

Recent advances in diagnostic technologies and their impact in autoimmune diseases

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Abstract

Conventional immunological methods for the detection of serum autoantibodies have been an essential tool for the diagnosis of autoimmune diseases for 40 years: in the last decade autoantibody tests have become accepted criteria for the diagnosis and classification of the main systemic and organ-specific autoimmune diseases.

The high degree of purification reached by the autoantigens used in these methods has allowed high diagnostic sensitivity and specificity, especially in the case of some new autoantibodies of particular clinical significance, such as anti-nucleosome, anti-transglutaminase, anti-TSH receptor and anti-citrullinated protein autoantibodies.

In the last 5 years the advent of proteomic technology, which allows the simultaneous measurement of a number of autoantibodies (multiplexing), has opened up new horizons in the diagnosis of autoimmune diseases.

Multiplexing is particularly interesting for clinical laboratories, for organisational, logistical/managerial, physiopathological and research reasons.

The emerging technologies are represented by systems based on planar or non-planar (suspension) arrays: the latter include methods which use addressable microbeads or nanobarcoded particles.

Within a few years, the new methods will allow testing of individual autoantibody profiles, which will probably improve understanding of the physiopathology of autoimmunity, allow early diagnosis (due to the predictive value of autoantibodies), and drive the diffusion of antigen-specific therapies in autoimmune diseases.

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Abbreviations: RF, rheumatoid factor; IIF, indirect immunofluorescence; CF, complement fixation; PA, passive agglutination; ID, immunodiffusion; CIE, counterimmunoelectrophoresis; IPA, immunoprecipitation assay; IB, immunoblot; DB, immunodot; RIA, radioimmunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemoluminescent immunoassay; FIA, fluoroimmunoassay; AnuA, anti-nucleosome antibodies; TSHR, TSH receptor; tTG, tissue transglutaminase; CCP, cyclic citrullinated peptides.

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1. Conventional immunoassay methods for the detection of autoantibodies: the ‘old’ technologies

For four decades (1957–1997), conventional immunoassay methods were the main tool used for laboratory diagnosis of autoimmune diseases. Although some autoantibody detection tests date back to 1948 [1,2], immunological assays were first developed in 1957 [3–5]. Since then, several technologies have been developed and used in clinical practice (Tables 1 and 2):

- *immunochemical methods*: indirect immunofluorescence (IIF), complement fixation (CF), passive agglutination (PA), immunodiffusion (ID), counter-immunoelectrophoresis (CIE), immunoprecipitation (IPA), immunoblot (IB) and immunodot (DB);
- *immunometric methods*: radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA), fluoroimmunoassay (FIA) and chemiluminescent immunoassay (CLIA).

Immunochemical methods have allowed qualitative research, defining the presence or absence of autoantibodies in the serum of patients, whereas immuno-

metric methods have allowed quantitative measurement of antibody concentrations.

During the 1990s, the application of immunometric methods to instruments increasingly advanced in terms of analytical reliability and automation led to widespread use of autoantibody testing, with increase in the volumes of tests performed at each clinical immunology laboratory, and improvement in turn-around times.

A wide range of analytical systems with different degrees of complexity and throughput have been validated [6–9] (Table 3).

2. The use of single autoantibody tests in the diagnosis and classification of autoimmune diseases

As the production of high-affinity specific autoantibodies represents a hallmark of autoimmune diseases, the use of immunoassay detection methods has gradually entered clinical practice as a tool for the diagnosis of these diseases, to the extent that one or more laboratory tests constitute classifying/diagnostic criteria, e.g. antinuclear antibodies (ANA), anti-dsDNA, anti-Sm and anti-phospholipid antibodies (aPL) for systemic lupus erythematosus (SLE), RF for rheumatoid arthritis (RA), anti-Ro

Table 1
Conventional immunological methods for autoantibody detection (for acronyms, see text)

Immunochemical methods	Autoantigen source(s)	First Ab detected with this method	Actual automation level	Laboratory use	Main (first) reference(s)
IIF	Cryostatic tissue sections, human or protozoarian cultured cells (HEp-2, <i>Crithidia luciliae</i>)	ANA	+	+++	Friou [3], Holborow (1957)
CF	Purified native antigens	dsDNA, TMA, CPA	–	–	Robbins (1957), Trotter [4], Anderson (1957)
PA	Purified native antigens	dsDNA, Tg	–	–	Miescher (1957), Witebsky (1957)
ID	Purified native antigens	dsDNA, Tg, Sm	–	++	Ceppellini [5], Seligmann (1957), Doniach (1957), Tan (1966)
CIE	Purified native antigens	ENA	–	++	Kurata (1976), Walravens (1996)
RIPA	Purified native or recombinant antigens	RNP, GAD 21-OH	–	–	Lerner (1979), Hagopian (1993), Colls (1995)
IB	Purified native or recombinant antigens	rP, Ro 60/52	–	+++	Towbin (1979), Ben-Chetrit (1985)
DB	Purified native or recombinant antigens	ENA	–	+++	Stott (1989), Hermens (1997)

