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## The role of the autoimmunity laboratory in autoimmune diseases

SS Hasson\*, MS Al-Balushi, AA Al-Jabri

Department of Microbiology and Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, P.O. Box 35, Muscat, Oman

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### ABSTRACT

Laboratory testing is of great value when evaluating a patient with a suspected autoimmune disease. The results can confirm a diagnosis, estimate disease severity, aid in assessing prognosis and are useful to follow disease activity. Components of the laboratory exam include complete blood count with differential, comprehensive metabolic panel, inflammatory markers, autoantibodies, and flow cytometry. Currently, autoimmunity laboratories are very vibrant owing to the constant and increasing availability of new tests, mainly due to the detection of new autoantibodies. The main characteristic that differentiates the autoimmunity laboratory from other laboratories is the use of immunoassays such as enzyme-linked immunosorbent assay (ELISA), as basic techniques which determines antibodies (autoantibodies) and not antigens. For this reason, immunoassay techniques must employ antigens as reagents. However, over the last few years, a significant trend at autoimmunity laboratories has been the gradual replacement of immunofluorescence microscopy by immunoassay. Nowadays the revolution of new technology has taken place significantly, for examples; recombinant DNA technology has allowed the production of large quantities of antigens for autoantibody analysis. Flow cytometry for the analysis of microsphere-based immunoassays allows the simultaneous measurement of several autoantibodies. In the same way, autoantigen microarrays provide a practical means to analyse biological fluids in the search for a high number of autoantibodies. We are now at the beginning of an era of multiplexed analysis, with a high capacity of autoantibody specificities. The future tendency in this field will include immunoassays with greater analytical sensitivity, specificity, simultaneous multiplexed capability, the use of protein microarrays, and the use of other technologies such as microfluidics.

## 1. Introduction

The prevalence of autoimmunity in the general population is contentiously rising. The situation is aggravated because of the broad range and partial overlap of the various clinical symptoms which make it difficult in establishing a definite diagnosis. For this reason, the development of adequate and improved diagnostic techniques is essential and vital.

Currently, autoimmunity laboratories are in a very vibrant situation owing to the constant and increasing availability of new and developed tests, mainly due to the detection of new autoantibodies and demonstration of their clinical usefulness. Continuous improvement of the biochemical and molecular methods has allowed rapid dissection of the autoantigens associated with specific autoimmune diseases. Collectively, the autoimmune diseases can generally be classified into two groups: those that are systemic in nature

with varieties of autoantibodies which are highly specific for certain diseases, including anti-dsDNA, anti-Sm, anti-ribosomal P autoantibodies in SLE, anti-topoisomerase I (Scl-70) in scleroderma, anti-CCP in rheumatoid arthritis, anti-SS-A/Ro, anti-SS-B/La in Sjögren's syndrome (SjS), anti-U1-RNP, anti-PM-Scl in mixed connective tissue disease (MCTD) or anti-Jo-1 in polymyositis or dermatomyositis; and those that are more organ or tissue directed which are associated with autoantibodies specific to the main affected organ, like thyroglobulin (TGA) and thyroid peroxidase enzyme (TPO) in thyroiditis, insulin and glutamic acid decarboxylase autoantibodies in T1D and anti-mitochondrial autoantibody in primary biliary cirrhosis.

The detection of such autoantibodies may represent a status of disease activity or at least predict a future pathogenic condition. However, each of these groups presents unique problems to the diagnostic laboratory, complicated by the fact that they may occur in combination with each other. And this in return will reflect that the autoimmunity laboratories should analyze and measure an increasing number of autoantibodies employing a

\*Corresponding author: Dr. Sidgi Hasson, Department of Microbiology and Immunology, Division of Immunology, College of Medicine and Health Sciences, Sultan Qaboos University, P.O. Box: 35, Code: 123, Muscat, Oman.

