Karsten Conrad, Edward K.L. Chan, Luis E.C. Andrade,
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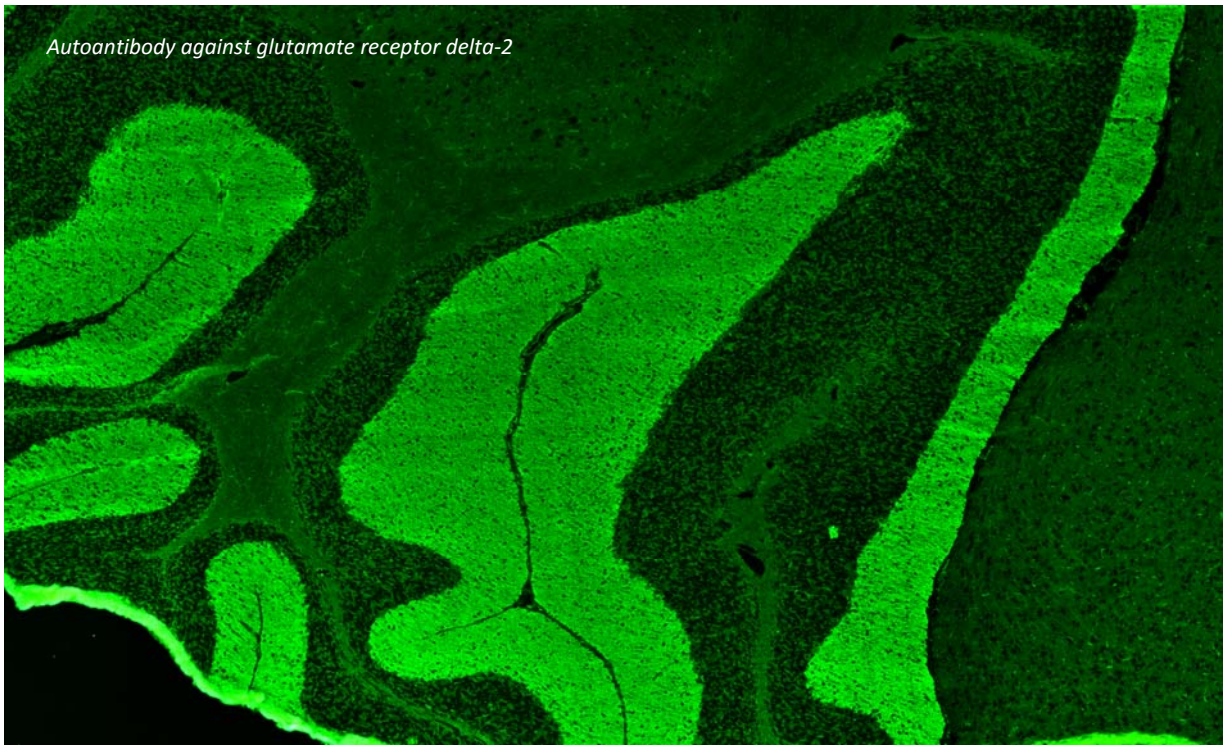
Report on the 12th Dresden Symposium on Autoantibodies, September 23-26, 2015



AUTOANTIGENS, AUTOANTIBODIES, AUTOIMMUNITY
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Preface

Testing for autoantibodies (AAB) becomes more and more relevant not only for diagnosing autoimmune disease (AID) but also for the differentiation of defined AID subtypes with different clinical manifestations, course and prognosis as well as the very early diagnosis for adequate management in the context of personalized medicine. A major challenge to improve diagnostic accuracy is to harmonize or even standardize AAB analyses. The 10th volume of the book series "AUTOANTIGENS, AUTOANTIBODIES, AUTOIMMUNITY" presents the results of the 12th Dresden Symposium on Autoantibodies that focused on several aspects of improving autoimmune diagnostics. Topics that are addressed include the International Consensus on ANA Pattern (ICAP) and the International Autoantibody Standardization (IAS) initiatives, the optimization of diagnostic algorithms, the description and evaluation of novel disease-specific AAB as well as the development and introduction of novel assays into routine diagnostics. The current volume also highlights important developments of the last years, most notably the improvement in diagnosing and predicting the course of rheumatoid arthritis, systemic sclerosis, idiopathic inflammatory myopathies, and of autoimmune neurological, gastrointestinal and liver diseases; the potential diagnostic role of anti-DFS70 antibodies; and tumor-associated AAB. Furthermore, some hot topics in autoimmunity regarding disease pathogenesis and management are described.

Hopefully, the data and information described and discussed in this volume will further stimulate initiatives for the improvement in diagnostics and management of AID.

The editors

Autoantibody testing and methodological platforms: a story with several versions

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Keywords

autoantibodies, autoantibody testing, laboratory standardization, diagnosis

Abbreviations

Addressable laser-based immunoassay (ALBIA), antinuclear antibodies (ANA), indirect immunofluorescence assay in HEp-2 cells (ANA-HEp-2), Ankylosing spondylitis (AS), Disease Specific Autoantibodies (DSA), Food and Drug Administration (FDA), Chronic hepatitis (CH), Enzyme-linked immune sorbent assay (ELISA), Healthy individuals (HI), histidyl-tRNA synthetase (Jo-1), Liver kidney microssome (LKM), Osteoarthritis (OA), Pathologic controls (PC), Polymyositis (PM), Rheumatoid arthritis (RA), Ribonucleoprotein (RNP), DNA-topoisomerase I (Scl-70), Systemic lupus erythematosus (SLE), Smith antigen (Sm), Sjögren syndrome type A (SS-A), Sjögren syndrome type B (SS-B), Systemic sclerosis (SSj), Sjögren's syndrome (SSj)

All is version
(Paulo Francis, Brazilian journalist; 1930 – 1997)

Disease specific autoantibodies

Autoantibodies are important elements in several aspects of autoimmune diseases. Some autoantibodies occur in the context of diverse autoimmune diseases whereas some others are specific diagnostic biomarkers for a restricted set or a single systemic autoimmune disease. These are designated Disease Specific Autoantibodies (DSA) [1-3]. Some DSA are specifically associated with specific sub-phenotype of these diseases. For some DSA, there is close association between serum titer and disease activity, which may be helpful in monitoring disease treatment.

The reputation of DSA is based on high specificity and not on high sensitivity

DSA associated with systemic autoimmune diseases usually occur at low to moderate frequency (Table 1) [4]. This is in frank contrast to DSA associated with organ-specific autoimmune diseases, which usually occur at higher frequency (Table 2) [5]. This dual behavior entails that DSA are not particularly sensitive biomarkers for systemic autoimmune diseases, but are quite sensitive biomarkers for organ-specific autoimmune diseases. However, in both classes of diseases they are very specific biomarkers. In fact, the high clinical specificity has been the very argument that built up the time-honored reputation of these biomarkers. Since the old days in which these biomarkers were originally detected by immunodiffusion and precipitation in agarose gels, some DSA display very modest sensitivity in systemic autoimmune diseases [6-9]. For example, anti-Sm antibodies were observed in no more than 15% of SLE patients, anti-Scl-70 (DNA topoisomerase 1) antibodies in no more than 20% of systemic sclerosis patients, and anti-Jo-1 (histydil tRNA synthetase) antibodies in no more than 30% of polymyositis patients [4,5,10]. Despite presenting low specificity these DSA achieved a high reputation as very useful biomarkers and some of them figure as part of the classification criteria for some of these diseases [11-13].

One may wonder why these low-sensitivity biomarkers hold such a high standing in terms of laboratory diagnosis. Since this reputation was not based on their sensitivity, it must be related to some other indicator of good diagnostic performance. In fact, that is the case. DSA are very specific biomarkers and represent an objective parameter to define the immunological identity of several systemic autoimmune diseases. It is well known that systemic autoimmune diseases are quite pleomorphic and vary considerably in terms of the spectrum of severity of each clinical feature. In addition, there is no established specific etiologic agent defined for them. In such scenario, DSA represent valuable specific surrogate biomarkers in clinical investigation and in the day-to-day management of these patients.

Table 1. Clinical associations and sensitivity of autoantibodies associated with systemic autoimmune diseases

Autoantibody	Clinical association	Sensitivity
Centromere	Systemic sclerosis	12-43%
	Primary biliary cirrhosis	10-30%
Citrullinated peptides	Rheumatoid arthritis	50-70%
Double stranded DNA	Systemic lupus erythematosus	40-70%
Fibrillarin	Systemic sclerosis	5-10%
Jo-1 (histidyl-tRNA synthetase)	Polymyositis	30%
Ki (SL)	Systemic lupus erythematosus	8-21%
Ku	Polymyositis/scleroderma	5-25%
	Systemic lupus erythematosus	5-10%
	Sjögren's syndrome	5-15%
Mi-2	Dermatomyositis	10-30%
Nucleosome (chromatin)	Systemic lupus erythematosus	50-75%
PCNA	Systemic lupus erythematosus	3-5%
PM/Scl (PM-1)	Polymyositis/scleroderma	20-25%
	Systemic sclerosis	3-15%
Ribosomal P	Systemic lupus erythematosus	10-20%
SS-A/Ro	Sjögren's syndrome	30-60%
	Systemic lupus erythematosus	25-30%
	Subacute cutaneous lupus	50-70%
	Neonatal lupus	90%
SS-B/La	Sjögren's syndrome	10-40%
	Systemic lupus erythematosus	5-15%
Scl-70 (topoisomerase 1)	Systemic sclerosis	18-30%
Sm	Systemic lupus erythematosus	5-15%
To/Th	Systemic sclerosis	4-10%

In which clinical scenario is a DSA test most useful?