Table 2

Conventional immunometric methods for measuring autoantibodies (for acronyms, see text)

Immunometric methods	Autoantigen source(s)	Early detected Abs	Automation level	Laboratory use	Main (first) reference(s)
RIA	Purified native or recombinant antigens	dsDNA, Tg, TPO, TSHR	++	++	Wold (1968), Peake (1974), Rees Smith (1974)
EIA	Purified native or recombinant antigens	dsDNA, Tg, TMA/TPO	+++	+++	Engvall (1976), Voller (1980), Endo (1980)
FIA	Purified native or recombinant antigens	dsDNA	+++	+++	Jones (1981), van der Sluijs Veer (1996)
LIA	Purified native or recombinant antigens	AGA	+++	+++	Merridew (1995)

and anti-La for Sjögren's syndrome, aPL and anti- β_2 glycoprotein I (β_2 GPI) for antiphospholipid syndrome, anti-liver kidney microsomal (LKM), anti-smooth muscle (SMA) and ANA antibodies for autoimmune hepatitis, and anti-thyroperoxidase (TPO) for Hashimoto's thyroiditis. The introduction of quantitative methods for the measurement of antibody concentrations also allowed monitoring of the clinical course of autoimmune diseases, especially their activity and response to treatment, as in the case of PR3-ANCA in Wegener's granulomatosis, anti-dsDNA antibodies in SLE, and anti-TSH receptor antibodies (TSHRAb) in Graves' disease.

In the 1990s, the discovery of new autoantigens and the progressive refinement of purification techniques increased the sensitivity and specificity of antibody tests, improving the diagnosis and classification of some important autoimmune diseases. Some examples of this technological development are the methods of assaying anti-chromatin (nucleosome) antibodies (ANuA) for the diagnosis of SLE, anti-citrullinated peptide (anti-CCP) antibodies for the diagnosis of RA, anti-transglutaminase (anti-tTG) antibodies for the diagnosis of celiac disease, and TSHRAb for the diagnosis of autoimmune thyroid diseases, especially Graves' disease (Table 4).

The availability of purified antigen preparations, constituted by histone H1-free chromatin, has significantly increased the analytical and diagnostic sensitivity of ANuAs to 85–90% [10,11], exceeding the values obtained with first-generation techniques, which used the intact nucleosome.

The introduction of second-generation methods, which use a mixture of citrullinated peptides rather than the single peptide, has also enabled the diagnostic sensitivity of anti-CCP to be increased to 85% in RA [12].

The situation is similar for second-generation anti-tTG Ab and anti-TSHR assay methods: since recombinant antigen preparations have become available, these autoantibody tests have presented analytical sensitivity rates close to 100% in the diagnosis of celiac disease [13] and Graves' disease [14,15].

It should be emphasised that these results were achieved without reducing the diagnostic specificity of the tests, leading to great advantages in clinical practice.

3. Multiplex technologies for the study of autoantibody profiles: the 'new' technologies

Conventionally, the study of the autoimmune response has always been conducted by analysing the presence or concentration of individual autoantibodies in biological fluids. The objective is to identify a few autoantibodies (often only one) as specific markers for each autoimmune disease, and to attribute to each one a central role in the physiopathology of each syndrome.

The possibility of simultaneously measuring a number of correlated analytes (multiplexing) overcomes some of the limitations of conventional methods, and appears to be very interesting for analytical reasons (volumes of samples, reagents and low costs), logistical/managerial reasons (association of markers currently measured with different methodologies) and physiopathological reasons (combination of markers in disease-oriented or organ-oriented profiling), and for research purposes. In this case, the aim is to study the entire autoimmune process rather than its individual components.

The limitations of genomics have made it necessary to study gene products, namely RNA and proteins.

