Toward a new autoantibody diagnostic orthodoxy: understanding the bad, good and indifferent

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Abstract Rapid advances in diagnostic technologies used to detect autoantibodies have made it difficult for even the most modern laboratory to keep abreast of the changing approaches and platforms, not to mention the clinicians who are hard pressed to keep abreast of evolving diagnostic paradigms attended by these newer techniques. While autoantibody testing is traditionally considered to be primarily serving the realm of diagnostic medicine, there is little doubt that autoantibodies are also being recognized as an approach to providing prognostic and therapeutic information. Accordingly, along with related proteomics, genomics and metabolomics, it is taking on increasing importance in the realm of personalized medicine. In today’s world of autoantibody diagnostics, overarching concerns about false-negative and false-positive autoantibodies tests cannot be summarily dismissed by citing pros or cons of any one technology or diagnostic platform, but often point to persisting gaps in our knowledge about, and understanding of, the origin and roles of autoantibodies. Before we can hope to completely understand the enigmas that attend the results of autoantibody diagnostic tests, perhaps it is time to step back and re-examine long-accepted paradigms and beliefs. This review will address some of the issues that impact on autoantibody detection technologies and some of the considerations and issues that will attend a new orthodoxy of autoantibody diagnostics. These issues will be addressed in the context of “bad” (pathogenic), “good” (protective) or “indifferent” (no apparent role in disease) autoantibodies.

Keywords Autoantibodies · Autoantigens · Diagnostics · Anti-nuclear antibodies

Overview: autoantibody detection in systemic autoimmune rheumatic diseases

The history of autoantibodies (aab) dates back more than a century to Ehrlich’s description of “horror autotoxicus” [1] and subsequent observations that sera from syphilis, systemic lupus erythematosus and other inflammatory conditions reacted with Treponema-related components, including cardiolipin, in the Wasserman and the subsequent Venereal Diseases Research Laboratory (VDRL) tests [2–5]. Despite this long history, advances in the detection of antibodies were slow or spotty for about 50 years (i.e., 1900–1950) and was largely relegated to infectious diseases and, to a certain extent, organ-specific autoimmune diseases [6], but then achieved prominence in the following 50 years (i.e., 1950–2000). The tremendous surge in aab testing dates to the seminal observation of the lupus erythematosus (LE) cell phenomenon by Hargreaves and his colleagues [7] and then the development and wide use of the LE cell test [8]. With the notion that aab in human sera and other biological fluids could have diagnostic value beyond the realm of infectious diseases, a number of techniques such as immunofluorescence, immunodiffusion, hemagglutination and complement fixation were developed and refined in the following 20 years (reviewed in [9]). Indirect immunofluorescence (IIF), first described by Coons, Kaplan and Weller in the early 1950s [10, 11] almost stands alone as one of those early techniques that has stood the test of time as an important screening test in the diagnosis of systemic autoimmune rheumatic (SARD) and other diseases (reviewed in [12]).
While the first IIF protocols utilized a variety of substrates, cryopreserved sections of rodent organs became the mainstay for ~20 years [13–16] until the mid-1970s, when it was discovered that human tissue culture cells, such as HeLa and HEp-2, derived from various malignancies were superior to organ sections primarily because they were easier to produce in large numbers, had larger nuclei (of value at a time when nucleolar antigens were a particular focus) and expressed novel antigens in various stages of the cell cycle [17, 18]. In retrospect, it is curious that carcinoma cell lines were chosen as the substrate of choice and, for reasons that are not clear, the development of IIF techniques for SARD diagnostics eventually relied almost entirely on HEp-2 rather than HeLa cells; this despite subsequent evidence that some presumed HEp-2 cell lines may actually be HeLa cells anyway [19, 20]. In any event, the adoption of HEp-2 cell substrates led to what might be regarded the “golden age” of aab detection in SARD. The increased sensitivity of IIF techniques on HEp-2 cells and a plethora of HEp-2 based IIF diagnostic kits became an issue in interpreting IIF anti-nuclear antibody results. It took almost a decade to finally appreciate that the serum dilutions used to detect aab on rodent cryosections were not appropriate on HEp-2 substrates. This led to a study by a Serology Subcommittee of the International Union of Immunology Societies/World Health Organization/Arthritis Foundation (IUIS/WHO/AF) who recommended a serum screening dilution of 1/160 to achieve a balance of sensitivity and specificity in the serological diagnosis and classification of adult SARD [21]. In retrospect, it is obvious that this advice was not widely heeded, or perhaps accepted, because to this day many laboratories still screen at dilutions that they feel provide the appropriate balance of sensitivity/specificity.

