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SHORT ANALYTICAL REVIEW

Accepting clocks that tell time poorly: Fluid-phase versus standard ELISA autoantibody assays

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Abstract The predominant autoantibody assays employed in basic immunologic studies are variations of solid-phase assays where autoantigens are bound to 96-well plates. Though the assay format is convenient and often appropriate for studies of induced immune responses in inbred strains of mice, we will argue that this assay format usually, but not always, leads in clinical medicine to what should be unacceptable false positive results as well as lower sensitivity compared to the current generation of high throughput fluid-phase radioassays. Utilizing simple *in vitro* transcription and translation labeling of autoantigens, it is now possible to rapidly create fluid-phase radioassays for most (but not all) autoantigens, thereby allowing direct comparison between the different assay formats. In addition, adding a fluid-phase competition step to both solid-phase ELISA assays and even fluid-phase radioassays can enhance specificity. Development in a field of such assays with excellent specificity and sensitivity (e.g. studies of type 1A diabetes) is fostered by Societies sponsoring workshops where blinded samples are evaluated with “competing” assay formats for sensitivity, specificity, and reproducibility.

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Introduction

The basic parameters defining assay performance are precision, accuracy, specificity, and sensitivity. Precision refers to the ability of an assay to consistently reproduce a result using sub-samples of the same specimen. Accuracy refers to the ability of the test to measure known amounts of a test sample. Specificity is defined as the percent negative in a reference “control” population with $1 - \text{specificity}$ the false positive rate. Sensitivity is the percent positive in a reference “case” group with $1 - \text{sensitivity}$ the false negative rate. The positive and negative predictive values, the parameters of primary

importance to a clinician and their patient are dramatically influenced by the prior probability of the specific disease or state being studied (Bayes’ theorem). There are specific factors affecting autoantibody assays: (1) Although in the aggregate, autoimmune diseases are common, each single autoimmune disease is usually uncommon in general populations (<1%). Lower prior probability of disease mandates higher specificity assays, or else a large percentage of positives will be false positives. (2) Autoantibodies are usually polyclonal with a mixture of autoantibodies varying in affinity, capacity, isotype, and the epitopes recognized. Thus there is not a gold standard for direct comparison to determine the accuracy of measurement (that one is measuring what one thinks one is measuring) such as in a mass spectrometry determination of an analyte, or a cesium atomic clock in a vault at the Naval Observatory. (3) Autoantibodies can be

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present in the absence of disease, and in particular, can even precede the development of disease. (4) Finally of primary importance, some autoantibodies react with a given autoantigen but are not indicative of disease. It is probably this latter phenomenon that has led to the acceptance of assays with poor specificity with the assumption that one could not distinguish disease-associated autoantibodies with accuracy and that a large population of normal individuals have autoantibodies that cannot readily be distinguished for highly specific disease-associated autoantibodies.

A number of fields have progressed to the stage of having validated specific and sensitive fluid-phase assays and combination of assays providing high specificity, sensitivity, positive and negative predictive values, indicating that a field does not have to accept assays with either low specificity (false positive rates greater than 1%) or sensitivity [1]. In addition, in a number of fields, it has been possible to develop formats similar to ELISA formats where either by initial fluid-phase capture of the autoantigen or by performing an additional parallel assay with fluid-phase competition with the autoantigen, high specificity and sensitivity can be achieved. The field of type 1A diabetes provides perhaps the best example of such progress and we will emphasize studies for this disorder and then illustrate other disease applications. Perhaps the most important part of the process of developing and applying such autoantibody assays is the realization that standard ELISA autoantibody assays usually (not always) are substandard and by testing in organized workshops the current generation of assay methodology, a field can progressively improve their diagnostic tests.