Despite considerable clinical heterogeneity, each systemic autoimmune disease is associated with a distinctive collection of clinical and laboratory features. Careful and judicious selection of sets of these features have enabled investigators to design efficient classification criteria that are mostly useful for keeping a minimum homogeneity in series of patients taking part in clinical research. These criteria have also been used at variable extent as an aid for clinical diagnosis. The need to define classification criteria is indicative of the difficulty in establishing a definite diagnosis for patients with systemic autoimmune diseases. Although some patients display a clear and typical clinical presentation that poses no difficulty or doubt in diagnosis, that is not always the case with other patients. Diagnosis may be particularly difficult

at early stages of disease, when the most characteristic clinical features are still not evident. The same is true for patients that never develop the most typical traits of the disease.

In such cases, the clinical scenario is rich in equivocal findings that hampers a firm decision. In this situation, the least thing one needs is one more non-specific parameter. That is when a specific biomarker is quite valuable and this is why DSA achieved a high clinical reputation, despite their relatively low sensitivity.

Table 2. Clinical associations and sensitivity of autoantibodies associated with organ-specific autoimmune diseases

Autoantibody	Clinical association	Sensitivity
Acetylcholine receptor	Miastenia gravis	80-90%
F-actin	Type 1 autoimmune hepatitis	60-80%
Adrenal cortex	Autoimmune polyglandular syndrome	85-100%
Aquaporin 4	Neuromyelitis optica	85-90%
Endomysium	Celiac disease	>95%
Glutamic acid decarboxylase	Type 1 diabetes mellitus	70-90%
	Stiffman's syndrome	>90%
Glomerular basement membrane	Goodpasture's syndrome	>95%
Insulin	Type 1 diabetes mellitus	>90%
Islet cell	Type 1 diabetes mellitus	80-90%
LKM-1	Type 2 autoimmune hepatitis	80-90%
Mitochondria	Primary biliary cirrhosis	>95%
Tissue transglutaminase	Celiac disease	95-98%

Antibodies as complex and heterogeneous analytes

Most analytes identified in the clinical laboratory have a homogeneous molecular nature. That means that the same molecule is present in biological samples from all individuals in that species and even across different species. Examples of inter-species homogeneous analytes are glucose, potassium, nitric oxide, oxygen and lactic acid. Examples of intra-species homogenous analytes are albumin, alkaline phosphatase, and insulin.

In contrast to homogeneous analytes, antibodies to any given antigen represent a polyclonal humoral response and comprise a host of different molecules that share the unique characteristic of binding to that given antigen. Importantly, these polyclonal antibodies vary with respect to isotype, affinity, isoelectric point, heavy chain glycosilation, and serum concentration. In addition, there is considerable heterogeneity in the molecular epitopes they target within the same antigen molecule. This concept is illustrated in figure 1. This vast heterogeneity means that no two individuals will mount an identical polyclonal response to the same antigen. In other words, the collection of anti-Sm antibodies in SLE patient A must be different from anti-Sm antibodies from SLE patient B.

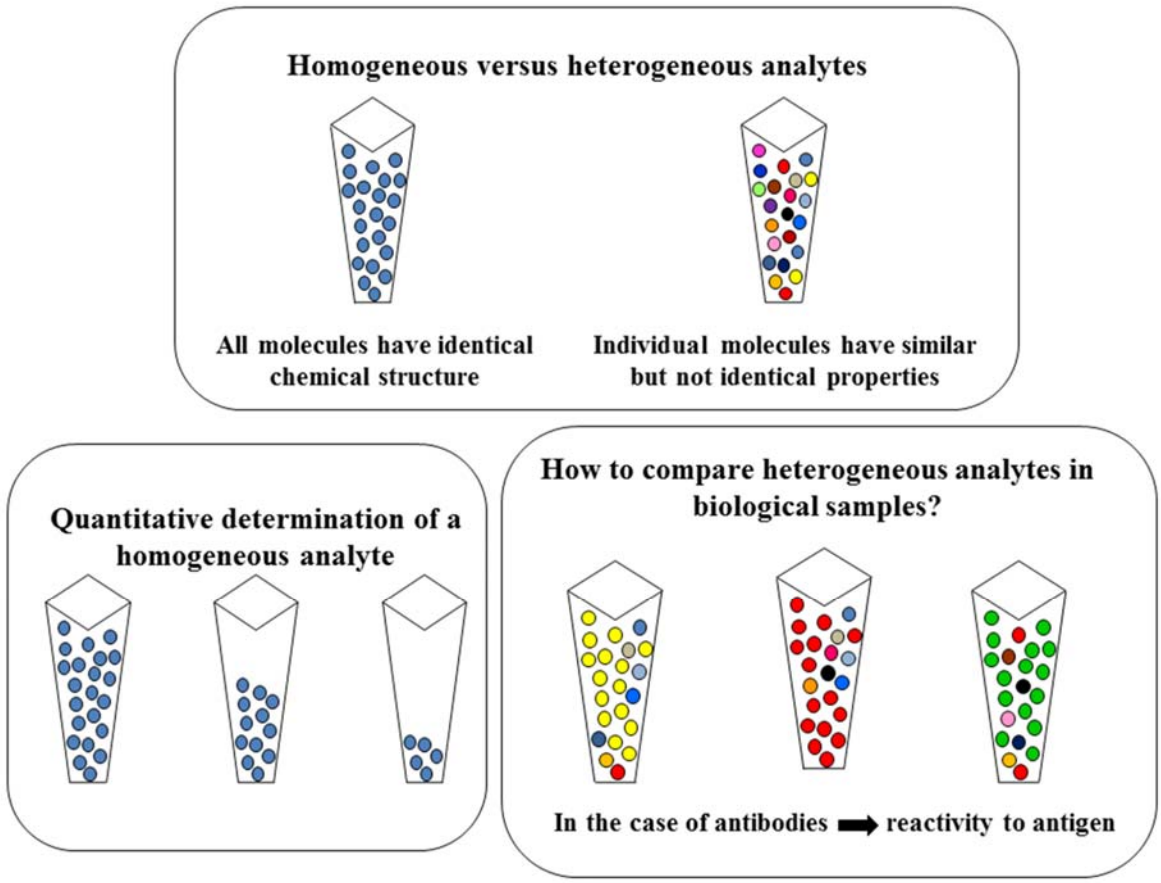

Figure 1. Heterogeneous nature of the "analyte autoantibody"

Among other peculiarities, the heterogeneous nature of antibodies poses some difficulty in designing assays for their laboratory determination and quantification in biological samples. Any methodological platform for determination of antibodies to a given antigen will privilege the detection of a certain collection of antibodies in detriment of others. As a corollary, a sample from any given patient may present different reactivity when assayed for antibodies to the same antigen in different methodologies. That means that any given laboratory assay may correctly detect and quantify the relevant antibodies in some samples, but not in others. It also means that no laboratory method will have optimal performance for samples from all patients.

Natural autoantibodies, pathologic autoantibodies and the continuous gradient between health and disease

It has been well established that immunoglobulins with the capacity to react with self-antigens are present in the circulation of normal individuals since intra-uterine life [14, 15]. These so-called natural autoantibodies depict some differences in comparison to pathologic autoantibodies present in the context of systemic autoimmune diseases (Table 3). The function of natural autoantibodies is not definitely established, but there is some evidence that they contribute to the first line of defense against microorganisms, autoantigen clearance, and immune regulation [16, 17]. As any other biological variable,

the serum concentration, avidity and spectrum of natural autoantibodies vary among individuals, so that the level of individual physiologic "auto-reactivity" is variable.

Table 3. Differences between natural and pathologic* autoantibodies

NATURAL	**PATHOLOGIC***
Mainly IgM, but occasionally IgG or IgA	Mainly IgG
Poly-reactivity	Restricted reactivity
Low affinity	**High affinity**
Low titer	**High titer**
Derived from germ line	Somatic mutation

* The designation "pathologic autoantibodies" refers to the fact that these are antibodies associated with disease and does not imply that they necessarily have a pathogenic effect.