Tel: +968 24143549

E-mail: [shyahasson@squ.edu.om](mailto:shyahasson@squ.edu.om)

broad spectrum of techniques and methods<sup>[1]</sup>. On the other hand, it has been clearly shown that autoantibodies that are associated with autoimmune diseases not only play a significant role as diagnostic markers, but that their occurrence may also be used to make a well-founded prediction. For example, in the past, when autoantibodies were found to be in a patient who apparently showed no signs of disease, this was generally assumed to be a false positive result at that time. However, thanks to some excellent studies done at that time which have been stored for documentation purposes, it is well known now that autoantibodies can occur 10 to 20 years before the outbreak of autoimmune disease, and in some cases even earlier. The most striking example of this is with primary biliary cirrhosis, where the typical anti-mitochondrial antibodies (AMA) may be identified 30 years before the occurrence of the first symptoms. Anti-dsDNA antibodies precede the development of systemic lupus erythematosus (SLE) by 5 to 10 years.

## 2. The role of the autoimmunity laboratory in autoimmune diseases

Autoimmunity laboratories use immunoassays as the basic technique for the determination of autoantibodies and not the antigens<sup>[1]</sup>. Important antigens have been well described and they are applied in methods that are used to detect autoantibodies. The detection from autoantibodies to antigens for examples; SS-A/Ro, SS-B/La, Sm, RNP, Scl-70, PM-Scl and Jo-1 are clinically useful in systemic autoimmune diseases. There are a number of other antigens which have been used in assays for the detection of autoantibodies associated with specific systemic or organ specific diseases. The clinical usefulness of the analysis' results depends on the quality of the laboratory tests. Hence an ideal diagnostics test has both, high sensitivity and specificity. It also identifies all patients with diseases and is not positive in those who do not have diseases<sup>[2,3]</sup>.

## 3. History

In 1947 Hargraves introduces the first method that used to detect antinuclear antibody (ANA) using "LE cell" preparation which linked autoimmunity to the systemic lupus erythematosus diseases<sup>[4]</sup>. Subsequently in 1957 the immunofluorescence technique was designed to detect ANA, which denotes specific subtypes based on the nuclear or cytoplasm component<sup>[5]</sup>. In the years following, the first enzyme immunoassay method was introduced in 1972, and since that time several different forms of enzyme immunoassays have been introduced successfully<sup>[6,7]</sup>. Other assays based on hemagglutination reactions, immunodiffusion and to some degree, immunofluorescence are increasingly being replaced since then by less demanding tests, *i.e.*, immunoblotting techniques or enzyme immunoassay (EIA) based on detecting the presence or concentration of individual autoantibodies in biological fluids.

## 4. Techniques used for the detection of autoantibodies

Requests of screening tests for autoantibody detection have risen remarkably, mainly due to the increased understanding of the nature of autoantibodies. The main analytical techniques used in the autoimmunity laboratory are listed in Table 1.

**Table 1**

Analytical techniques used in the autoimmunity laboratory.

1	Indirect immunofluorescence (IIF)
2	Immunoassays
3	Enzyme immunoassays (ELISA)
4	Immunoradiometric assay (IRMA)
5	Immunoblot
6	Western blot
7	Dot blot
8	Agglutination (Haemagglutination and latex agglutination)
9	Immunoprecipitation
10	Turbidimetry
11	Nephelometry
12	Double immunodiffusion
13	Counterimmunoelectrophoresis

Over the last few years, the most significant trend at the autoimmunity laboratory has been the gradual replacement of microscopy (IIF) and other manual methods such as double immunodiffusion and counter immuno-electrophoresis, by immunoassays such as enzyme immunoassay (EIA), which requires less skilled operators. This immunoassay is more objective, and can easily be automated. The following sections illustrate the common techniques that are in use nowadays in the autoimmunity laboratories.

## 5. Enzyme immunoassay

At present, enzyme immunoassays offer a basic technique and it is widely used for identifying specific autoantibodies to nuclear or cytoplasmic antigens of different group of organ-specific disorders, such as Grave's disease, primary biliary cirrhosis, insulin-dependent diabetes mellitus or systemic affecting different organs like systemic sclerosis, Sjögren's syndrome, mixed connective tissue disease or rheumatoid arthritis, in clinical laboratories<sup>[8,9]</sup>. Of the several different forms of enzyme immunoassays, the enzyme-linked immunosorbent assay (ELISA) format is the one most used.