Type 1A diabetes

We can now predict the development of type 1A diabetes (immune mediated diabetes) such that large trials of prevention (to date unsuccessful) are underway. Type 1A diabetes occurs in approximately 1/300 children of the general US population, 1/20 first-degree relatives of a patient with type 1 diabetes and 1/2 twins (identical to proband with type 1 diabetes). Multiple genes conferring susceptibility have been defined, with by far the most important within the major histocompatibility complex (41% of the 48% of familial aggregation currently genetically defined related to MHC class II genes) [2]. It is now possible to predict appreciable genetic risk even in the general population with HLA class II typing. The persistent expression of ≥ 2 of three "classic" islet autoantibodies (autoantibodies to GAD65, IA-2 or insulin) is associated with almost complete progression to diabetes given long enough follow up, and a fourth major islet autoantigen has recently been discovered [3]. Hundreds of thousands of individuals have been screened for islet autoantibodies and such screening forms the basis for preventive trials.

The discovery of "cytoplasmic" islet cell autoantibodies with immunohistochemical staining of frozen sections of normal human pancreas initiated the utilization of anti-islet autoantibodies and was rapidly followed by the realization that type 1A diabetes is a chronic autoimmune disorder that could be predicted [4,5]. A series of workshops improved the measurement of cytoplasmic islet cell autoantibodies but

also highlighted difficulties in standardization of an assay dependent upon "observer" and pancreatic tissue sections. The discovery that insulin autoantibodies were present prior to insulin injections that induce insulin antibodies initiated the studies of what has been termed "biochemical autoantibodies" [6]. The first assays demonstrating insulin autoantibodies utilized ^{125}I -labeled insulin in a fluid-phase radioassay. (the discovery of the radioassay by Berson and Yalow utilized insulin antibodies.) Shortly after the report of insulin autoantibodies at the onset of diabetes prior to insulin therapy, dozens of laboratories set up assays and an immediate controversy developed as to whether insulin autoantibodies predicted diabetes. Remarkably clear results from an insulin autoantibody workshop ended the debate [7,8]. Fluid-phase radioassays detected insulin autoantibodies of prediabetic and new onset patients while ELISA assays only detected antibodies induced by insulin injection. The first insulin autoantibody assays utilized relatively large volumes of serum and polyethylene glycol precipitation to separate labeled insulin bound to antibody from free insulin. Polyethylene glycol is a non-specific precipitant and factors in sera, particularly in cord blood thus could produce low levels of signal. Subsequent assays introduced competition with unlabeled insulin to improve specificity and sensitivity and micro-assays utilizing protein A precipitation were developed [9]. Insulin autoantibody expression is dramatically age-related amongst individuals developing type 1A diabetes with almost all children presenting prior to age 5 having such antibodies, but markedly fewer diabetics expressing insulin autoantibodies at diagnosis after age 12 [10]. Prospective studies from birth indicate that not infrequently insulin autoantibodies are present years before diagnosis, but then can be absent at diagnosis even though other anti-islet autoantibodies have appeared.

Following the initial application of insulin autoantibody assays, assays for autoantibodies reacting with GAD65 and IA-2 (and related IA-2 beta molecule) autoantibodies were introduced [11,12]. These assays were facilitated by the general application of in vitro transcription and translation reticulocyte kits to produce labeled autoantigen with usually ^3H -leucine for ^{35}S -methionine incorporated into the protein autoantigen. Given a cDNA for a given protein antigen, within a week appropriate labeled autoantigen can be produced and an assay run. Assays can be run in 96-well plates with protein A beads (or isotype-specific beads) and filtration to separate bound from free autoantigen. The majority of new assays work the first time they are run, but for those that do not work, perhaps due to improper folding of a protein antigen or lack of essential post-translational modifications of the antigen, it is very difficult to appropriately modify the antigen. Of the three standard assays, the insulin autoantibody assay has remained the most difficult for new laboratories to perform and to standardize. This assay is typically run with a competitive step with unlabeled insulin blocking binding of ^{125}I -insulin to the autoantibody while the GAD65 and IA-2 autoantibody assays do not require such competition. The difficulties with the insulin autoantibody radioassay probably relate to the very low concentrations of insulin autoantibodies, with a very small difference between approximately 1/2 of positive patients and normal controls. It appears that insulin autoantibodies of man cannot react with insulin bound to plates, hence the importance of the fluid-phase format. Given the presence of these

autoantibodies in man, workshops evaluating animal models have concluded that only specific insulin autoantibodies are present in the spontaneous model of diabetes, the NOD mouse. Insulin autoantibody levels are as high in the NOD mouse as the youngest children developing diabetes and follow a classic course, usually decreasing to negative about the time of onset of diabetes (Fig. 1).