Laboratory assays for autoantibody determination take advantage of the fact that pathologic autoantibodies usually depict higher serum concentration and higher avidity than natural autoantibodies (figure 2A). Therefore, the analytical threshold level for autoantibody detection should be above the serum avidity and concentration ranges of natural autoantibodies, so that the majority of reagent samples will belong to patients with the relevant autoimmune disease (figure 2B). It is important to emphasize that the analytical threshold is determined not only by the cut-off in serum concentration but also by the cut-off in terms of antibody avidity. In assays with very high sensitivity (ability to detect antibodies in low serum concentration and low avidity), a significant fraction of samples from individuals without the relevant autoimmune disease will be reagent (positive) due to the reactivity of natural autoantibodies and cross-reactive antibodies (figure 2C).

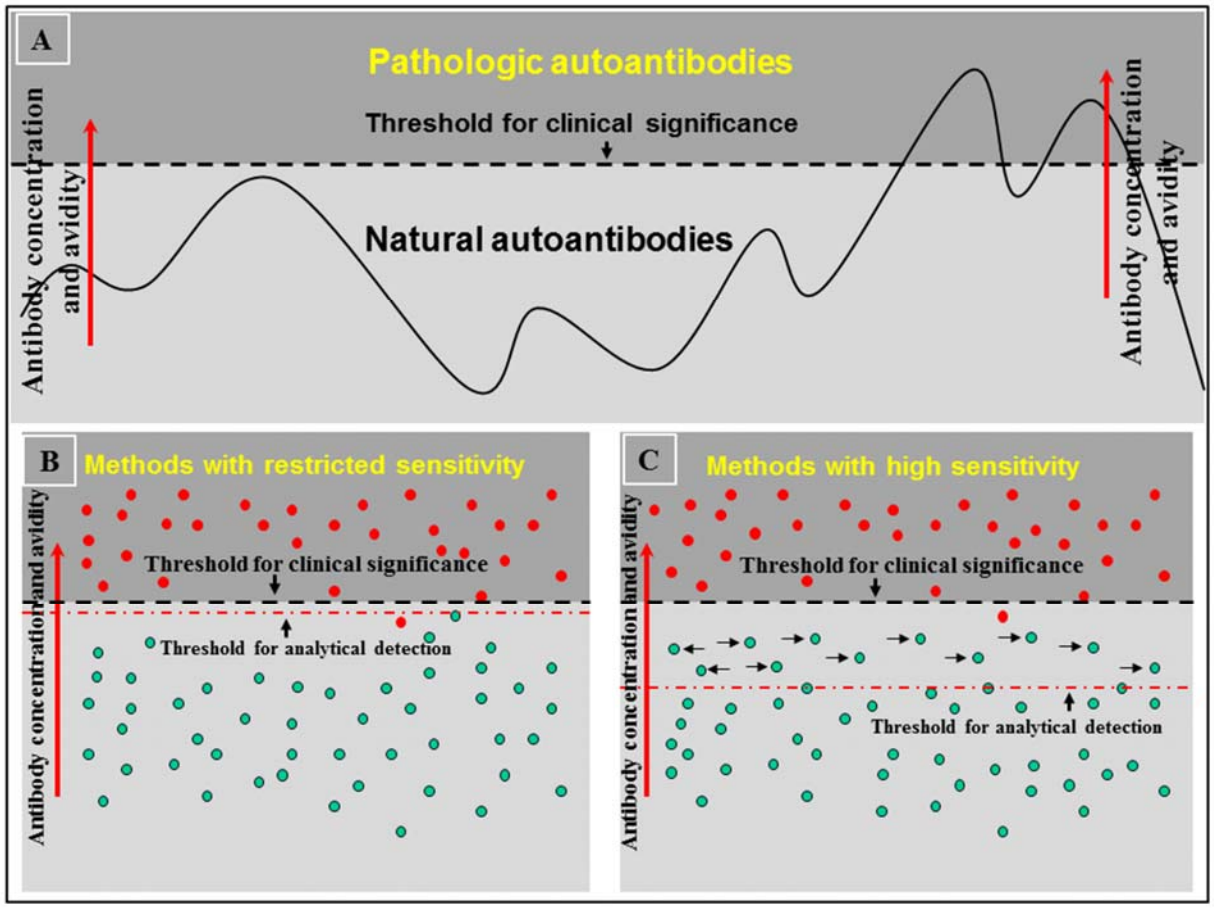

Figure 2. Concentration and avidity of autoantibodies – relevant aspects in designing a clinically useful immunoassay

The impact of the development of new methodological platforms

In the early days of autoantibody testing, in-house assays were developed in academic laboratories dedicated to research in autoimmunity [6-9]. The possibility of determining autoantibodies was restricted to a few laboratories and there was low degree of inter-laboratory standardization. Tests were performed only by expert personnel. Immunodiffusion and precipitation in agarose gel was one of the predominant methods for assaying autoantibodies in systemic autoimmune rheumatic diseases. The known clinical associations and the clinical sensitivity and specificity of most of these autoantibodies were established at those days based on this methodology.

Progressively, other methodological platforms were established based on an array of solid phase assays, including enzyme-linked immune-sorbent assay (ELISA), hemagglutination, western blot, dot bot and addressable laser bead immunoassays (ALBIA). Increasing involvement of private companies gave origin to a plethora of kits for the determination of a wide range of autoantibodies using various methodological platforms [18-22]. The availability of commercial kits ready to use by the general laboratory technician turned the procedure of testing for autoantibodies possible to general laboratories with no particular expertise in testing for autoimmune diseases. More patients and physicians have now access to the determination of autoantibodies.

In fact, diagnostic reagents companies have contributed enormously to autoantibody testing around the world. However, this widespread and independent activity generated some problems. One of them comes from the fact that companies develop their own assays in a completely independent way. Several companies have well equipped research and development facilities and a qualified staff of PhD innovators. Frequently there is collaboration with academic clinical investigators that provide the clinical background and biological samples necessary to perform the adjustment of the assays aiming for optimal diagnostic performance. The end result is the production and commercialization of a vast variety of kits after approval of regulatory agencies. However, this independent and prolific activity generates multiple products that have not been calibrated using a common standard. The arbitrary units, cut-off levels and range of detection are unique to each brand. In addition, assays from different brands destined to determine a given autoantibody display different sensitivity and specificity [23, 24]. In fact, the wide variability in the dozens of technical parameters that comprise these immunoassays necessarily entails that kit A aimed to determine autoantibodies to a given autoantigen will detect a set of autoantibodies that is different from kit B, and so on and so forth. This was demonstrated in a recent study from our group in which seven FDA-approved kits were tested in a cohort of patients with well-defined diagnosis [25]. The result for six DSA showed an extremely high degree of non-agreement in positive results (figure 3).

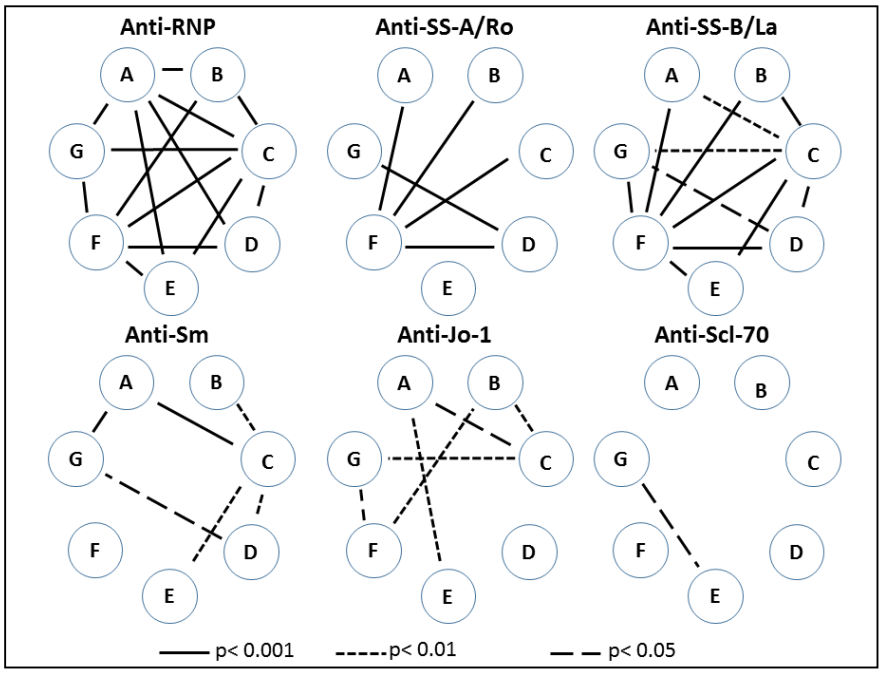

Figure 3. High disagreement rate in positive results obtained with different brands of solid-phase immunoassays for disease specific autoantibodies (DSA)

Each letter represents one brand solid-phase immunoassay. Concordance rate of positive results for each disease specific autoantibody was assessed by McNemar test. Kits showing disagreement are linked by lines with patterns according to the level of statistical significance. Brand immunoassays that are concordant for positive results are not linked by lines. Adapted from Pereira KM, Dellavance A, Andrade LEC: Clin Chim Acta. 2014;437:203-10. doi: 10.1016/j.cca

Another problem, which affects the first one, is the fact that solid phase immunoassays have very high sensitivity. ELISA and related methods are able to detect antibodies in lower serum concentration and with lower avidity than gel immunodiffusion/precipitation methods. As a result, solid phase assays have a lower analytical threshold, which widens the gap with respect to the threshold of clinical significance (figure 2). Industry developers may believe that these assays achieve better performance because they yield higher sensitivity. However, the increase in sensitivity is associated with a substantial decrease in specificity and in positive predictive value. Our study with seven FDA-approved kits for determination of six DSA on clinically well-defined samples showed a very high frequency of clinically unexpected (and unacceptable) results (Table 4) [25].

