New diagnostics in autoimmunity: a review and our experience

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Introduction

The detection of autoantibodies is useful in the diagnosis and/or classification of autoimmune diseases as systemic lupus erytehmatosus (SLE), rheumatoid arthritis (RA), vasculitis and others. Indeed, the search for a number of autoantibodies is an essential requirement mentioned in the classification criteria of several autoimmune diseases. In light of the fundamental pathogenic role played by autoantibodies huge efforts were made in recent years to develop more sensitive and specific identifying methods. The immunoassay methods developed in the first instance to improve the results of scientific research are available today in the laboratories for routine clinical practice. The observation of the LE phenomenon by the common technique of light microscopy dated back to the early 50's, is the first to be recognized as a phenomenon linked to the presence of autoantibodies (1). Thus, since then the interest on immunodiagnostic has turned on, starting by borrowing and rearranging techniques used in microbiology.

The first assays were represented by conventional (or monoplex) analytical methods capable of determining single autoantibodies. The first autoantibodies observed in the serum were the antinuclear antibodies (ANA) by using indirect immunofluorescence (IIF), first on antigenic microspots coated slides (2) and then on cellular substrates (3). The indirect immunofluorescence (IIF) was therefore the first method applied to the detection of autoantibodies and for a long time it has been the best method in laboratory practice. However, the need for an expert morphologist, the subjectivity of interpretation and the low degree of standardization and automation does not make possible to take full advantage of the IIF potential (4, 5).

To overcome these limitations different systems have been developed over the years. Nonetheless, IIF remains (6) the gold standard in the determination of anti-nuclear antibodies (ANA) (7), anti-dsDNA and

anti-neutrophil cytoplasmic (ANCA) (8-10), especially thanks to the technical innovations made in identifying more than 60 autoantibodies simultaneously and to detect over 26 different cellular patterns by automated methods (11).

The automation of this method indeed gives the chance to reduce the variability of the results between laboratories, to increase the accuracy of results and to improve the correlation of staining patterns with corresponding autoantibody reactivities.

Other conventional monoplex methods include double immunodiffusion, complement fixation, passive agglutination, radio immunoprecipitation, and western blot.

With the last years between 1970 and 1980a new generation of monoplex methods, defined quantitative immunometric assay (IMA), was developed. This includes radioimmunoassay, immunoenzymatic assay, chemiluminescence immunoassay and flurometric immunoassay.

However, regarding the ANA, anti-dsDNA, and ANCA antibodies, the IMA monoplex methods does not represent a substitute for IIF since the literature shows that IMA does not provide the same analytic accuracy as IIF(12-15). Indeed, the results of the research have demonstrated a high percentage of false negative results (more than 35%) in seeking rare autoantibodies. In addition, when researching anti-dsDNA, ELISA has not been shown to have good specificity in differentiating single-stranded DNA (anti-ssDNA) from anti-dsDNA antibodies.

In recent years, new tools have allowed us to develop multiplex platforms that can investigate the presence of tens of molecules simultaneously, even if presenting small quantities in biological samples (16). The great sensitivity of these methods capacitate them for the study of the large amount of molecules involved in the activation of the immune system, thus, to study the entire concert of the autoimmune process rather than the single component. The methods developed can be divided into planar and non-planar autoantigenic arrays. The planar arrays systems use microspots on glass slides, polystyrene microplates, nitrocellulose membranes or linear immunoblot systems on nitrocellulose membrane. Among the non-planar arrays there are systems that use microparticles recognized by laser nephelometry, and laser fluorimetry in flow cytometry (17, 18). The diffusion of these new multiplex platforms, however, has revealed some limitations of different nature that limit their use. The problems relate to different aspects: analytical, logistical /managerial and pathophysiological (19, 20). The biomedical industries have therefore decided to develop autoantibody profiles already consolidated for the principal autoimmune diseases in multiplex versions to achieve diagnostic usage limited to the most common autoantibody pattern. This would allow to know the specific structure of the antibody in the patient, to monitor their status and to implement appropriate therapeutic strategies (21).