Evaluation of standard ELISAs for anti-islet autoantibodies in multiple workshops indicated a lack of sensitivity, specificity, or both compared to the fluid-phase radioassays. Recently a modified ELISA format for GAD65 autoantibodies that involves fluid-phase reactivity of autoantibody with GAD65 performed as well as the best fluid-phase radioassays. In this format, labeled fluid-phase GAD65 bound to antibody that subsequently bound to GAD65 on the ELISA plate [13].

The evolution of assay formats in the field of type 1 diabetes illustrates the utility of workshops that occur approximately every 2 years with evaluation of 100 control sera and 50 sera from patients with new onset diabetes, as well as sera to evaluate assay reproducibility. In general, groups are allowed to utilize any methodology they want and it is the comparative results that rapidly determine which methodologies are accepted. The appearance of "biochemical" autoantibodies is used as an intermediate phenotype in studies of the genetics and environmental factors preceding diabetes as well as entry criteria for preventive and new onset trials. Even with the recent discovery of a fourth major islet autoantigen, there likely exist additional specific islet autoantibodies, given some rare sera that are positive on sections of pancreas but negative for the radioassays. In contrast, the cytoplasmic ICA test cannot detect insulin autoantibodies, and misses most low positive GAD65 and IA-2 autoantibodies.

There is a subset of individuals with single anti-islet autoantibodies, particularly GAD65 and insulin that do not progress to diabetes with more than 10 years of follow-up. It appears that with a single autoantibody risk of diabetes for relatives of patients is only approximately 20%. In contrast the great majority with ≥ 2 autoantibodies progress. Recent evidence indicates that the affinity of "single" anti-insulin autoantibodies in young children that do not predict diabetes are considerably lower than the insulin autoantibodies associated with diabetes risk. In young children, insulin autoantibodies are usually the first to appear [14] (Fig. 2).

It is likely that the assay formats utilized in type 1 diabetes and the ability to develop highly predictive assays (99% specificity) with multiple autoantigens should be applicable to many fields. We would suggest that an essential feature is not to assume that lack of prediction by an assay reflects the basic biology, but rather the possible dynamics of the assay. We would also suggest that high-affinity autoantibodies reacting with native antigens not bound to solid phases with autoantibody-specific reagents (e.g. protein A) should be the format used in reference assays.

Celiac disease

This is a field where the ELISA assays are very good and excellent radioassays are available for comparison. Measurement of IgA antibodies against tissue transglutaminase (TG) is the single best autoantibody assay for the prediction of celiac disease, and quantitative levels of the autoantibody reflect degree of intestinal injury. Changing the substrate from guinea pig TG to human recombinant TG led to significant improvements in the performance of this assay [15]. This autoantibody assay is typically performed