Table 4. Unexpected positive disease specific autoantibody (DSA) results as detected by seven EIA/ALBIA kits

Disease	Total samples tested	Unexpected positive samples					
		anti-Sm	anti-RNP	anti-SS-A/Ro	anti-SS-B/La	anti-Scl-70	anti-Jo-1
In samples with systemic autoimmune rheumatic diseases (SARD)							
SLE	45	NA	NA	NA	NA	3*	3
SSc	29	8	14	12	9	NA	10
PM	17	3	6	NA	2	3	NA
RA	29	3	12	7	11	3	1
SjS	7	4	6	NA	NA	1	3
In samples with no systemic autoimmune rheumatic diseases (Non-SARD)							
OA	26	3	8	5	7	1	2
CH	28	6	9	5	7	10	5
AS	20	3	6	4	3	0	1
PC	NA	30/156 (19.2%)	47/110 (42.7%)	33/132 (25.0%)	39/149 (26.2%)	21/172 (12.2%)	25/184 (13.6%)
HI	47	1 (2.1%)	5 (10.6%)	4 (8.5%)	11 (23.4%)	3 (6.4%)	3 (6.4%)
Total	248	31	66	38	50	24	28

Adapted from Pereira KM, Dellavance A, Andrade LEC: Clin Chim Acta 2014;437:203-10. doi: 10.1016/j.cca

* Absolute number of positive samples for each autoantibody in at least one of the EIA/ALBIA kits; samples with positive results in more than one kit were considered only once.

SARD: systemic autoimmune rheumatic disease; SLE: systemic lupus erythematosus; SSc: scleroderma; PM: polymyositis; RA: rheumatoid arthritis; SjS: Sjögren's syndrome; OA: osteoarthritis; CH: chronic viral hepatitis; AS: ankylosing spondylitis; PC: Total of pathologic controls; HI: healthy individuals; NA: not applicable, meaning that the autoantibody is expected in this clinical condition.

Clinical laboratories worldwide buy and use these kits. In many of these laboratories, there is no critical analysis on the underlying methodology and intrinsic properties of the product. Test reports on a given

autoantibody issued by different laboratories mirror the heterogeneity of commercial kits available. Physicians reading the laboratory report on a given autoantibody have no idea of what is behind the process, and interpret the findings inspired on what they have learned from textbooks and original investigative studies.

At this point, it is important to ask what physicians and patients expect from tests for DSA. Does the physician really seek for higher sensitivity at the expense of specificity? As discussed above, autoantibodies are clinically useful in cases where the clinical scenario is obscure and ill-defined. In such occasions, one does not need additional dubious information and rather values a high specificity parameter. LESS (*sensitivity*) means MORE (*specificity*). In the natural competition among the diagnostic reagents companies, the driving force should be the quest for assays with higher clinical specificity and positive predictive value rather than assays with higher sensitivity.

Shift from qualitative to quantitative paradigm

As mentioned earlier, DSA in systemic autoimmune rheumatic diseases were originally established mainly using gel immunodiffusion/precipitation assays [6-9]. Since this methodology provides a qualitative output, the established paradigm has been towards a qualitative report of DSA tests. Patients have been quoted as positive or negative for a given DSA. Classification criteria for some diseases include the presence or absence of DSA. This has not been the case with autoantibodies that were originally established using quantitative or semi-quantitative methods, as was the case for anti-phospholipid antibodies. The quantitative dimension of anti-cardiolipin antibodies has been appreciated in the clinical practice and in the establishment of classification criteria, where serum levels above 40GPL or 40MPL are required for fulfillment of the serologic criterion.

Gel immunodiffusion/precipitation methods have an inherently restricted sensitivity where antibodies are detected only above a certain concentration threshold. In contrast, solid phase immunoassays are able to reveal antibodies at a much lower concentration and with lower avidity. Using the qualitative approach, different samples with marginal, moderate and strong reactivity in ELISA are equally classified as “positive”. This is not the most appropriate way to report results obtained in solid phase immunoassays, especially because the clinical meaning of marginally positive and strongly positive samples tends to be quite different. In our study with seven FDA-approved solid-phase immunoassays, we noticed that clinically unexpected positive results tended to be in the low range of reactivity as opposed to the strongly positive results observed in samples within the expected clinical context [25].

Therefore, a shift from qualitative to quantitative paradigm is required for the reporting of autoantibodies assayed by sensitive solid phase immunoassays. This paradigm shift involves a mindset twist in scientists, laboratory technicians, physicians and even patients, all of whom are used to deal with a positive/negative parameter in terms of DSA. This strategic realignment will be facilitated by the fact that these professionals are acquainted with a quantitative appreciation of numerous other laboratory and clinical parameters.

The challenge of harmonizing multiple versions generated by distinct DSA assays

The increasing availability of a wide variety of commercial immunoassays for the determination of DSA has largely contributed to the widespread use and popularization of these invaluable biomarkers all over the world. However, the independent development of assays based on a variety of methodological platforms by dozens of companies has created an important problem. As mentioned earlier, the repertoire of antibodies to a given antigen varies considerably from individual to individual. On the other hand, different immunoassays are heterogeneous in several intrinsic characteristics, such as antigen concentration and

purity, molecular nature of the antigenic substrate (purified versus recombinant, fragment versus whole molecule, native versus denatured), the profile of available epitopes, matrix scaffold, buffers, conjugates and development strategies, etc. As a consequence, polyclonal antibodies from a given patient will react better in some immunoassays than in others. In other words, any biological sample may yield very different results according to the brand assay used. A series of patients with a given disease may present a varied frequency and profile of positive results for the related autoantibody according to the brand assay used. This implies that the measuring instrument (immunoassay) affects directly the result of the measurement (autoantibody reactivity).

Intermezzo

This scenario is vaguely reminiscent of the perplexity that baffled scientists in the area of subatomic physics in the beginning of the 20th century. Physicists were puzzled with the difficulty to define precisely the nature and mechanical relationships among subatomic particles. In order to handle the fuzzy reality of subatomic particles, quantum physicists postulate a dual nature (particle and wave) for the subatomic elements and admit that it is impossible to precisely determine the momentum and position of a particle at the same time. This is known as the Heisenberg's uncertainty principle. Another subtle concept of quantum physics is the superposition of alternate states at the atomic and subatomic scale. Alternate states collapse into a single state when an observer looks at the phenomenon. That implies that a given object (e.g., an atom) is simultaneously at alternate states (activated versus inactive), but when the observer looks at it, it assumes either one of the two states.

Conclusion

Autoantibodies, as we reckon, represent a virtual concept that simplifies a much more complex reality. Anti-Jo-1 antibody, for example, is a virtual concept that represents thousands of possibilities of combinations of heterogeneous immunoglobulins that share the property of binding to histidyl tRNA synthetase. The instruments (immunoassays) used to detect and measure anti-Jo-1 antibodies vary in their capacity to match each of these thousands of possibilities. Therefore, the immunoassay strongly influences the reading that one has of the reality. The dozens of immunoassays available throughout the world generate their own versions of what is measured as autoantibody to a given autoantigen.

The potential confusion and heterogeneity in autoantibody testing can be attenuated by promoting worldwide harmonization in standards for DSA. Ongoing international quality assessment and standardization initiatives have achieved some concrete results [26-28]. Recently, an International Autoantibody Standardization (IAS) workshop was held during the 12th International Workshop on Autoantibodies and Autoimmunity in Sao Paulo. This workshop aimed to stimulate harmonization and coordination of the independent efforts of the several cognate initiatives and to promote an approximation between academic and industry scientists. The 2nd IAS is scheduled to take place in the framework of the 12th Dresden Symposium on Autoantibodies and is expected to consolidate and advance the deliberations and proposals of the 1st IAS. The common goal is to promote standardization and improvement in laboratory diagnosis of autoimmune disease. Biotechnology companies are strongly encouraged to join in this enterprise. Hopefully these efforts will bring together the several "quantum" versions of autoantibodies.