Table 3

Commercial analytical systems for autoantibody detection

Systems	Commercial instrumentations	Main (first) reference(s)
Conventional immunoassays, semi-automated (microplates)	DSX, Triturus, Alisei, Evolis, etc	Tampoia (2005)
Conventional immunoassays, full-automated	Cobas Core, Immulite, Elecsys, EliA, etc	Bayer [6], Bohuslavizki [7], Sapin (2003), Villalta [8], Villalta [9], Gonzalez (2005)

Table 4

1st and 2nd generation conventional immunoassay methods for autoantibody detection

Autoimmune disease	'Old' antigen 1st generation	'New' antigen 2nd generation	Detected autoantibodies	Main (first) reference(s)
Systemic lupus erythematosus	Intact nucleosome	H1-stripped nucleosome	ANuA	Ghirardello [10], Villalta [11]
Rheumatoid arthritis	CCP1 (1 peptide)	CCP2 (10 peptides)	CCP-IgG	Van Gaalen [12]
Celiac disease	Guinea pig tTG	Human recombinant tTG	tTG-IgA tTG-IgG	Collin [13]
Graves' disease	Porcine TSHR	Human recombinant TSHR	TSHR-IgG	Costagliola [14], Villalta [15]

Proteomics, the science that studies the expression, function and interaction of proteins on a large scale, seems to represent the key to comprehensive research into some acquired human diseases in the fields of oncology, infectious diseases, immunology, etc. Like genomics and ribonomics, proteomics allows parallel analyses to be conducted on hundreds of different antigens/antibodies in minimal quantities of biological fluids, with the aim of identifying the proteins or polypeptides encoded by messenger RNA transcripts, which are up- or down-regulated in association with a particular disease [16].

Among the numerous systems developed in recent years, some microarray assays are being used to study the autoantibody profiles of autoimmune diseases. Of all the variations now available, planar and non-planar autoantigen arrays (suspension arrays) have attracted particular attention from manufacturers and researchers.

Planar arrays include systems constituted by microspots on slides, polystyrene microplates or nitrocellulose membranes, and linear immunoblot systems on nitrocellulose membranes (Table 5).

Microspot assays are based on the technique originally devised by Ekins [17], and are likely to be very important in view of their simplicity and flexibility once some technical problems have been solved; commercial applications are not yet available, but numerous experiments validating their reliability are known [18–21].

Linear immunoblot systems are based on the linear western-blot technique (line immunoblot), with electrophoretic cell migration to nitrocellulose membranes; this method has numerous commercial applications, and clinical validation testing have been conducted on a large number of patients, some of which originate from multicentric studies [22–24]. In general, the line immunoassay shows a level of diagnostic and clinical accuracy comparable to, if not greater than that of conventional immunometric methods.

Non-planar arrays (Table 6) include suspension arrays which use microparticles recognized by laser nephelometry [25] or laser fluorimetry in flow cytometers [26]. This latter technology has numerous commercial applications, is rapidly expanding, and has been extensively validated in numerous clinical trials conducted all over the world.

Table 5

Planar autoantigen arrays for autoantibody detection

Support	Format	Detection system	Autoantibodies tested (no. and type)	Estimated throughput (test/h)	Commercial product	Reference
Glass slide	Microspot	L	18	~8.000	–	Joos (2000)
Glass slide	Microspot	L	115	~10.000	–	Haab (2001)
Glass slide	Microspot	F	196	~10.000	–	Robinson [18]
Glass slide	Microspot	F	650	~10.000	–	Hueber (2003)
Glass slide	Microspot	F	225	~10.000	–	Hueber [19]
Microplate	Microspot	C	15	ND	–	Feng [20]
Nitrocellulose membrane	Microspot	C	30	ND	–	Hentschel [21]
Nitrocellulose membrane	Line-blot	C	15 (ENA)	~50	Inno-LIA	Pottel [22]
Nitrocellulose membranes	Line-blot	C	13 (ENA)	~50	RecomLine	Eissfeller [23]
Nitrocellulose membrane	Line-blot	C	11 (ENA)	~50	ANA-LIA	Damoiseaux [24]

L, chemiluminescence; F, fluorescence; C, colorimetry; ND, not determined.

Table 6

Non-planar (suspension) autoantigen arrays for autoantibody detection

Support	Format	Detection system	Autoantibodies tested (no. and type)	Estimated throughput (test/h)	Commercial product	Reference
Microbead	Polystyrene well	LN	3 (ENA)	~200	Copalis	Bizzaro [25]
Microbead	Polystyrene wells	LF	9 (ENA)	~1.000	FIDIS	Rouquette [27], Bouliard (2005)
Microbead	Polystyrene wells	LF	3 (AGA/ITG)	~200	FIDIS	Tozzoli [28], Yiannaki (2004)
Microbeads	Polystyrene wells	LF	2 (Tg/TPO)	~200	FIDIS	Tozzoli (2006), Gonzalez [29]
Microbead	Polystyrene wells	LF	5 (ENA)	~1.000	AtheNA	Gilburd (2004), Shovman [31]
Microbead	Polystyrene wells	LF	5 (ENA)	~500	QuantaPlex	Ghillani (2004), Martins [32]
Barcoded particle	Polystyrene wells	LF	9 (ENA)	~10.000	UltraPlex	Smith [35], Pang [36]
Microbead	Full automation	LF	15 (ENA)	~2200	BioPlex 2200	Shovman [33], Binder [34]

LN, laser nefelometry; LF, laser fluorescence.