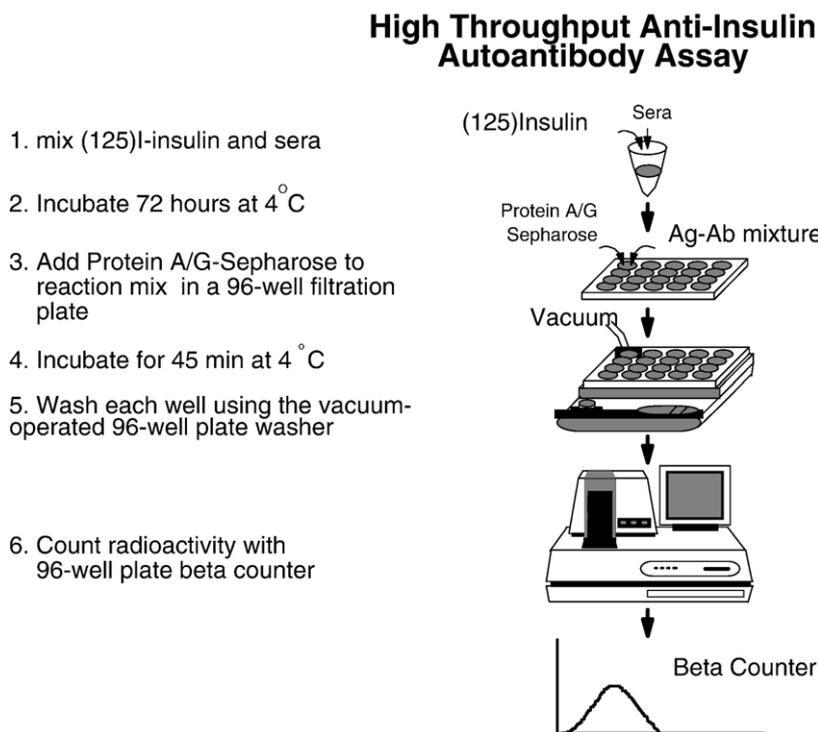


Figure 1 General outline of high-throughput filtration autoantibody assays. From www.barbaradaviscenter.org Teaching Slides, Immunology of Diabetes, Ed. G.S. Eisenbarth.

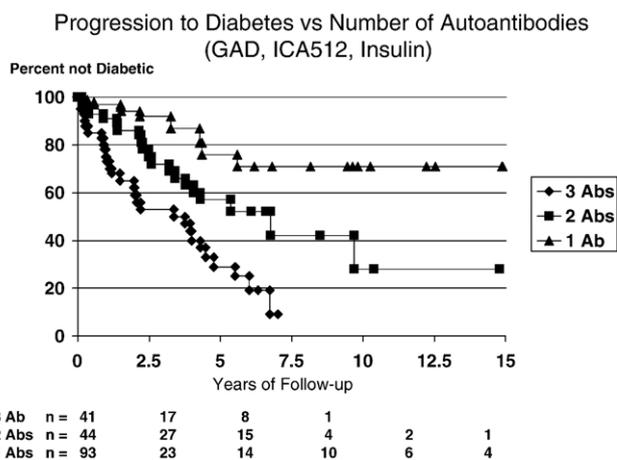


Figure 2 Progression to diabetes of first degree relatives of patients with type 1 diabetes subdivided by expression of one to three anti-islet autoantibodies measured with fluid-phase radioassays. Modified from Verge et al. [3].

in the solid phase, but can also be performed in the fluid phase using radioactive detection methods. Tiberti et al. proposed that the fluid-phase radiobinding assay was superior to standard ELISA assays but larger and direct comparisons to improved ELISA assays is needed [16]. A head-to-head comparison of a radioassay with 5 commercial ELISAs showed that marked qualitative differences were obvious amongst all of the assays, even though increasing the cutoff for positivity uniformly improved the predictive value for celiac disease [17]. Since quantitative assessment of TG levels is important (and also reflects the degree of intestinal pathology), this study underscored the need for standardization of the assays amongst all commercial and research laboratories. A TG autoantibody workshop designed after the diabetes autoantibody workshops is currently underway with the aim of comparison and improvement of the transglutaminase autoantibody assay amongst commercial and research laboratories, with the future goal of standardization.

Part of the underlying pathogenesis of celiac disease involves specific deamidation of amino acids in ingested gliadin proteins to enhance immunogenicity. A new antibody assay against specific previously deamidated gliadin peptides (DGP) has recently become available, as a marked improvement over the original anti-gliadin antibodies. Both IgA and IgG antibodies against DGP are measured by standard ELISA, and our examination of these antibodies demonstrate that the anti-DGP antibodies parallel (quantitatively) the course of anti-TG autoantibodies (measured by radioassay) as they are followed prospectively over time through serial measurements in children at risk for celiac disease. Anti-DGP antibodies become undetectable sooner than anti-TG autoantibodies when treatment with a gluten-free diet is instituted, which could either reflect the removal of the offending antigen in a patient's diet, or due to differences in sensitivity between the two assays (Fig. 3). Further study into the DGP antibody is important since it likely reflects the specific immune response against exogenous antigen, namely gliadin, rather than the autoimmune response of TG. It remains to be determined whether the appearance

of multiple antibodies to indicate celiac autoimmunity can someday improve the predictive value for intestinal lesions in celiac disease, to replace intestinal biopsy as the future "gold standard."

