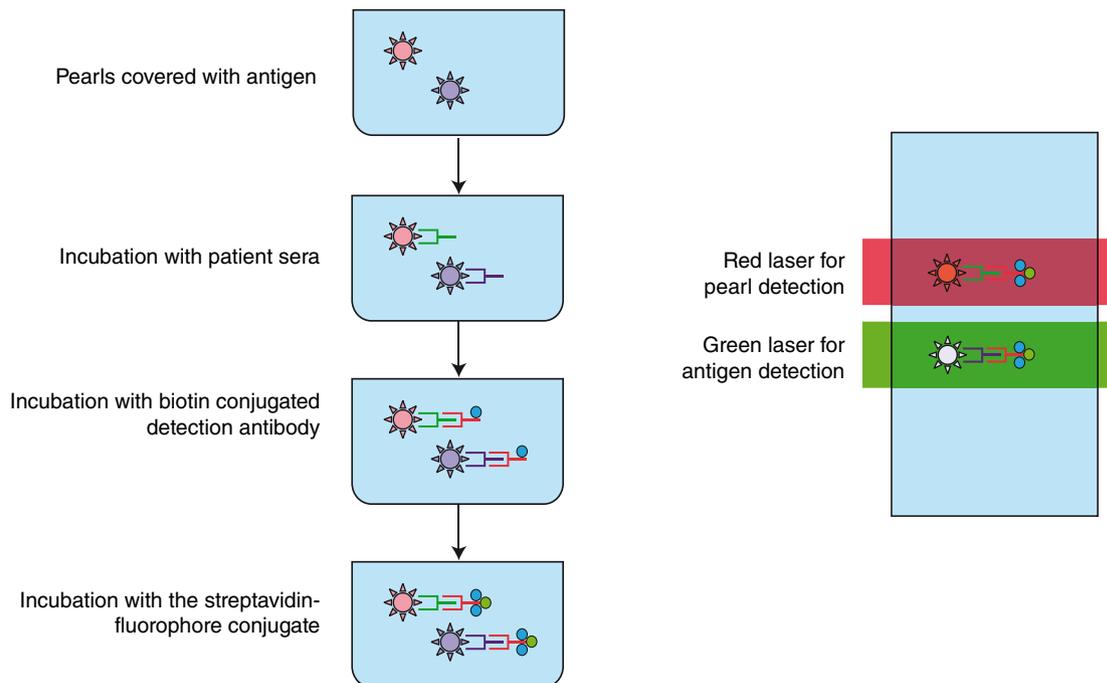


Figure 5. Multiple luminometric assay. Specific antigen covered microspheres are recognized by the antibodies present in the patient's sera. Antigen-antibody interaction is revealed by the formation of an anti-human Fc streptavidine-PE complex which, when passing through the luminometer is excited by a laser that passes through 2 filters: one of 635 nm, allowing to discriminate between pearls and, another of 488 nm, which allows to quantify the mean intensity of fluorescence, in other words, the binding of patient's antibodies to antigen.

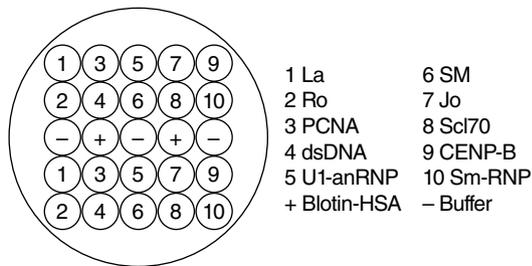


Figure 6. Image showing a nanometric multiple assay or NMA (top part). In the lower part of the image there is a well; as can be seen, each well contains duplicated 10 antigens and 2 controls. Antigens are shown in the right lower part of the image. Controls, localized in the middle part of the well are: buffer (negative control) and human serum albumin attached to biotin (Biotin-HSA, positive control of the system). McBride, 2008.

antigens recognized by autoantibodies present in sera of patients with autoimmune diseases.

- Among the immunological techniques that support the diagnosis of autoimmune diseases we find IFI, ELISA, EIT and multiple tests (MLA and NLA).
- ELISA, EIT and MLA are tests that confirm the reactivity of antibodies in patient samples. Their sensitivity and specificity are higher compared with initial screening tests.

Disclosures

The authors have no disclosures to declare.

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