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Harmonization and Standardization of Autoantibody Testing

The international consensus on standardized nomenclature of antinuclear antibody HEp-2 Cell patterns (ICAP) initiative - Current state and perspectives

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Abbreviations

anti-cell (AC), antinuclear antibody (ANA), Centers for Disease Control and Prevention (CDC), indirect immunofluorescence (IIF), International Union of Immunological Societies (IUIS), rods and rings (RR)

Introduction

The determination of antinuclear antibodies (ANA) is frequently used for the screening of autoantibodies in systemic autoimmune diseases and indirect immunofluorescence (IIF) has been the main methodology for ANA determination.[1,2] Since the early 1970's, the immortal HEp-2 cell line has progressively replaced the initially used rodent tissue slices as the substrate for the assay. HEp-2 cells grow easily as a monolayer and exhibit relatively large intracellular structures. These features contribute for optimal visibility and ready recognition of several subcellular structures. In addition, it is quite a stable cell line what favors uniformity of substrate for universal application.

The establishment and distribution of autoantibody reference reagents was a cornerstone in the development of autoantibody standardization[3]. Over the past 35 years, the Autoantibody Standardization Committee (www.AutoAb.org), a subcommittee of the International Union of Immunological Societies (IUIS) Quality Assessment and Standardization Committee, has coordinated a joint initiative with the Centers for Disease Control and Prevention (CDC) and other agencies to provide autoantibody reference standards (also known as CDC ANA reference standards, or IUIS ANA reference standards). Currently, 17

reference standards are available free of charge to any qualified clinical or commercial laboratory and research investigator[4]. These efforts have largely contributed for education, training, and laboratory quality control of ANA and related autoantibody testing. Despite substantive contribution of these reference standards in promoting inter-laboratory standardization in ANA and disease-specific autoantibody testing, there is a critical need for improving standardization in ANA pattern nomenclature and reporting. This point is particularly important in view of recent discussions on the appropriate use of the ANA assay[5-7].

During the 12th International Workshop on Autoantibodies and Autoimmunity (IWAA) held in São Paulo, Brazil, a full day workshop was promoted to build up the first International Consensus on ANA staining Patterns (ICAP). Brazil has pioneered the initiative of ANA pattern consensus with their first workshop held in Goiania in August, 2000. Reports on the successive rounds of ANA consensus were published in 2001[8], 2003[9], 2009[10] and 2013[11]. The first ICAP initiative was framed mainly on the template of the Brazilian consensus on ANA nomenclature, but other initiatives with focus on nomenclature consensus in ANA on HEp-2 cells[12-15] were also taken into consideration.

The ICAP agenda was focused on the discussion of ANA patterns regarding four cell compartments: nucleus, nucleolus, cytoplasm, and mitotic apparatus. Each session was coordinated by two experts, who presented a preliminary proposal that was subsequently discussed by the assembly that counted with 63 participants from several countries. The report on the first ICAP has been recently published in Frontiers in Immunology[16] and is available online at the IWAA 2014 website with a link to the ICAP website: (www.ANApatterns.org). It has been acknowledged that the establishment of ANA consensus must be a continuous process seeking universal harmonization and improvement in the standard of ANA testing. Therefore, successive ICAP rounds are expected in the next years and the second ICAP workshop is scheduled for September 22, 2015, one day before the 12th Dresden Symposium on Autoantibodies.

ICAP home page: www.ANApatterns.org

The ICAP home page provides a short introduction to the history of this initiative as well as briefly describes how the consensus on pattern classification and nomenclature was constructed (figure 1). The key toolbar shows links to the nomenclature and classification tree (figure 2), listings of designated nuclear, cytoplasmic, or mitotic patterns (figure 3), access to patterns based on the alphanumeric codes (figure 4), and a search function for patterns based on keywords. The classification tree is a summary of all the consensus patterns based on their main staining feature fitting best to the nuclear, cytoplasmic, or mitotic compartments (figure 2). All 28 ICAP patterns are shown in the classification tree as designated from AC-1 to AC-28. Boxes with amber background are recommended as competent-level reporting, whereas those with olive green background are considered for expert-level reporting. Competent-level patterns are those that should be readily recognized versus patterns that would be more challenging and distinguishable only when observers or technologists have attained the expert-level. The distinction between competent-level versus expert-level patterns is based on at least two considerations. First, clinical relevance is a major consideration to ensure that important clinical implications are recognized. Second, easily recognizable patterns should be included even when the clinical relevance is less clear at this time. The competent-level patterns are placed at the top levels starting from the left. The assignment of the different AC codes generally flows from left to right, and top to bottom. Thus, the classification tree shows 11 competent-level reportable patterns. The six competent-level reportable nuclear patterns include homogeneous (AC-1), speckled (AC-2, 4, 5), dense fine speckled (AC-2), centromere (AC-3), discrete nuclear dots (AC-6,7), and nucleolar (AC-8,9,10). Five competent-level reportable cytoplasmic patterns are fibrillar (AC-15, 16, 17), speckled (AC-18,19,20), reticular/mitochondrion-like (AC-21), polar/Golgi-like (AC-22), and rods and rings

(RR, AC-23). The RR pattern is not recognized in certain commercial ANA substrates as these structures are only seen consistently in slides from some manufacturers[17-19].

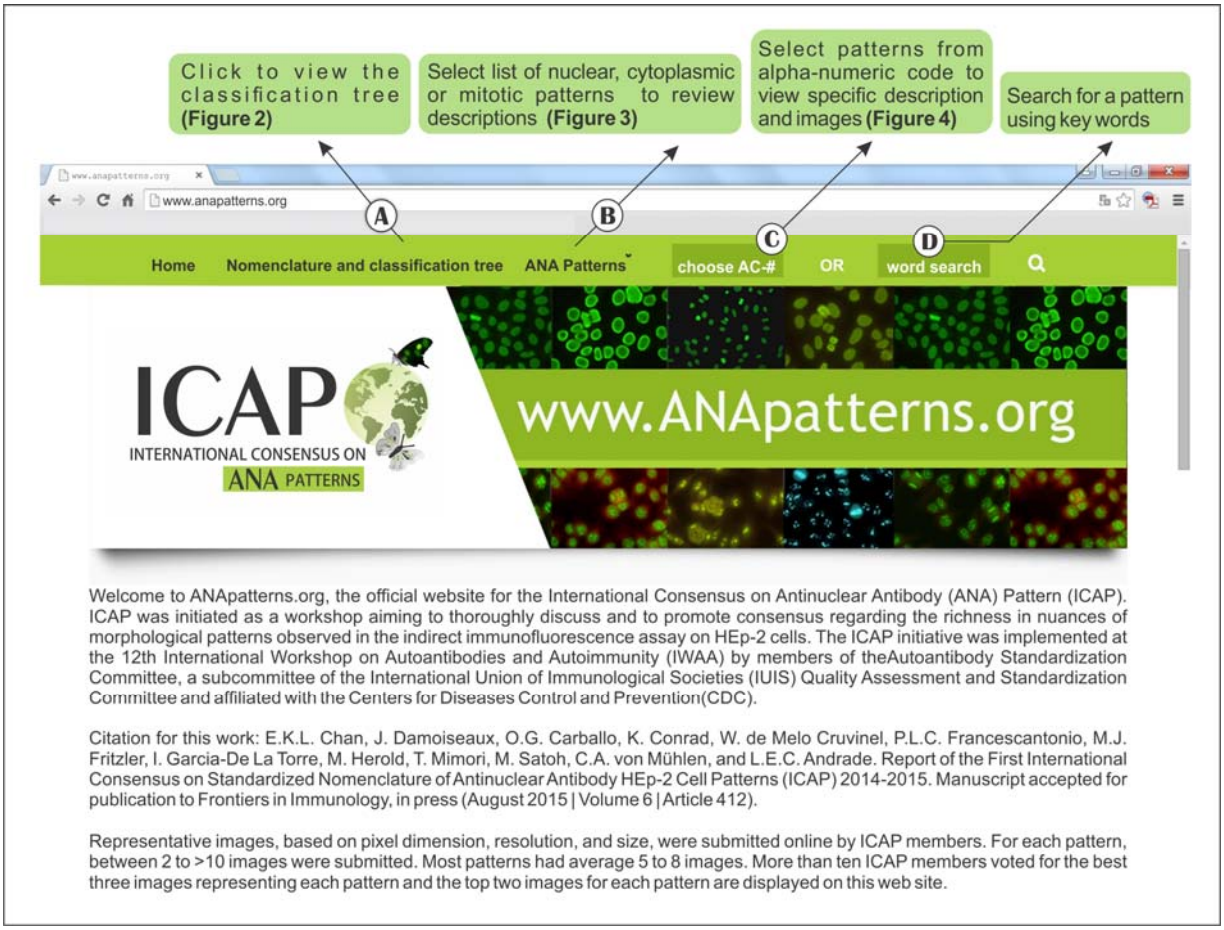

Figure 1. The ICAP home webpage at www.ANApatterns.org. The important web links (A-D) on the toolbar are indicated connecting to other figures in this chapter.