Lupus erythematosus

Anti-dsDNA autoantibodies measured by standard ELISA have relatively poor sensitivity and low specificity. In a recent comparative study with a standard commercial ELISA kit, the specificity amongst 50 normal controls was only 80%, and thus a false positive rate of 20% [18]. The Farr assay, measured in the fluid-phase with ammonium sulfate precipitation, utilizing radiolabeled ¹²⁵I had high specificity but lower sensitivity compared to a fluid-phase radioassay. The reported fluid-phase filtration radioassay for anti-dsDNA, adopted from standard techniques to measure anti-insulin autoantibodies allows both high sensitivity and specificity [19]. This modified assay also utilizes ¹²⁵I-DNA with protein A/G precipitation and demonstrates that the fluid-phase filtration radioassay format for measuring autoantibodies can be generalized to measure other autoantibodies, and should be considered when optimizing current assays.

The difficulty of precisely correlating autoantibodies against ribonuclear proteins (anti-RNP) with rheumatologic diseases may be partly due to the standard method of detection, which is likely suboptimal. Using similar method described above with radiolabelled, in vitro transcribed/translated proteins, detection of a diverse panel of anti-RNP autoantibodies was superior compared to standard detection assays [20]. The radioassay showed much greater specificity and sensitivity in a large group of controls versus rheumatic conditions. With improved assay techniques, clinical correlation of such autoantibodies to the disease condition might become better defined.

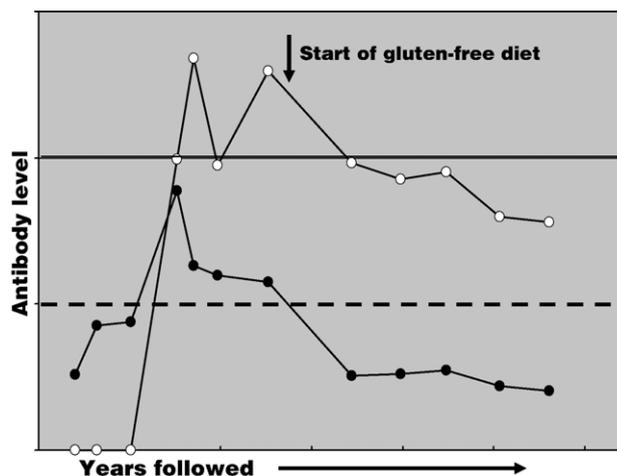


Figure 3 Natural history of anti-TG and DGP antibodies in a child followed prospectively because of a genetic risk for celiac disease. Arrow indicates small intestinal biopsy confirming celiac disease along with the initiation of treatment with a gluten-free diet. Open symbols indicate anti-TG autoantibodies. Closed symbols indicate anti-DGP antibodies. Dashed line indicates cutoff for positivity for both assays.

APS-1 (autoimmune polyendocrine syndrome type 1)

APS-1 is a rare autoimmune disorder resulting from autosomal recessive mutations of the autoimmune regulatory (*AIRE*) gene and associated with mucocutaneous candidiasis, Addison's disease and hypoparathyroidism. Antibodies to interferon-alpha ($\text{IFN-}\alpha$) were recently found in a large number of Finnish and Norwegian patients with autoimmune polyglandular syndrome type 1 (APS1) with a prevalence approaching 100% utilizing a bioassay [21]. Similar autoantibodies were originally described in patients with myasthenia gravis and mucocutaneous candidiasis, and patients treated with interferons also develop anti-interferon antibodies. Using a Europium-based solid-phase assay, anti- $\text{IFN-}\alpha$ antibodies in patients with APS1 can be detected. However, to enhance assay specificity, fluid-phase competitive inhibition with soluble $\text{IFN-}\alpha$ can be performed. Such high-specificity assays may be valuable in the rapid diagnosis of this rare disorder (preceding definitive *AIRE* gene sequencing). The simple step of adding extra wells to an ELISA assay with fluid-phase competition with the autoantigen, and using the "delta" of the wells with and without competition as the specific signal might greatly reduce current specificity problems for many ELISA assays.

