Autoimmune Diabetes Mellitus: The Importance of Autoantibodies for Disease Prediction and Diagnostic Support

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Abstract: More than three decades ago, screening for autoantibodies associated with immune mediated type 1A diabetes was limited to measuring antibodies to cytoplasmic antigens of islet cells (ICAs). After a period of identification and sequencing of the major autoantigens involved (insulin, glutamic acid decarboxylase and tyrosine phosphatase IA-2) the recombinant technology allowed the quasi-quantitative assessment of the respective specific autoantibodies (IAA, GADA and IA-2A). In addition, the beta-cell-specific zinc transporter isoform 8 (ZnT8) has recently emerged as another major autoantigenic target of type 1 diabetes. The first useful assay for measuring islet autoantibodies was an indirect immunofluorescence assay with frozen sections of human pancreas as substrate. Other methods for the determination of islet autoantibodies (markers) are the radioligand binding assay (RBA) and ELISA. The current fluid phase RBA utilizes low levels of tracers, usually labeled with ^{125}I, ^{35}S-methionine or ^{35}S-cysteine. After a series of international workshops and proficiency programs starting in 1985, the first Diabetes Antibody Standardization Program (DASP) was run in 2000. The predictive value of markers assays was shown in several series of prospectively followed first-degree relatives of type 1 diabetes patients. On the other hand, islet autoimmunity is frequent in adult patients considered to have type 2 diabetes (Latent Autoimmune Diabetes of Adults, LADA). The presence of autoantibodies in such patients may forecast the need of insulin administration, thus sparing these patients from months of inadequate metabolic control. Signals above a cutoff value were usually employed as the criterion for marker positivity, whereas in other studies determination of titer, epitope specificity, and IgG subclass were included to improve diabetes prediction. Recently it was suggested that combining affinity and titer of specific antibodies significantly improves the sensitivity, specificity, and concordance of markers measurement between laboratories. These new contributions on stratification of type 1 diabetes risk on the basis of markers characteristics will be invaluable in the design, implementation and interpretation of multicenter intervention trials. Moreover, marker testing is likely to be increasingly used in clinical practice, and may need to be performed in nonspecialized laboratories.

Keywords: Diabetes mellitus, autoantibodies, methods.

DEFINITION AND CLASSIFICATION OF DIABETES MELLITUS

Diabetes Mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The chronic hyperglycemia of diabetes is associated with long-term damage, dysfunction, and failure of various organs. Several pathogenic processes are involved in the development of diabetes. These range from autoimmune destruction of pancreatic beta cells with consequent insulin deficiency to abnormalities that result in resistance to insulin action [1].

A major requirement for epidemiological and clinical research and for the clinical management of diabetes is an appropriate classification system to identify and differentiate its various forms and stages. The current American Diabetes Association classification system defines four major forms of Diabetes Mellitus: type 1, type 2, diabetes due to other known causes, and gestational diabetes (Table **1**). Type 1 diabetes is defined in etiologic terms, i.e. as diabetes caused by the destruction of insulin-producing beta-cells, the consequence of which is an absolute dependence on insulin for survival. Type 1 diabetes is further subdivided according to whether beta-cell destruction is immunologically mediated (type 1A) or due to other processes (type 1B) [2].

Type 1 diabetes, formerly known as either juvenile-onset diabetes (because of the early age of onset) or insulin-dependent diabetes mellitus (because of the clinical need for insulin), is now widely thought to be an organ-specific autoimmune disease. It is now recognized that as many adults develop type 1A diabetes as children, and the classification of adults with clinical manifestations of type 2 diabetes who express anti-islet autoantibodies is debated. Although the later patients have been included in separate subgroups, such as LADA (latent autoimmune diabetes of the adult), they have human leukocyte antigen (HLA) alleles associated with typical type 1A diabetes, and exhibit an accelerated loss of C-peptide secretion [3]. There is evidence that some patients have insulin resistance as well as anti-islet autoimmunity, and there is no reason to exclude a potential coexistence of type 2 and type 1A diabetes. The genes controlling the development of type 1A diabetes do not increase the risk of type 2 diabetes, and the most important genetic polymorphism related to type 2 diabetes, which affects the TCF7L2 (transcription factor 7-like 2) gene, is not associated with the risk for type 1A diabetes [4].

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Table 1. Etiologic Classification of Diabetes Mellitus

Types of Diabetes	Old Nomenclature	Etiology
Type 1 Type 1A Type 1B	Juvenile-onset, Insulin-dependent diabetes mellitus	Beta-cell destruction Immune mediated Idiopathic
Type 2	Adult-onset, Non-insulin-dependent diabetes mellitus	Insulin resistance/beta cell loss
Gestational		Onset during pregnancy
Other types	Secondary diabetes	Specific genetic defects, pancreas disease, endocrinopathies, infections, etc.