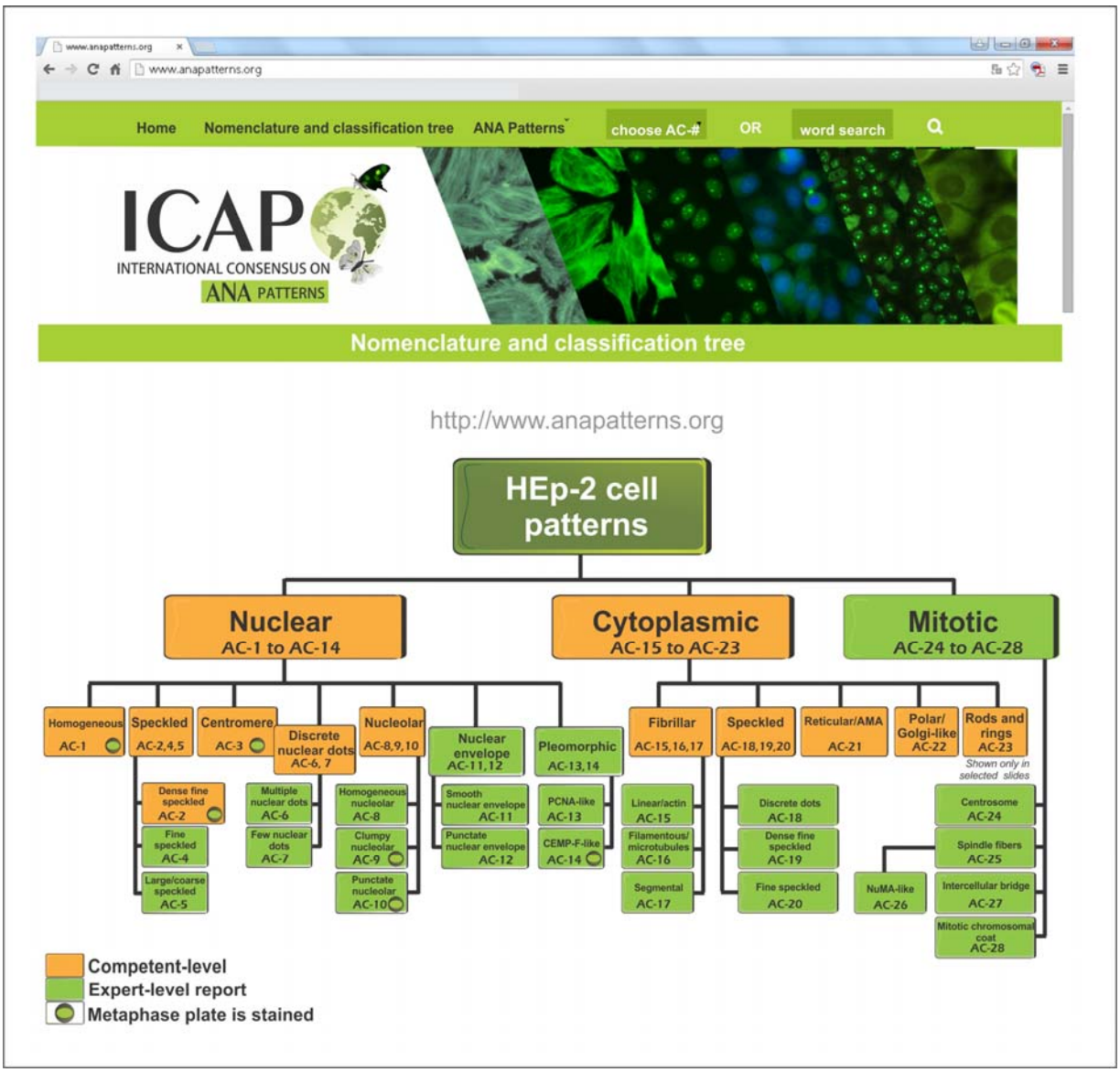

Figure 2. The nomenclature and classification tree for all HEp-2 cell patterns as shown at www.ANApatterns.org. There are total of 28 ICAP patterns designated with alphanumeric AC code for each from AC-1 to AC-28. Boxes with amber background are recommended as competent-level reporting, whereas those with olive green background are considered for expert-level reporting. AC, anti-cell.

Figure 3 shows an example of the list of ICAP nuclear patterns. Similar lists are provided for ICAP cytoplasmic and mitotic patterns. Each pattern is shown with the AC code, name of the pattern, other common names in use (synonym), a general description, and two small IIF icons of full representative images. Selecting each pattern links to information as shown in figure 4, which also provides additional information on antigen association and disease association. In figure 4, when one of the two images is selected, the corresponding full image is shown.

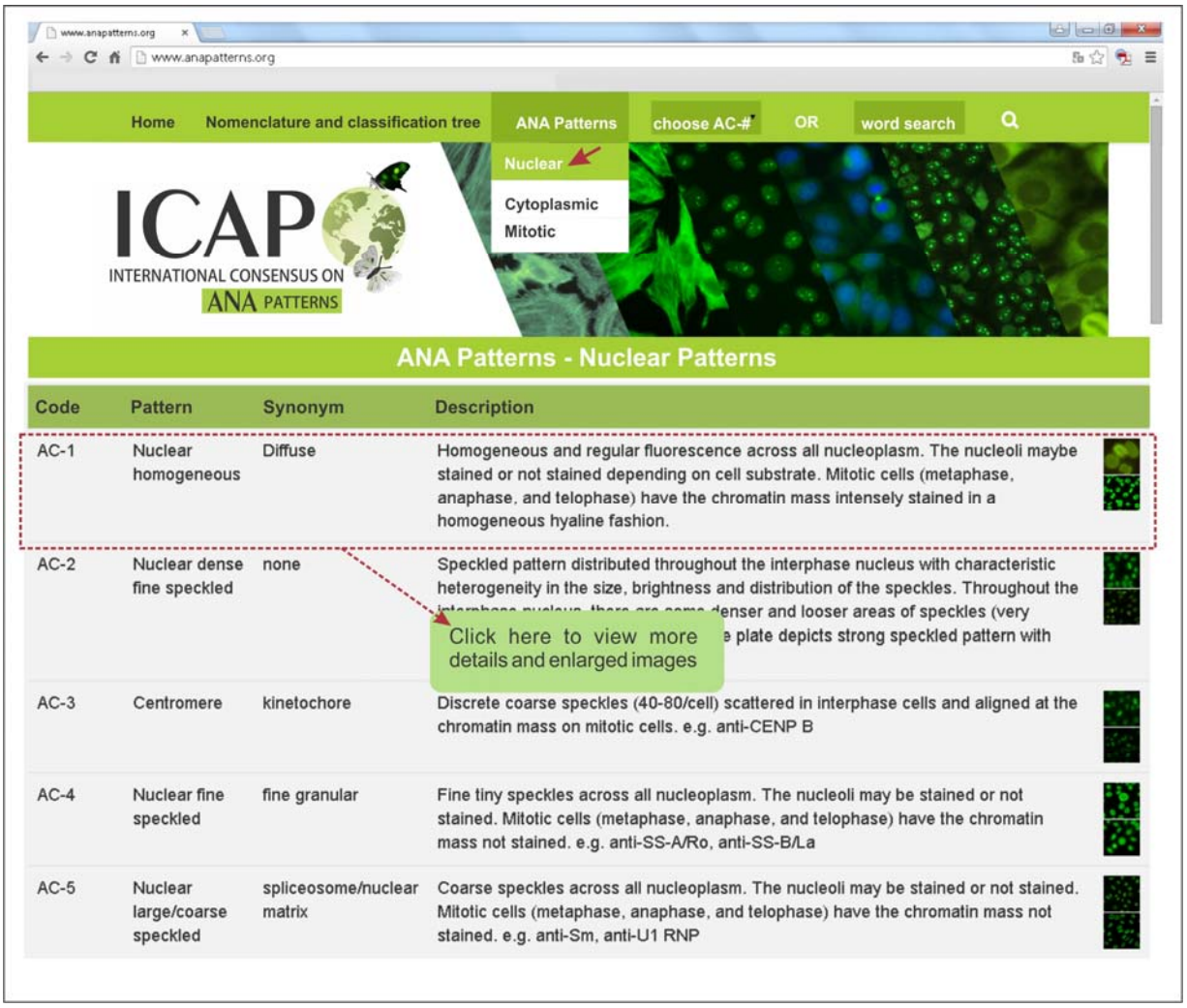

Figure 3. An image of the webpage providing a partial list of the ICAP nuclear patterns.
Two representative images for each pattern are shown as icons on the right column. Click each pattern to view its synonyms, antigen associations, disease associations, and detail description.