One of the unanticipated advantages of IIF on HEp-2 cells was the ability to identify target autoantigens that were weakly expressed, selectively expressed or not expressed at all in differentiated organs such as rodent liver or kidney. This led to a virtual explosion of publications identifying novel antigen targets that had hitherto not been appreciated and to the point where there are now over 150 aab described in systemic lupus erythematosus (SLE) [22, 23] and over 30 in scleroderma [24]. These included anti-proliferating cell nuclear antigen (PCNA) and other cell cycle related targets (reviewed in [25]), anti-centromere (reviewed in [26]), and a number of targets in nucleoli (reviewed in [27, 28]), nuclear envelope [29] and cytoplasm (reviewed in [30]). The use of IIF to identify these novel targets was fortuitous because, in parallel, new techniques in cell and molecular biology such as immunoprecipitation, immunoblotting, expression cloning and rapid and economical DNA sequencing opened up the entire field of molecular biology that facilitated the identification of the more precise targets of human aab and in many cases completely novel proteins or nucleic acids that were unknown to basic cell and molecular biologists [31].

The tremendous strides in identifying the molecular targets of human aab quickly led to the next iteration of diagnostic technologies that used the molecular information to design, develop and market antigen-specific immunoassays using novel platforms that included enzyme-linked immunoassays (ELISA) [32–34], dot blots [35, 36], line immunoassays (LIA) [37, 38] and then multiplexed immunoassays such as addressable laser bead immunoassays (ALBIA) [39, 40], antigen arrays on planar surfaces [41–43], nanobarcodes [44] and chemiluminescence [45]. Such advances have been a boon to the modern diagnostic laboratory, because these platforms have facilitated automated, high throughput, increasingly inexpensive and rapid turnaround time test results.

In the mix of emerging technologies to detect aab and the widespread familiarity and reliance on IIF, it is clear that, depending on the assay used, the results that one can get from the new technologies can be at considerable variance. This has led to debates of the relative value of old and new diagnostic platforms where the implications of false-negative versus false-positive test results are being considered (reviewed in [46]). There has been a tendency to adhere to things that are easily understood, fit within existing diagnostic paradigms and seem to have immediate clinical relevance. Hence, some prefer IIF on specified substrates (i.e., HEp-2 and certain tissue sections) as the “gold standard” for autoantibody testing [47]—one of the associated claims being that this class of substrates contains well over 100 different target antigens, whereas newer screening technologies may be limited to <20 [48]. While that may be true, it is abundantly clear that IIF does not detect all aab even when they are directed to an autoantigen that is highly expressed in HEp-2 cells and otherwise reacts with the same aab that are found in other sera. For example, a significant proportion of sera that have antibodies directed against Jo-1 [49], ribosomal P proteins [50, 51], PCNA [25], GWB [52] and PM/Scl [53] (to name a few) are not detected by IIF on HEp-2 substrates. While the facile argument is that these ‘false-negative’ IIF results are likely attributable to autoantibody titers, hidden or cryptic epitopes, the IIF technique itself or characteristics of the substrate (i.e., cell density, growth media, fixation protocols), the evidence to support such conclusions is far from uniform or convincing. A case in point is aab to proteinase 3 (PR3), which are detected by conventional techniques such as ELISA and generally recognized as a cANCA IIF pattern on human neutrophil substrates. However, in a recent international study of sera from ulcerative colitis patients who typically show an atypical ANCA (aANCA or
Autoantibodies: the bad, good and indifferent

It is clear that the terminology and concept of “horror autotoxicus” coined by Nobel Laureate Paul Ehrlich has had an indelible impact on our view of aab as being “bad” through presumed involvement in the genesis and pathogenesis of the SARD (reviewed in [54]). This view has been abetted by evidence that anti-acetylcholine receptor aab in myasthenia gravis were a key to the clinical expression and pathogenesis of the disease (reviewed in [55]) and more recent evidence that aab binding the water channel aquaporin 4 are pathogenic in neuromyelitis optica and transverse myelitis (reviewed in [56–58]). Accordingly, there has been a long and arduous search for evidence that aab in systemic lupus, systemic sclerosis, rheumatoid arthritis and other SARD are pathogenic [59]. Early evidence that aab directed to double-stranded DNA participate and/or initiate lupus nephritis has waxed and waned along with fairly compelling evidence that antibodies to the protein components of chromatin are possibly more directly related to the disease process (reviewed in [60]). Similarly, anti-DNA antibodies that cross-react with N-methyl-D-aspartate receptor (NMDR) have been demonstrated to be pathogenic and related to neuropsychiatric lupus [61, 62], although these observations have not been widely corroborated. Similarly, aab directed to platelet derived growth factor were promoted to contribute to a unifying paradigm in antibody-mediated pathogenesis of systemic sclerosis [63, 64], although other unpublished studies have yet to successfully corroborate that evidence (personal communication, Dr. M Servant, McGill University). The list could go on, but the point is despite what we might have been taught in introductory immunology, whereas the evidence supporting antibody-mediated pathogenesis in certain organ-specific autoimmune diseases is substantiated, the evidence supporting autoantibody-mediated disease in SARD is far from compelling.