Diagnosis of the antiphospholipid syndrome

The antiphospholipid syndrome (APS) is a pro-thrombotic autoimmune disorder that can affect both the venous and arterial circulation of any tissue and organ without signs of vessel wall inflammation (22). The major clinical manifestations of APS include obstetric complications, such as unexplained death of one or more morphologically normal fetuses at or beyond the 10th week of gestation, the premature birth of one or more morphologically normal neonates before the 34th week of gestation because of either eclampsia or severe preeclampsia, and three or more unexplained, consecutive spontaneous abortions before the 10th week of gestation (23).

In 2006, the Sapporo classification criteria for APS diagnosis were updated, and the main innovation was the introduction of the detection of specific autoantibodies as an essential criterion for the diagnosis. In fact, the disease is characterized by the presence of a heterogeneous population of autoantibodies against mainly negatively charged phospholipid-binding proteins.

Historically, the term ‘‘lupus anticoagulant’’ (LA) was first coined by Feinstein and Rapaport in 1972 (24). The authors observed that in some patients the disorder was associated with another autoimmune disease, systemic lupus erythematosus (SLE), and that in plasma of these patients laboratory testing showed an anticoagulant effect (24). Afterwards, Harris et al. developed a radioimmunoassay for detection of anticardiolipin antibodies (aCL). The results of this assay on serum samples of patients with SLE and thrombotic complication showed a strong correlation between CL levels, LA, and the development of thrombotic manifestations (25). Deepening the studies, it was hypothesized the presence of a cofactor that was critical for the autoantibody binding to anionic phospholipids. Doubts arose after the observation that the LA effect in up to two-thirds of patient plasma samples was ‘‘augmented’’ by the addition of normal plasma (26) and that anticardiolipin antibodies do not bind to immobilized cardiolipin if plasma is not used in the assay (27).

The results of more research led to the identification of the plasma protein β2-glycoprotein-I (βGPI) as an essential cofactor (28, 29).

Hereafter, antibodies directed against other anionic phospholipids (e.g., phosphatidylserine, phosphatidic acid, etc.), against other phospholipid-binding plasma proteins (e.g., protein C, protein S), and IgA isotype-specific antibodies against cardiolipin and β_2GPI have also been identified in a number of patients with APS (30).

Since the pioneering work of Graham Hughes' group in the ‘80s, coining the term anticardiolipin syndrome later referred to as APS, antibody assessment in APS serology has mainly focused on plastic surfaces employed as solid phases for phospholipid or corresponding cofactor immobilization in various assay types (31, 32).

To date, according with the revised classification criteria, antiphospholipid antibodies (33) are recommended to be assessed by ELISA and by a functional clotting test detecting aPL antibodies interfering with phospholipid-dependent steps in the coagulation cascade, the so called lupus anticoagulant (LAC) (34-36).

Considering the poor standardization and lack of international reference standards and international consensus guidelines for aPL antibody ELISA, several attempts have been made to standardize aCL, LAC, and anti-β_2GPI tests including international workshops: an European forum convened for that purpose, the Australasian Anticardiolipin Working Party (AAWP), the College of American Pathologists (CAP), the National External Quality Assessment Scheme (NEQAS), and the Standardization Subcommittee (SSC) on LAC and phospholipid-dependent antibodies of the International Society of Thrombosis and Haemostasis (ISTH).

Consensus guidelines for the detection of LAC were first published in the 1990s and they have been recently revised and modified by the SSC on LAC and phospholipid dependent antibodies of the ISTH (37).

Despite these efforts, a considerable degree of inter-laboratory variation still exists mainly due to laboratories performing aPL assays. Regarding aCL test, the efforts to standardize the method began in the 80’s, but only in the past six years it has reached a good level of inter-laboratory agreement. In addition, it was recognized that the identification of isotype and the level of positivity were important because IgG isotype at higher levels was more closely associated with APS.

Given the recently reported lack of standardization and harmonization regarding these tests, in the last APLA 2010 task force (37) the performance of different ELISA and other immunoassays for the detection of aCL and anti- β2GPI antibodies (IgG, IgM) was tested.

A pool of sera were tested with different aCL and anti- β2GPI ELISA. In the APhL ELISA_ (an assay that utilizes a mixture of negatively charged phospholipids instead of cardiolipin, Louisville APL Diagnostics [LAPL]) and in three fully automated methodologies: HemosIL_ AcuStarAntiphospholipid assay panel, a chemiluminescent immunoassay panel on the ACL AcuStarTM (Instrumentation Laboratory [IL]); a fluoro-enzyme immunoassay (Phadia); and in the BioPlex 2200, random access, multiplex testing immunoassay system (Bio-Rad), using either automated or 'manual' platforms.