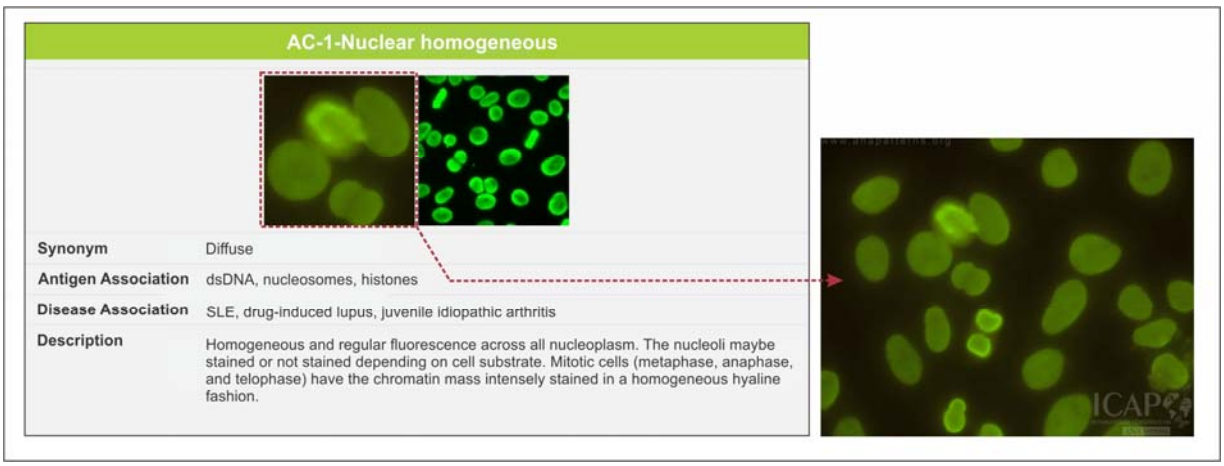

Figure 4. Example of information shown on the web page for the nuclear homogeneous pattern AC-1. A click of the smaller image links to the full ICAP image.

Agenda for second ICAP meeting

An aggressive agenda with several items is planned for the second ICAP meeting to be held in Dresden, Germany. It is acknowledged that the first ICAP report should be followed up with actions to ensure that the consensus is progressively adopted worldwide. In addition, it is recognized that several points in the first ICAP need further discussion. What are the practical limitations for laboratories to meet the recommended competent-level reporting? What is needed to encourage laboratories to gain ability to reach the expert-level? The www.ANApatterns.org site will contribute towards this goal. Should the ultimate target be global standardization so that everyone is capable to identify all patterns? It has been proposed that an on-line assessment tool for users of www.ANApatterns.org will help to train technicians and investigators to develop technical competence. Are there available tools ready to be adapted?

In the first ICAP report, one limitation is the lack of consideration for composite patterns as a separate category. Discussion will focus on what are the most important composite patterns and how to distinguish them effectively. ANA patterns association with diseases is listed in three of the tables in the Frontiers in Immunology ICAP report. What do clinicians want to know from the HEp-2 patterns? Can we come up with a more practical presentation for what comes after the pattern is reported?

Recommendations for standardized ANA pattern reporting will be discussed. This can be an important development to establish an international standard. To become a true international consensus, plans are needed to translate ICAP website into other languages. Appropriate discussion is needed regarding the strategy and guidelines to achieve an effective outcome.

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The International Autoantibody Standardization (IAS) initiative. Current state and perspectives.

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Keywords

autoimmunity, systemic autoimmune diseases, diagnostic tests, autoantibodies, reference material, harmonization.

Introduction

The measurement of autoantibodies in rheumatic diseases is an important diagnostic tool in particular when an early diagnosis is needed for a prompt and aggressive treatment to ultimately improve the patient's prognosis (1). Quantification of autoantibody concentrations may also help in monitoring the disease activity and/or the response to therapy. In addition, some autoantibodies represent valuable biomarkers predictive for specific organ damage. Hence, reliability and harmonization of autoantibody testing are becoming more and more important not only in the initial diagnosis, but also in identifying subgroups of patients who require specific and more aggressive treatment or primary prophylactic therapies (1).

The heterogeneity of the techniques, including the new methodologies, and the intrinsic variability of analytes and reagents may explain the inter-laboratory variations and the discrepancies in the results. A wrong or missed diagnosis are the main consequences of inaccurate autoantibody testing; in addition, unreliable assays and inaccurate results may also be responsible for additional costs due to the repetition

of unnecessary confirmatory tests, unjustified further diagnostic investigations, and complications from inappropriate diagnosis and treatment (1).

Standardization of autoimmune testing has become a strong need and should take into account several aspects such as the definition of the target analytes, the pre-analytical stages, and the calibration procedures, which directly influence reporting of the results (1, 2). The availability of suitable reference materials for calibration and quality control represents a fundamental point and efforts have been recently made in order to obtain new reference materials for autoantibody testing (1). Several separate international initiatives have been independently developed with the aim of providing elements for standardization and harmonization in autoantibody testing (1-5). However, potential problems could arise from the operation of such separate and non-coordinated initiatives. In response to this perception, a group of specialists launched the first International Autoantibody Standardization (IAS) workshop, which was held during the 12th International Workshop on Autoantibody and Autoimmunity in Sao Paulo in 2014. The preliminary discussions undertaken at the 1st IAS workshop represented a stimulus for ongoing debate and the scheduling of the 2nd International Autoantibody Standardization (IAS) Workshop to be carried out in conjunction to the 12th Dresden Symposium on Autoantibodies. The present paper will review the most recent initiatives in the field and discuss the main points to be addressed at the 2nd IAS Workshop.

Challenges in autoantibody testing

The detection of autoantibodies by manual, qualitative, or semi-quantitative assays is gradually being replaced by quantitative, automated, high-throughput solid-phase assays. These new methodologies are now moving from specialized centres into routine service laboratories in order to satisfy the increasing request by clinicians. Such a shift in paradigm made even more urgent the issues related to quality control, quality assurance, standardization, analytical sensitivity and specificity, and clinical sensitivity and specificity of autoantibody diagnostic assays. All these aspects are relevant for the ultimate clinical interpretation and usefulness of the tests.

Table 1. Main methodological issues that can affect autoantibody test results.

Analytical issues:

- Definition or characterization of the target analyte (antigen)
- Antigen preparation, coating and blocking systems
- Calibrators
- Matrix effects
- Methods e.g. ELISA, multiplex
- Variability of the analyte (selectivity, avidity, subclass, glycosylation, …)
- Validation/verification of new assays: the problem of using biological samples with complete clinical records in order to correlate the results of the assay with the clinical picture
- Lack of a "gold standard" method required for validation and verification of new assays

Post-analytical issues:

- Clinical specificity and sensitivity and interpretation of the new assays in comparison to the existing or even older ones (e.g. counterimmunoelectrophoresis vs ELISA, chemiluminescence vs ELISA, range of sensitivity)
- Setting of the cut off: different distribution among healthy subjects matched for sex and age; pathological controls (including other autoimmune diseases and infectious diseases, "sticky samples")
- Reporting of the results (quantitative versus qualitative; stating cut-off values)

The effort of the international community for autoantibody standardization

Strong with high concentrations of the relevant autoantibodies from patients with well characterised and relevant diseases were initially selected as standards in order to validate the laboratory assays. The first initiative on autoantibody standardization was done by a group chaired by Dr Eng M. Tan under the umbrella of the joint committee of the Arthritis Foundation, the World Health Organization (WHO), the Centres for Disease Control and Prevention (CDC), and the International Union of the Immunological Societies (IUIS) at the beginning of the 1980's (3,4). The group selected sera monospecific for a given auto-antigen from rheumatic patients. These samples were then stored at the CDC and are available upon request for laboratories that want to check the reliability of their own assays as well as companies interested in validating new commercial assays. The group is still active and information on the most recent activities can be found at www.autoab.org. Reference standards for 17 autoantibody specificities are available free of charge for any qualified academic of private laboratory or company.

In 2002 a European group (European Autoimmune Standardization Initiative – EASI, chaired by A. Wiik) started a similar initiative but with further objectives. In addition to the purpose of harmonizing the technical assays for autoantibodies, both clinicians responsible for direct patient care and laboratory scientist working in autoimmunity were invited to join efforts in order to improve both the appropriateness of requesting and the interpretation of the results (www.easi-network.com) (5).

The European Consensus Finding Study Group on autoantibodies (ECFSG) is another group working in the context of the European League Against Rheumatism (EULAR) organization. More than 40 European laboratories involved in serological diagnostics in rheumatic diseases have participated in the initiative to achieve common consensus and harmonization in autoantibody testing since 28 years. Each year a collection of ten sera from clinically characterized patients with systemic autoimmune diseases is

distributed to participant laboratories for testing of a variety of autoantibodies. The goal is to achieve comparable laboratory results among the different laboratories and to relate any differences in analytical outcome to the clinical data of the patient donating the sera. Detailed results of the last annual exercise can be available at the following websites: www.sanquin.nl/en/products-services/diagnostic-services/ecfsg/ and http://www.eular.org/investigative_rheumatology_study_groups.cfm.