PREVALENCE OF TYPE 1A DIABETES

Type 1A diabetes accounts for approximately 90% of childhood onset diabetes, and for 5–10% of adult onset diabetes. About 40% of patients with type 1A diabetes develop the disease before 20 years of age, thus making it one of the most common severe chronic diseases of childhood. In the USA, where 30,000 new cases occur each year, type 1A diabetes affects 1 in 300 children and as many as 1 in 100 adults. Diabetes (of all causes) is a leading cause of end-stage renal disease, blindness and amputation, and a major cause of cardiovascular disease and premature death in the general US population [5]. Approximately one-half of patients develop type 1 diabetes within the first two decades of life, but an increasing number of cases are being recognized in older individuals. The geographic incidence varies widely from less than 0.1/100,000 per year in China to more than 37/100,000 in Finland [6]. Of note, the incidence of type 1A diabetes is increasing in many Western countries, doubling approximately every 20–30 years [7].

PATHOGENESIS OF TYPE 1A DIABETES

Type 1A diabetes develops slowly, and progressive abnormalities in beta-cell function herald what appears to be a sudden development of hyperglycemia. Increasing HbA1c values within the normal range [8], impaired fasting blood glucose or glucose tolerance, as well as loss of first-phase insulin secretion usually precede overt diabetes. The exact beta cell mass remaining at diagnosis is poorly defined, and there are almost no studies on the presence of insulitis before diabetes onset [9]. For patients with long-term type 1A diabetes there is evidence of some beta cell function remaining (C-peptide secretion), although beta cell mass is usually decreased to less than 1% of normal [10]. At present, methods to image/quantitate beta cell mass and insulitis are only beginning to be developed. In particular, positron emission tomography scanning using a labeled amine (dihydrotetrabenazine) may provide the first method to image islet mass [11], and is now being evaluated in humans. In addition, a number of techniques are being evaluated to image insulitis [12].

A large body of evidence indicates that the development of type 1A diabetes is determined by a disruption of the balance between pathogenic and regulatory T lymphocytes [13, 14]. A fundamental question is whether there is a primary autoantigen for initial T cell autoreactivity with subsequent recognition of multiple islet antigens. Using the non obese diabetes (NOD) mouse model which spontaneously develops type 1 diabetes, a number of researchers have addressed the importance of immune reactivity to insulin with the dramatic finding that the elimination of immune responses to insulin blocks the development of diabetes and insulitis and, importantly, of immune responses to downstream autoantigens such as the islet-specific molecule IGRP (islet-specific glucose-6-phosphatase-related protein) [15]. Knocking out both insulin genes (mice, in contrast to humans, have two insulin genes) followed by the introduction of a mutated insulin with alanine rather than tyrosine at position 16 of the insulin B chain prevents the development of diabetes [16]. Recognition of this B chain peptide of insulin by T lymphocytes depends on a "nonstringent" T cell receptor with conservation of only the α chain sequence (Vα and Jα) and not the N-terminal region of the α-chain or the β-chain [17].

A recent study of pancreatic lymph nodes from two patients with type 1 diabetes found a conserved T cell receptor, with T cells reacting with amino acids 1–15 of the insulin A chain [18].

NATURAL HISTORY OF TYPE 1A DIABETES

The clinical presentation of type 1A diabetes is preceded by an asymptomatic period of highly variable duration. Aggressive beta cell destruction may lead to disease manifestations within a few months in infants and young children, while in other individuals the process may continue for years (more than 10 years in some cases) before the presentation of overt disease. The appearance of autoantibodies is the first detectable sign of emerging beta cell autoimmunity. There are four disease-related autoantibodies that have been shown to predict clinical type 1A diabetes [19]. These include classical islet cell antibodies (ICA), insulin autoantibodies (IAA), autoantibodies to the 65 kD isoform of glutamic acid decarboxylase (GADA), and autoantibodies to the protein tyrosine phosphatase-related IA-2 molecule (IA–2A). The zinc transporter Slc30A8 has recently been identified as a novel autoantigen in type 1A diabetes [20], and the use of an antigen construct spanning the C-terminal amino acids 268-369 resulted in an autoantibody assay useful for the prediction of type 1 diabetes. The number of detectable islet autoantibodies is unequivocally related to the risk of progression to overt type 1A diabetes both in family studies and also in surveys based on general population cohorts. In family studies positivity for three to four autoantibodies is associated with a 60–100% risk of developing clinical type 1A diabetes over the next 5–

10 years [21]. Studies in the general population indicate that the predictive value of multiple autoantibody positivity is close to that observed among first-degree relatives [22].

MOLECULAR GENETICS OF TYPE 1A DIABETES

The largest contribution to genetic susceptibility from a single locus comes from several genes of the major histocompatibility complex (MHC) located on chromosome 6p21.3 [23], accounting for at least 40% of the familial aggregation of this disease. Important residues for the structure of the peptide-binding groove of both HLA-DQ and DR molecules are associated with disease susceptibility and resistance [24]. The highest risk genotype for type 1A diabetes includes the HLA alleles DR3/4-DQ8 (DQ8 is DQA1*0301, DQB1*0302).