All the assays, but in particular the AcuStar chemiluminescent immunoassay panel and the BioPlex 2200 assays, showed an excellent intra-assay variation (<10% CV). All aCL and anti- β2GPI tests showed excellent clinical sensitivities, specificities, and positive predictive values and good agreement with respect to the levels of IgG and IgM antibodies, regardless to assay type, or whether tests were done using automated or 'manual' systems.

Nonetheless, further efforts must be put in the search for standardization, simplicity and economy.

Automation development in immunoassay: from smoke to flesh ZENIT

Zenit RA is a fully automated immunoanalyzer provided by A. Menarini Diagnostics, based on a two-step indirect chemiluminescent immunoassay (CLIA) (38).

The daily approach in autoimmunity laboratories is to perform multi-parametric tests in a short time and automatization: besides reducing times, this attitude can improve the reproducibility and reduce inter-laboratory variations (39).

The assay uses antigen-coated magnetic particles as a solid phase and, as detection marker, antibody tagged with a dimethyl acridinium ester. Different cartridges of reagents can stay on board in a refrigerated area with stability during eight weeks, in addition each calibrator is stable for 2-3 weeks. Several studies about the detection technique of many different autoantibodies have shown that Zenit RA analyzer exhibits a good diagnostic reliability, regarding sensitivity, specificity and positive and negative predictive values. Specifically, the reliability of the assay in determining anti-cyclic citrullinated peptides (CCP), and anti-aCL IgG and IgM, the ENA, anti-dsDNA, MPO and PR3 has been tested and compared with ELISA (37, 38).The results have demonstrated a good global agreement between Zenit RA and conventional tests (90-98%). Regarding anti-CCP, a global concordance of 96% was found and a very good correlation was available between CLIA and ELISA, so that Zenit RA anti-CCP assay has proven to have a satisfactory diagnostic value useful for clinical use. In determination of APS related antibodies global agreements between Zenit and ELISA were 90-94%. Clinical specificity was similar and high for both tests, but clinical sensitivity was lower in Zenit than ELISA for aCL IgG and higher for aCL IgM and for anti-β_2GPI. These differences between tests may arise from the disparities of qualitative and/or quantitative antigens, the potential alteration of epitopes structure during coating and the amount of each antigen (40).

Finally a good agreement was found for each test in the determination of anti-dsDNA (94%), anti-ENA (97%), anti-MPO (98%) and anti-PR3 (95%).

In conclusion, Zenit RA analyzer seems to be an attractive alternative to ELISA, in virtue of the complete automation and flexibility of work modalities, that reduce labor as well as assay time.

HELIOS: immediate diagnosis in the physician office

The important role of the IIF in immunological and immunometric assays has already been debated. As seen, despite attempts to use new methods for the determination of certain antibodies, IIF still is the best method. Indeed, following the recent statement made by the American College of Rheumatology that "the IIF technique should be considered as the standard screening method for the detection of ANA" (41), the biomedical industries have proposed technological solutions to remedy the problems presented by the standard IIF. Thereafter, an extensive evolution to develop technological solutions for autoimmunization of IIF was initiated, including devices for substrates (slides) preparation as well as for their interpretation.

These new systems are based on the use of automated microscopes, robotized slide trays, high-sensitivity video cameras, and software dedicated to acquisition and analysis of digital images (42, 43).

Helios is the first fully automated IIF processor including an integrated optical system for automatic slides reading aimed at positive/negative sample discrimination. The results can be recorded, saved and transmitted to the laboratory information system and validated remotely (44).

The expected advantages of the automatization are several, the most important concern the reduction in frequency of false negative and false positive results, the reduction of intra- and inter-laboratory variability and improvement in the correlation of staining patterns with corresponding autoantibody reactivities.

The agreement between ANA determination by the HELIOS system and the results obtained by expert observers reached 92%, of which a concurrence of 97.6% was observed in the ANA negative group and 90% in the ANA positive group (45).