ELISAs for ANAs show good sensitivity and a high negative predictive value, so they can be used to eliminate samples without ANA, although because of their low positive predictive value, positive samples must be analysed by IIF to confirm the presence of ANA and to determine the final result. However, it should be noted that new horizons are continually being found for EIA in the autoimmunity laboratory, addressing a question of whether this new era of ANA screening might mean farewell to the microscope<sup>[10]</sup>. ELISA is also used to measure anti-dsDNA antibodies<sup>[11–13]</sup>. In general, anti-dsDNA ELISAs have very good diagnostic sensitivity, but with low diagnostic specificity, because they detect high- and low-avidity antibodies; the latter considered of low clinical relevance.

When using ELISA as a screening method for SLE for example, another test such as IIF should be used to increase diagnostic specificity, and/or the Farr assay, which only

detect high-avidity antibodies. Nucleosomes have been considered a major autoantigen in SLE and the measurement of anti-nucleosome antibodies has been proposed as more sensitive and specific than that of anti-dsDNA antibodies for SLE and drug-induced lupus<sup>[13,14]</sup>. Antinucleosome autoantibodies react with epitopes comprised of the native DNA structure found in chromatin, the native histone epitopes exposed in chromatin, and epitopes made up of the histone-DNA complex found in chromatin. Specifically excluded are reactivities to non-histone proteins such as centromere and DNA topoisomerase II<sup>[14]</sup>. However, based on experience anti-nucleosome antibodies and anti-dsDNA antibodies provide similar information in established SLE<sup>[15]</sup>. Currently ELISA is also being used for the measurement of anticentromere antibodies using recombinant antigen<sup>[16]</sup>. Several ELISA methods have been described for the detection of anti-neutrophil cytoplasmic antibodies, using purified and recombinant antigens. Attempts to obtain recombinant PR3 have not been successful for obtaining a protein reactive to human antibodies. Immobilisation of native PR3 by coating plastic plates can provide partial denaturation, with changes to conformational epitopes, which can produce a loss of reactivity with autoantibodies. This phenomenon has been observed in some patients treated with anti-PR3 antibodies and could be overcome by using a capture monoclonal antibody to immobilize PR3 protein<sup>[9]</sup>.

## 6. Immunofluorescence

The IIF technique, which uses various tissue sections or the human larynx epithelioma cell line (HEp-2) that have larger nuclei and nucleoli than rodent tissue cells as an antigenic source, has had major implications for the diagnosis of autoimmune diseases in a routine laboratory setting<sup>[9]</sup>.

Autoantibodies give characteristic fluorescence images called fluorescence patterns. The staining pattern provides some indication of the specificity of the antibodies in the sample. Hence the IIF staining pattern of a positive sample can be used to evaluate which appropriate antigen specificities to look for. In the first applications of this technique, slices with fixed tissues, for example, Hep-2 from mouse or rat tissue were incubated with serum samples and the binding of serum autoantibodies to their corresponding antigens in the tissues is detected by using fluorescent-labelled anti-immunoglobulin antiserum. It is well known that the Hep-2 cells used for the detection of autoantibodies do not have a satisfactory ability to give positive IIF results for antibodies to SS-A/Ro-52 and Jo-1 (histidyl-tRNA synthetase)<sup>[6]</sup>. Many serum samples give speckled or grainy homogenous staining patterns which cannot be clearly identified as one of the known patterns<sup>[9]</sup>. Other drawbacks associated with IIF are the substrate variations, manual performance, subjective result interpretation, low reproducibility and a lack of standardisation. In order to overcome these limitations, there were some solutions being introduced. The first one is that for most IIF assays, the laboratory should select a screening dilution, because undiluted serum gives a background staining due to the non-specific binding of clinically non-significant levels of circulating autoantibodies. Increasing the screening dilution produces a less sensitive and more specific assay. However, the greater sensitivity of ANA

assays with Hep-2 cells as compared to rodent tissues is associated with lower specificity. Thus, more patients with diseases other than SLE and healthy people show positive results. To overcome this problem a 1/160 titer has been recommended as the most acceptable cut off to separate normal from abnormal sera<sup>[17]</sup>.