Autoimmune hepatitis

The hallmark of detecting autoimmune hepatitis remains the identification of non-organ-specific autoantibodies with characteristic histologic features in the liver. The major nonorgan-specific antibodies used in clinical diagnosis remain anti-nuclear antibodies (ANA), anti-neutrophilic cytoplasmic antibodies (ANCA), anti-smooth muscle antibodies (SMA), and anti-liver–kidney microsomal (LKM) antibodies.

These antibodies are present in varying frequencies and titers in affected patients and controls. Therefore, it might be considered that these autoantibodies are indicators of general immune activation. Currently, one cannot employ autoantibodies as a single marker to diagnose AIH [22] and approximately 10% of patients do not express any known autoantibodies. The relative unreliability of such autoantibodies in the past may have been related to the conventional method of detection, which utilized indirect immunofluorescence. The International Autoimmune Hepatitis Group (IAIHG) acknowledged this deficiency and recognized the lead that has been taken by the diabetes community for standardization efforts, and subsequently established an internationally representative committee to define guidelines and develop procedures and reference standards for more reliable testing [23]. With the identification of the molecular targets for anti-LC1 autoantibodies [formiminotransferase cyclodeaminase (FTCD) liver-cytosolic protein type 1], anti-asialoglycoprotein receptor (ASGP-R), and soluble liver antigen/liver–pancreas antigen (tRNP), molecularly based assays can or have been designed including those by radioassay [24]. In addition, when the target for anti-LKM autoantibodies was identified as CYP2D6, a radioassay with relative high sensitivity/specificity using *in vitro* translated ^{35}S -CYP2D6 was developed that eliminates the possibility of overlap by indirect immunofluorescence signals

with other autoantibodies such as anti-LC-1 [25]. Thus, it is possible that with improved assay formats for detection, the role of such autoantibodies in diagnosis and management might become mainstay.

Newer assays

Other novel assays under development include protein arrays designed for multiplex analysis of autoantibodies that allows autoantibody screening of a panel of autoantigens. Potential applications include screening for candidate autoantigens, autoantigen epitope mapping, and antibody isotype usage [26]. Such assays could allow for rapid screening of new and existing autoantibodies to create individualized autoantibody profiling in the future. Further studies will be needed to define the performance of such assays designed for screening "en masse." Given that such assays are performed in solid-phase format, we would suggest evaluation of adding competitive inhibition with fluid-phase antigens to improve performance. Another assay method recently described involves the novel use of radiolabeled, folded self-antigens arranged in a tetrameric fashion utilizing a streptavidin core. Antibody binding occurs in the fluid-phase, and has the potential benefit of increased valency of the target autoantigen, which could permit low-affinity antibody binding. This self-antigen tetramer is able to identify conformation-dependent autoantibodies against myelin oligodendrocyte glycoprotein (MOG) in individuals with acute disseminated encephalomyelitis [27].

Conclusion

This short review has illustrated the development and utilization of autoantibody assays from a limited number of fields and many additional examples could have been chosen. For some fields, ELISA assays (e.g. celiac disease) have appropriate sensitivity and specificity, but for many clinical disorders, this is not the case. We believe that for many diseases, autoantibody assays can be greatly improved if assays with relatively poor specificity (false positive rates greater than 1%) are not accepted, and both greater sensitivity and specificity can be achieved by utilizing assays that depend upon fluid-phase interaction of autoantibody with autoantigen. This can be achieved by utilization of simple high-throughput fluid-phase radioassays, incorporating parallel competition with fluid-phase unlabeled autoantigen in an ELISA format, or with development of novel assays where labeled autoantigen is captured in the fluid phase by an autoantibody that also binds to plate bound autoantigen. It is likely there are additional methods to achieve high specificity and sensitivity and a key step in developing such assays is development of workshops and disease specific Societies defining the characteristics of "the best" assays for their discipline. We believe the concept that autoantibodies are common in normals and high specificity/sensitivity assays cannot be developed is primarily dependent upon the assay methodology, and that with continued refinement, diagnostic accuracy, both positive and negative predictive values for disease can be remarkably high. Such diagnostic accuracy will be essential for a series of autoimmune disorders for the implementation of personalized medicine, with intervention

very early in the natural history of the disease, and potentially prior to symptoms for disease prevention.

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