More recently in 2009, another group was started in the framework of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC): the Working group on Harmonisation of Autoantibody Tests (WG-HAT) chaired by J. Sheldon. The task of this initiative was to prepare certified reference materials, where possible, for autoantibodies in close cooperation with the Institute for Reference Materials and Measurements (JRC-IRMM) of the Joint Research Centre of the European Commission (www.ifcc.org/ifcc-scientific-division/sd-working-groups/harmonisation-of-autoantibody-tests-wg-hat/). The IFCC WG-HAT and JRC-IRMM have conducted feasibility studies that showed that it is possible to make measurement results more comparable for autoantibody testing, provided that reference materials are used that meet requirements in terms of commutability, homogeneity and stability. IgG anti myeloperoxidase (MPO) and IgG anti beta2-glycoprotein I (β2GPI) were used as model systems for developing the general approaches for producing certified reference materials according to the requirements of ISO Guide 34 (General requirements for the competence of reference material producers).

General approach for the harmonization of autoantibody testing using reference materials

The variability of the results observed in autoantibody testing has many different causes. Some of them can be addressed with recommendations valid for most antigens and antibodies, but others are specific for a given antigen/antibody system. Hence, developing a reference material and guidelines for the assays needs to be done separately, taking into account the particularities of each individual test.

Reference materials can be used for direct assay calibration, or to set a common measurement scale but also for assay quality control. All these uses can are important to reduce lot-to-lot variation for reagents in the manufacture stages but also in the analysis in the laboratories. In addition, reference materials can serve for keeping values equivalent over time, and between different methods. Whatever materials are developed, they should meet requirements such as homogeneity, stability and commutability (6, 7) and these parameters should be detailed in the characteristics of the material.

The first step should be focused to verify that all assays do indeed measure the same analyte, and give correlating results. This is not always the case in autoantibody testing, since some autoantibody subpopulations can be preferentially detected by one technique but not by another. This is especially relevant because the autoantibody response is polyclonal in nature and exhibits wide heterogeneity among patients with the same disease. For example sandwich ELISA versus capture ELISAs may yield different results because antigens coated in different ways in solid phase assays may offer diverse sets of conformational and linear epitopes. In case different assays give correlating results they may be harmonised, even if the results are on different scales. Assays that are *de facto* measuring different analytes can still be harmonised if the analytes are present in a constant ratio in all patient samples. If the results from various assays do not correlate they cannot be harmonised as such, and one needs to look again at the clinical significance of each assay. If non-correlating assays have a good, but different clinical significance, efforts should focus on a better definition of the analytes (7) and spreading the correspondent information to clinicians and laboratory personnel.

The next step is to test different formats of reference materials for stability, homogeneity and commutability. The commutability of reference materials is a measure of the extent to which they behave in the same manner as patient samples in two or more assays. Once commutable matrix reference materials have been identified it becomes possible to set a common scale for a particular analyte. This can either be done by assigning arbitrary values to a particular reference material (e.g. International Units) or by assigning values that are traceable to a stable reference like the Units of the International System. The latter option has the advantage of being reproducible over time, whereas in the first case each new generation of reference material needs to be value-assigned by comparison with the previous one, with a risk of drift in the values.

Finally, the use of reference materials for calibration requires careful validation of the manner in which they are used (e.g. the reconstitution of the reference material, the way dilutions are prepared, the need for multiple measurements and intermediate precision, and the manner in which uncertainties are treated).

Better characterization of some of the auto antigens and the use of antigen-affinity purified immunoglobulin fractions or the monoclonal antibody technology offer more reliable tools for standardization. Preliminary results of these efforts appear to be encouraging (1).

Ongoing recent initiatives

Anti-neutrophil cytoplasmic antibody (ANCA) reference materials

The detection of MPO and proteinase 3 (PR3)-ANCA IgG contributes to the correct diagnosis and follow-up of small vessels-associated systemic autoimmune vasculitides. Standardisation of available tests is still poor. The WG-HAT has been working on this topic for the last two years. For anti-MPO ANCA thirteen different commercial methods have been evaluated with a panel of sera from patients and controls and found to offer largely correlating results. A candidate reference material was selected, validated in term of commutability and used to improve the harmonization of MPO-ANCA testing; the results of such a process have been recently submitted for publication (First steps in the standardisation of MPO-ANCA measurements by Hutu et al. submitted). The reference material for MPO-ANCA IgG was certified and released in April 2015 (https://ec.europa.eu/jrc/sites/default/files/rm/ERM-DA476_report.pdf), and is distributed by JRC-IRMM and their authorised distributors. The EASI group (chaired by Y. Shoenfeld) already planned to distribute the MPO-ANCA reference material to the affiliated laboratories for a further validation in a setting closer to the real life. For anti-PR3 ANCA a comparable feasibility/correlation study has been performed. A candidate reference material has been processed and is being characterised.

Anti-β2GPI antibody reference material

Antibodies against β2GPI are widely accepted as the main anti-phospholipid antibody subpopulation responsible for positive results in formal diagnostic tests for the anti-phospholipid syndrome and also displaying a pathogenic effect. Despite a huge effort in assay harmonization, still the test displays discrepancies among laboratories. Both the AF/WHO/CDC/IUIS and the WG-HAT committees have collaborated in identifying monoclonal and polyclonal candidate reference materials in the last years. Polyclonal IgG/IgM anti-β2GPI were affinity-purified from high-positive anti-phospholipid syndrome sera and monoclonal IgG were obtained from culture supernatant of anti-human β2GPI HCAL, a chimeric antibody with human gamma constant regions and variable regions of mouse WBCAL-1 anti-human β2GPI antibody (8). Polyclonal/monoclonal reference materials were evaluated for linearity, unit equivalency and

commutability and both displayed good results representing the next candidates for harmonization of anti-β2GPI immunoassays (9).

Anti-double stranded (ds) DNA antibody reference material

The first standard for anti-dsDNA assays was established in 1985 (W1065/Wo80) (10). Its international units assignment was used to standardise commercial ELISA kits and to validate immunofluorescence tests. This standard was initially distributed by Sanquin Blood Supply (formerly Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands) under the umbrella of the WHO but when it was transferred to National Institute for Biological Standards and Control (NIBSC) in 2007, it was discovered that stocks were exhausted. A large amount of a candidate reference material for anti-dsDNA assays has been recently identified and its validation is on going through a collaborative effort of the WG-HAT, the NIBSC and the ECFSG.

Anti-Lens Epithelium-Derived Growth Factor (LEDGFp75) / Dense Fine Speckled p70 (DFS70) antibody reference material

Anti-LEDGF p75/DFS70 antibodies are responsible for a considerable fraction of high titre positive antinuclear antibody (ANA) assays in individuals with no evidence of systemic autoimmune disease (11). These autoantibodies yield a very characteristic ANA pattern that has been largely mistaken as the nuclear homogeneous pattern. However, these two patterns have opposite clinical significance and therefore the appropriate discrimination of both is mandatory. Since ANA pattern interpretation is highly subjective, appropriate training of technician is necessary and the availability of a reference reagent for anti-LEDGFp75/DFS70 is an urgent demand.

The Autoantibody Standardization Committee, affiliated with the IUIS and the WHO, has determined the development of an international anti-LEDGFp75/DFS70 standard based on the strategy of pooling hundreds of samples with isolated high anti-LEDGFp75/DFS70 reactivity. This initiative is ongoing since 2013 by retrieving such samples from the ANA workout in a large laboratory with a specialized autoimmune testing section and an average volume of 12,000 ANA tests a month. All selected samples yield the expected ANA pattern by indirect immunofluorescence on HEp-2 cells at 1/640 or higher titre and moderate to high reactivity in a specific chemiluminescent assay to anti-LEDGFp75/DFS antibodies. A strategy of gradual and successive pooling of samples (polling of 5 samples, followed by pooling four 5-sample pools, followed by pooling five 20-sample pools) with validation of reactivity of each intermediate pool has been implemented to guarantee a final pool with the expected reactivity. This “mega pool” strategy counts now with samples from over 800 individuals and shall be available for distribution by the IUIS Autoantibody Standardization Committee in the near future.

Conclusions

Standardisation and harmonisation of autoantibody testing is a great challenge. We are now entering a phase where we realise the importance of comparability of results from day to day, year to year and across methods. The development of standards and (better characterised) reference materials is the first step to a better understanding of the challenges in autoantibody testing and trying to address them. However, the complexity and variability of the antigens, the antibodies and the methods makes it unlikely that the introduction of standards will completely solve the all the issues. It is more likely that it will be the start of the process of defining the antigen, the antibody and the method. Ultimately, the goal is to reduce the variability in these tests to improve patient diagnosis and overall care.

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