IIF is also used for antineutrophil cytoplasmic antibodies (ANCA). This is an assay to detect autoantibodies directed against antigens found in cytoplasmic granules of neutrophils and monocytes<sup>[18]</sup>. ANCAs are closely associated with Wegener granulomatosis, microscopic polyangiitis, and Churg-Strauss syndrome<sup>[19]</sup>. Determination of ANCA by IIF using neutrophil preparations yields three patterns: cytoplasmic (C-ANCA), perinuclear (P-ANCA) and atypical (A-ANCA)<sup>[20,21]</sup>. C-ANCA is associated with anti-proteinase 3 (PR3) antibodies and P-ANCA with anti-myeloperoxidase (MPO) antibodies. A-ANCA is a perinuclear pattern obtained with ethanol-fixed neutrophils, but negative with formalin-fixed neutrophils. In A-ANCA, the main target antigens are lactoferrin, elastase and cathepsin G. The A-ANCA pattern is mainly observed in non-vasculitic diseases, such as rheumatoid arthritis, intestinal bowel diseases and infectious bacterial or viral diseases<sup>[20]</sup>. Other autoantibodies measured by IIF are anti-mitochondrial antibodies (AMA), anti-smooth muscle antibodies, anti-liver and kidney microsomal (LKM) antibodies, antiparietal cell antibodies, anti-adrenal cell antibodies and other organ-specific autoantibodies. The second solution was thought to introduce fully automated IIF interpretation systems with pattern-recognition software. However, as noted above, two of the objections to IIF are its subjectivity and therefore it cannot be completely automated. In contrast, some researchers have claimed that this technique is becoming out-of-date and that it should be replaced by enzyme immunoassays or multiplexed assays in the routine laboratory diagnostics<sup>[22]</sup>.

## 7. Detection of autoantibodies by proteomic's technology

Clinical proteomics offers opportunities to identify new disease biomarkers in body fluids, cells and tissues. The focus of clinical proteomics is on the analytical and clinical validation and implementation of novel diagnostic or therapy related markers<sup>[23]</sup>. Antigen microarrays allow the comprehensive analysis of autoantibodies directed against hundreds to thousands of antigens, including proteins, peptides, nucleic acids, and macromolecular complexes<sup>[24]</sup>. Among this future assays are the multiplexed immunoassays, microarray based assays and flow cytometry. As yet, arrays for autoantibodies have not been commercialized, but in the coming future this technology should be accessible to clinical autoimmunity laboratories.

## 8. Differences between laboratory diagnostic methods

With the development of new technologies, there is a need to evaluate and standardize the technologies or diagnostic kits in an appropriate clinical laboratory setting<sup>[25]</sup>. Many studies conducted under standardized conditions showed the analytical variability of different test systems<sup>[26]</sup>. Specificities and sensitivities of autoantibodies against different antigens are important for the diagnosis, but variability in results

depend on the source of antigen, assays reproducibility, precision and accuracy and clinical manifestation of diseases[27,28]. Some studies showed agreement between IIF and EIA[9,29] while others demonstrated differences in results[16,30]. However, the choice of test is highly dependent on the clinical setting and higher sensitivity and specificity strongly depend on the cut-off value[9].

Currently results obtained in diagnostic laboratories or in different clinical studies underline the need for a drastic standardization of the used procedures and the importance of independent calibrators or international standards. So that to it can challenge the available diversity of test methodologies reflects the complexity of assays standardization. Also to provide quantitative and definitive autoantibody measurements based on reliable and reproducible assays which can provide clinically useful information with high specificity and sensitivity.

### Conflict of interest statement

We declare that we have no conflict of interest.

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