Fortunately, the potential pathogenic role of aab is balanced by growing evidence that some aab are “good” or protective (reviewed in [65–67]). Indeed, the dogma that autoimmune processes lead to tissue damage has been refuted by a growing data in which autoimmune mechanisms in general, and aab specifically, have been demonstrated to be protective against disease genesis and processes. Even Paul Ehrlich himself was dispassionate about the notion of “horror autotoxicus” when he considered that anti-autotoxin antibodies might exist. However, as part of the contemporary immunology genre, Élie Metchnikoff and Alexander Besredka perpetuated the notion of autoimmunity and the notion that self-reactive antibodies could be controlled by anti-antibodies (reviewed in [68]).

To quote Arturo Casadevall, “the view that immunoglobulins function largely by potentiating neutralization, cytotoxicity or phagocytosis is being replaced by a new synthesis whereby antibodies participate in all aspects of the immune response, from protecting the host at the earliest time of encounter with a microbe to later challenges. Perhaps the most transformative concept is that immunoglobulins manifest emergent properties, from their structure and function as individual molecules to their interactions with microbial targets and the host immune system. Given that emergent properties are neither reducible to first principles nor predictable, there is a need for new conceptual approaches for understanding antibody function and mechanisms of antibody immunity” [69].

Evidence supporting the supposition that we all bear our own distinctive aab repertoire is supported by substantial evidence (reviewed in [68]). A recent excellent example is the observation that apparently healthy people harbor anti-PR3, myeloperoxidase and glomerular basement membrane type IV collagen antibodies [70]: aab that are generally characteristic of granulomatosis with polyangiitis (Wegener’s syndrome) and related vasculopathies [71]. These so-called ‘natural aab’ tend to be polyreactive, primarily IgM isotype, and react with both self and non-self targets. It is thought that some of these relatively non-specific and low-affinity binding natural aab may prevent
autoreactive cells from binding self-antigens by masking their antigenic determinants. These and other studies suggest that SARD may be prevented or ameliorated by protective “good” aab and that dampening the B cell response through anti-CD20 and related therapies may not be the best therapeutic approach. Hence, the identification and utilization of protective aab may serve as a much more interesting and potentially useful paradigm for novel therapies than current approaches that target cytokines, their cognate receptors or other multi-functional receptors as an approach to the treatment of SARD and other autoimmune diseases.

While the therapeutic paradigm of cytokine and cytokine receptor and other immune reactant blockade has had remarkable success (i.e., anti-TNF), there are likely more practical and perhaps less toxic ways to treat SARD in the future. For example, the administration of IgM anti-dsDNA antibodies into SLE-prone mice prevented the development of nephritis and the presence of rheumatoid factor (RF) in SLE was suggested by some to be protective against the development of lupus nephritis (reviewed in [65]).

Whereas some aab may be considered “bad” (pathogenic) or “good” (protective), it must also be considered that certain aab in an individual’s repertoire is “indifferent”—not related to pathogenesis or protection. This concept is supported by some studies in antibody responses to certain microorganisms where there is no apparent role for certain antibodies in isolation [69]. Perhaps reuniting with our “forefather” microbiologists and taking a systems biology approach to SARD and other autoimmune diseases is part of our desired future [72].