The main commercial systems, based on fluorescent microparticle technology, are represented by:

- the FIDIS system, BioMedical Diagnostics, Marne la Vallée, France;
- the AtheNA Multi-Lite system, Zeus Diagnostics, Raritan, NJ, USA;
- the QuantaPlex system, Inova Diagnostics, S. Diego, CA, USA;
- the BioPlex 2200 system, Bio-Rad Laboratories, Hercules, CA, USA.

The FIDIS system, which is currently the most widely used in clinical laboratories, allows the determination of 5 different autoantibody profiles, for rheumatic diseases, celiac disease, autoimmune thyroid disease, vasculitis and rheumatoid arthritis, and numerous validating clinical trials have been published [27–29].

The more recent AtheNA system allows the determination of 3 different autoantibody profiles, for rheumatic diseases, autoimmune thyroid disease and vasculitis, and presents a good level of analytical reliability [30,31].

The QuantaPlex system, still in limited use, allows the determination of various autoantibody profiles for rheumatic diseases, celiac disease, vasculitis and autoimmune liver disease, and demonstrates a high level of analytical reliability [32].

The very recently introduced BioPlex 2200 system, with full automation and high throughput, allows the determination of an autoantibody profile for rheumatic diseases; clinical validation experiments are available on very large numbers of patients [33,34].

A new multiplex system (UltraPlex, SmartBead Technologies, Cambridge, UK), which uses a different type of non-planar microarray (barcoded nanoparticles), was recently introduced onto the market, and some preliminary trials have already been conducted [35,36].

4. The predictive role of autoantibody profiles

Although a direct pathogenetic role of the autoreactive B lymphocytes has only been demonstrated for some organ-specific autoimmune diseases (Graves' disease, myasthenia gravis, etc.), autoantibodies appear at a very early stage in the natural history of autoimmune diseases, which feature a long pre-clinical period during which the symptoms are almost absent or very mild and vague. Moreover, autoantibodies accompany the clinical evolution of autoimmune diseases, often throughout their duration. The autoantibody response usually begins against one or a small number of autoantigens, and then extends to numerous other autoantigens or their epitopes. SLE, with its autoantibody multiplicity, is the most evident example of this phenomenon.

Detection of serum autoantibodies is considered to play a predictive role in autoimmune diseases, as autoantibodies reflect the presence, nature and intensity of the autoimmune response. Since the first pioneering study of the predictive value of thyroid antimicrosomal antibodies in post-partum thyroid dysfunction [37], specific autoantibodies have been closely associated with the pre-clinical stage of autoimmune diseases for over 20 years, and numerous studies of their role as risk factors have been conducted all over the world on different populations of subjects.

The predictive value of autoantibodies can be useful in the early diagnosis of disease in clinically healthy subjects or pregnant women, and for establishing the activity or severity of the disease [38]. For this purpose, multiple autoantibody determination systems can prospectively be used for preventive screening of open populations or at-risk groups, with significant practical implications (earlier diagnosis and treatment) and a correct cost–benefit ratio.

Autoantibody profiling will probably lead to new clinical correlations, allowing a more individual therapeutic approach (theranostics). Multiple determination systems could play a crucial role in this field too, allowing the identification in individual patients of autoantibody signatures able to drive the development and selection of antigen-specific therapies for autoimmune diseases [39], with the same results as obtained in the field of infectious diseases [40].

Take-home messages

- Detection of single antibodies in human sera represents a hallmark and a diagnostic criterion for autoimmune diseases.
- The conventional immunoassay methods for detecting autoantibodies present a high level of sensitivity and specificity.
- Newer diagnostic technologies, including microspots, line-blots, addressable microbeads and bar-coded nanoparticles, allow multiplex parallel testing of autoantibodies.
- Autoantibody profiles or signatures will enable early diagnosis and drive antigen-specific therapies in autoimmune diseases.

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Humanization of autoantigen

Transmissibility of characteristic lesions to experimental animals may help us understand the patho-mechanism of human autoimmune disease. Here, Nishie W. et al. (*Nat Med* 2007; 13: 378–83) show that human autoimmune disease can be reproduced using genetically engineered model mice. Bullous pemphigoid (BP) is the most common serious autoimmune blistering skin disease, with a considerable body of direct evidence indicating that the underlying autoantigen is collagen XVII (COL17). Passive transfer of human BP autoantibodies into mice does not induce skin lesions, probably because of differences between humans and mice in the amino acid sequence of the COL17 pathogenic epitope. Human BP autoantibody was injected into COL17-knockout mice rescued by the human ortholog. This resulted in BP-like skin lesions and a human disease phenotype. Humanization of autoantigens is a new approach to the study of human autoimmune diseases.