Furthermore, HELIOS system recognizes a broad range of fluorescence patterns, including one esoteric pattern. Thus, Helios system has proved to be able to discriminate correctly ANA positive/negative samples compared to manual microscopic IIF performed independently by two experts, and its introduction in clinical practice may reduce inter-laboratory variability and time required to perform this test especially in high throughput laboratories.

BIOPLEX 2200: the fast and furious multiple faces in diagnostics

The efforts to overcome the limitations of IIF and ELISA techniques led to the development of multiplex immunoassay platforms capable of measuring several autoantibodies simultaneously. The BioPlex 2200 system is a totally automated Luminex-based assay using multiple–labeled magnetic beads used for semi-quantitative detection of several autoantibodies in a single biological sample (46). To date, thanks to its high analytic productivity is employed to search different antibody profiles for rheumatic diseases, APS, vasculitides and celiac disease (47-50). Numerous clinical validation studies have been conducted to confirm its diagnostic reliability.

A study evaluated the concordance between BioPlex 2200 and ELISA in determining ENA screening and anti-dsDNA antibodies in patients with SLE or Sjögren's syndrome. The BioPlex results were comparable with those of the ELISA in 81.1-95.2% among total analyzed samples according to the respective anti-ENAs. Concerning anti-dsDNA antibodies, the BioPlex 2200 system has shown to have a sensitivity of 98.7%.

Thus, the BioPlex 2200 system has demonstrated comparable results with conventional ELISA, moreover, it is able to rapidly detect various antibodies to ENA and anti-dsDNA simultaneously from a sample within an hour, whereas ELISA requires manual works, 4 to 6 hours, and separate test for every single analyte. These aspects suggest a useful role of the Bioplex 2200 system for detecting multiple antibodies in patients with SLE and Sjögren'ssyndrome (51).

Concerning other autoantibodies classes, a recent study has compared the performance of the aCL GPL, MPL and anti- β_2GPI GPL, MPL by the fully automated multiplex platform BioPlex 2200 and routinely used aCL and anti- β_2GPI manual ELISA assays. Overall agreement between BioPlex2200 and ELISA for routine samples and for patients with APS ranged from 88% to 96% for aCL IgG and IgM respectively. Significant correlation was also found for anti- β_2GPI IgG and IgM antibodies, 90% to 94%, respectively. The demonstration that the system has the same reliability of ELISA represents a step forward in the diagnosis of APS, considering that a fully automated, universal platform for anti-phospholipid antibody measurement will lead to a better reproducibility and inter-laboratory agreement of these assays (52).

Finally, in the USA the introduction of a method capable of measuring several antibodies simultaneously lead to the hypothesis that they can be used as a screening platform for ANA testing as an alternative to IIF. However, the results are not yet adequate and the presence of false negative results with respect to IIF is quite similar to immunometric methods (53, 54).

OPTION-point of care

To date the new discoveries in the field of RA antibody profile have emphasized the fundamental role of the anti-CCP antibodies in the early diagnosis of the disease. Hence, there was the need to develop a method for the determination of anti-CCP, easy to perform, rapid, inexpensive and naturally reliable. Until now ELISA was the standardized method. Two serological point-of-care tests (POCT) for the early detection of RA have been very recently developed. The Rheuma-Chec test (Orgentec, Mainz, Germany) combines two biomarkers for the diagnosis of RA—rheumatoid factor and antibodies to mutated citrullinated-vimentin (MCV). Antibodies to CCP are detected with the CCPoint assay (Euro- Diagnostica, Malmö, Sweden) (55, 56). The main innovation of these tests is represented by the fact that they require only a single drop of whole blood and any general physician can perform them within minutes. The CCPoint assay is a colloidal gold based lateral flow immunoassay. Reactive CCP are immobilized as a discrete line on a porous membrane located in the test zone. The detection reagent (colloidal gold particles conjugated to anti-human IgG) is deposited within the device onto the conjugate pad.

A study by our group has evaluated the new point of care lateral flow device (CCPoint) and routinely used ELISA assay for anti-CCP antibodies screening in patients. The results were particularly encouraging. In fact, CCPoint has proven to have a high sensitivity and specificity in aCCP determination compared with ELISA (57).

Conclusion

To conclude, whether the method, the laboratory autoimmunologist has now new arrows at his bow aiming at diagnosing and monitoring autoimmune diseases. We need to practice more with those are now available to reach high standards of quality, accuracy, economy and feasibility.

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