**Breaking down paradigms**

The forgoing discussion should serve as an impetus to all stakeholders in aab diagnostics (educators, clinicians, diagnostic laboratories, regulators and diagnostic kit manufacturers) to reconsider what they have been taught and have come to believe about aab. In today’s world of diagnostics, overarching concerns about false-negative and false-positive aab tests that attend virtually all aab diagnostic platforms [46] should not be summarily dismissed by citing pros or cons of any one technology or diagnostic platform. Many of these issues point to persisting significant gaps in our understanding of the origin and roles of aab. Before we can hope to completely understand the enigmas that attend the results of aab diagnostic tests, perhaps it is time to step back and re-examine long accepted paradigms and beliefs. Some of the issues about aab that should be (re)considered in the future are discussed very briefly below and are outlined in Table 1.

| Table 1 | Future considerations of characteristics, functions and roles of autoantibodies that impact on their diagnostic relevance |
|-----------------|----------------------------------------------------------------------------------------------------------------------------------|
| • Autoantibody efficacy: a function of specificity, amount, isotype, host genetics/epigenetics |
| • Autoantibody isotypes and subclasses |
| • Reconsider the concept of “prozone” |
| • Autoantibody binding complement and/or other proteins |
| • Re-evaluate the potential importance of the autoantibody Fc |
| • Peptoid technology: define novel and disease relevant autoantigens and use them in diagnostics |
| • Sort the “wheat from the chaff” |

First, while aab testing is traditionally considered to primarily serve the realm of diagnostic medicine, there is little doubt that aab testing is rapidly being recognized as a discipline that will provide important **prognostic and therapeutic** information at the bedside. For example, the European consortium (EUSTAR) studying more than 3,600 systemic sclerosis concluded that the “clinical distinction seemed to be superseded by an antibody-based classification” [73]. And a recent report suggested that an autoantibody-based classification of SLE has clinical value [74]. Accordingly, along with other proteomic analyses (i.e., cytokine profiles), genomics and metabolomics, aab testing is taking on increasing importance in the realm of personalized medicine [75].

Second, in terms of differentiating “good” from “bad” or “indifferent” aab, it needs to be appreciated that the terms ‘protective’ and ‘non-protective’ are relative terms that depend on a number of factors: the chief among them being the host and the “trigger” that initiated the response [69, 76]. Indeed, inroads into our understanding of aab test results and aab functions will not be significantly advanced until they are understood in the context of the entire patient, and in particular any co-morbidities that may be present. Studies of clinical correlations of aab based only on simple diagnostic stratifications must take into account co-morbidities, which have an important influence both on the repertoire of aab produced and on the expression of autoimmune disease (i.e., disease phenotype). To achieve a more complete and meaningful serological profile, it will be particularly important to combine aab profiles with cytokine and other proteomic profiles in addition to genomics and metabolomics. While the amount of data generated in such studies can be overwhelming, bioinformatics is poised to permit such complex analyses and ‘paint’ a more comprehensive and realistic picture of clinical subsets of disease.

Third, the efficacy of aab must be considered. Based on primarily microbiological studies [69], the protective
efficacy of antibodies has been shown to be a function of specificity, amount, genetic and epigenetic characteristics of the host and, as presented in the next paragraph, isotypes. As implied earlier in this review, the same applies to defining and understanding pathogenic or indifferent aab.

Fourth, the isotypes and subclasses of aab in any given patient are very important. There is ample evidence in the broad aab literature that certain aab, expressed as certain isotypes or subclasses, make a big difference in terms of diagnostic, therapeutic and, by extension, prognostic importance. Not that many years ago, most autoimmunologists probably did not care that much about IgG4. However, today the emergence of clinical syndromes based on IgG4 aab and immune responses [77, 78] should be cause for reconsidering the entire spectrum of diseases that are both conventional and non-conventional autoimmune diseases. For one thing, antibody isotypes are considered to point to the triggering pathogen. For example, in C. neoformans infections IgG2a>IgG1>IgG2b>>IgG3, whereas in Mycobacterium tuberculosis IgG3>>IgG2 [69]. Such considerations in antigen or autoantigen driven or mediated aab responses may point to the elusive trigger(s) of autoimmunity.

Fifth, while the concept of “prozone” is generally thought of only in terms of in vivo or solution phenomena, this needs to be reconsidered and explored in the context of aab detection in both older and newer diagnostic platforms [79, 80]. Not all diagnostic platforms have the same antigen density available for aab binding or the same dynamic range. Newer platforms such as BioFlash that employ a bead-based chemiluminescence technology appear to provide advantages in this regard [45].

A sixth consideration is to determine if the aab in question binds complement and other proteins. A recent report indicating that synovial fluid complement, specifically, the membrane attack complex-mediated arm of complement, is crucial to the development of joint disease in three different models of osteoarthritis [81] may come as a surprise to some autoimmunologists, but does highlight the importance of re-evaluating the complement-binding and complement-activating capacity of not only aab, but other proteins (i.e., integrins) involved in immune mediate diseases. Such studies open up connections between adaptive immunity with numerous cellular components such as matrix metalloproteinases (MMPs), extracellular signal-regulated kinase (ERKs) and integrins. Recent evidence has provided renewed interest in complement as being a critical feature of ANCA-related vasculitis [82], anti-aquaporin 4-related neuromyelitis optica [83] and models of aab-induced hepatitis [84].

Seventh, while much attention has focused on the Fab-idiotype of aab, it is time to reconsider the potential importance of the Fc. There is now convincing evidence that the Fc component of antibodies has a dramatic effect on the reactivity of the Fab insomuch as Fc influences the fine specificity of antibody reactivity (reviewed in [69]). Contemporary views that aab are simply bifunctional molecules composed of independent Fab and Fc domains is no longer tenable, since it has been clearly shown that the V region of Fab and the C region of Fc act together to affect both affinity and specificity of antibody binding [85]. In part, this effect has also been attributed to observations that IgG Fc receptors have both stimulatory and inhibitory effects on cells [69].

Eighth, based on a lesson that could be learned from a major advance in the diagnosis of rheumatoid arthritis (RA), with the discovery of aab to citrullinated peptides by van Venrooij and his colleagues (reviewed in [86]), is the importance of autoantigens and analytes for aab detection that are related to the disease itself. In the past, approaches to identifying target autoantigens has had a modicum of success and in some cases major breakthroughs using conventional technologies employed by cell and molecular biologists. These have included immunoprecipitation of the target antigens followed by molecular analysis (i.e., mass spectroscopy), immunoscreening of recombinant proteins produced by expression clones and immunoscreening arrays of native and recombinant proteins and peptide arrays absorbed or ‘printed’ on solid phase substrates such as nitrocellulose or glass. The basic protocol for the latter included pouring index patient sera containing disease-specific antibodies of potential interest over the array and then seeing what sticks. The results were then compared to “controls” of an unrelated disease and normal persons, and “voila” you had your new candidate target autoantigen and biomarker for the disease of interest. In a related novel approach, Kodake and his colleagues at the Scripps Research Institute in Jupiter, Florida (USA) have reasoned that it is unlikely that aab from a person with a given disease would primarily bind to “normal” proteins [87]. In this paradigm, it is suggested that the immune system does not react to normal proteins simply because ‘tolerance to normal proteins has been broken’. Indeed, it seems more intuitive that the immune system reacts to foreign proteins of an invading microorganism or proteins altered in dying (i.e., senescent, apoptotic or necrotic) cells. Even more intriguing is the possibility that the target antigens are released from living cells as extracellular exosomes or microbodies [88, 89] during disease genesis when certain targets are altered either because of epigenetic effects, genetic mutations, microRNA regulation, proteasomal or exosomal dysregulation or simply post-translational modifications (i.e., citrullination) of the targets due to micro-environmental changes. Because the potential by-products of all of these events are logistically overwhelming, instead of using arrays of normal proteins, Kodake and his
colleagues employ a fairly random assortment of unrelated synthetic small molecules that bind to aab because of their chemical shapes (epitopes), allowing for a very broad and unbiased "fishing trip". The difference from earlier "fishing trips" that used large peptide/protein arrays is the "bait" (also referred to as 'peptoids') that is used [90]. So, nice idea, but does it work? As one early example, this approach has yielded novel aab targets in Alzheimer's disease [91]. Obviously, there is much more to be learned about this approach, not to mention the corroborated evidence of success, but it seems likely that peptoid chemistry will be a major advancement in the field of autoimmune diagnostics and therapeutics.

Ninth, if it is true that some aab are pathogenic and some are actually "good for you" or protective, and some may have less intuitive functions, then it is very important to "sort the wheat from the chaff" (quotation: Dr. Ian McKay). This is particularly critical in the future when aab detection not only will be more than a tool for early and accurate diagnosis, but also will provide key information about an individual patient's aab mosaic that reflects good, bad and, presumably, indifferent antibodies.

In summary, it appears that aab testing is on the threshold of new applications that extends beyond diagnostics into the realm of prognostics, therapeutics and personalized medicine. However, to move into this "new age", a much clearer and more thorough understanding of the genesis and roles of aab is required. This also includes appreciating that the two solitudes of innate B cell immunity and acquired immunity are a continuum, and this is attended by re-examining old paradigms and adopting new technological approaches to arrive at a new aab orthodoxy.

Conflict of interest None.

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