Polymorphisms of a variable nucleotide tandem repeat (VNTR) 5' of the proinsulin gene are associated with risk [25]. The long form of the VNTR is associated both with protection from diabetes and increased insulin messenger RNA expression within the thymus [26]. Thus diabetes risk is related to lower insulin expression within the thymus and is potentially related to the development of central T cell tolerance to insulin.

At a second level of importance, a single amino acid change in a gene termed PTPN22, a tyrosine phosphatase that influences T cell receptor signaling, also influences diabetes risk [27]. The same polymorphism increases the risk of a series of autoimmune disorders including rheumatoid arthritis and lupus erythematosus. Polymorphisms of another gene associated with T cell regulation, the CTLA-4 gene, have a minor influence on genetic risk [28, 29].

CELLULAR IMMUNITY IN TYPE 1A DIABETES

The following facts support the view that the destruction of beta cells is mediated by cellular immune responses: (i) T cells are present in insulitis, (ii) disease progression is delayed by immunosuppressive drugs directed specifically against T cells, and (iii) circulating autoreactive T cells can be detected in patients at clinical presentation of type 1A diabetes [30]. It has been postulated that the initial encounter of antigen-presenting cells (APC) and naïve self-reactive T cells takes place in the pancreatic lymph nodes. The activated T cells are capable of invading the islet, where they become reactivated by encountering cognate beta cell autoantigens and thereby initiate insulitis. The strongest genetic susceptibility is associated with MHC class II alleles, which supports the idea that autoimmune response is antigen-driven in type 1A diabetes.

Epitope recognition takes place during the generation of tolerance, during loss of tolerance as the disease process is initiated, and during epitope spreading as islet cell damage is perpetuated. Studies on human beta cell antigens have identified 155 epitopes for CD4 T cell recognition and 22 epitopes for CD8 T cell recognition [31].

There are limited data on the morphology of pancreatic islets in type 1A diabetes in humans. It has been shown that insulitis is characterized by mononuclear cell infiltration into the islets. The majority of the infiltrating cells are CD8 T cells, followed by macrophages, CD4 T cells and B lymphocytes, supporting the view that beta cell destruction is a cell-mediated disease [30]. Hyperexpression of MHC class I molecules has been observed on beta cells, whereas increased expression of MHC class II molecules has been detected on endothelial cells, and increased expression of intercellular adhesion molecule 1 has been detected in the vascular endothelium of the islet. It has been estimated that about 80% of the beta cells are already destroyed at the clinical presentation of type 1A diabetes.

Mathis *et al*. [32] have proposed two types of cellular mechanisms leading to beta cell death:

(a) The recognition-linked mechanism, in which cytotoxic T cells recognize autoantigens presented by MHC molecules on the surface of beta cells; this mechanism requires direct T cell/beta cell contact, and it would imply the recognition of MHC-I restricted antigens by CD8 T cells.

(b) The activation-linked mechanism, in which the T cells (either CD4 or CD8) recognize beta cell antigens presented by MHC molecules on APC in the proximity of islets; this mechanism is supported by the observation that MHC II molecules are not expressed on beta cells *in vivo*. This model results in the direct killing of bystander beta cells through cytokines and soluble death mediators produced by T cells and the activation of cytocidal functions of macrophages.

Final beta-cell death would occur *via* apoptosis in the first model, whereas soluble mediators and free radicals would destroy beta-cells in the latter model.

HUMORAL BETA CELL AUTOIMMUNITY

Type 1A diabetes is associated with a series of anti-islet autoantibodies and the autoantibodies can be present for years prior to the onset of hyperglycemia. Although type 1A diabetes is usually regarded as a T cell-mediated disease, there is evidence from studies in the NOD mouse model that antibodies and B lymphocytes contribute to pathogenesis. However, evidence is lacking in humans that transplacental passage of anti-islet antibodies increases disease risk.

More than three decades ago, screening for autoantibodies associated with immune mediated, type 1A diabetes was limited to measuring antibodies to cytoplasmic antigens of islet cells (ICAs). After a period of identification and sequencing of the major autoantigens recognized by the humoral autoimmune response (insulin, glutamic acid decarboxylase and tyrosine phosphatase IA-2) the recombinant technology allowed the quasi-quantitative assessment of the respective specific autoantibodies (IAA, GADA and IA-2A). In addition, the beta cell-specific zinc transporter isoform 8 (ZnT8) has recently emerged as another major autoantigenic target of type 1A diabetes.

ISLET CELL AUTOANTIBODIES (ICA)

Since the discovery of islet cell antibodies (ICA) in 1974 [33], subsequent work demonstrated the presence of these autoantibodies not only at diagnosis or in type 1A diabetes but also prior to the symptomatic onset of the disease [34].

ICA are polyclonal autoantibodies that react with all cells of the islet. ICA are detected by indirect immunofluorescence utilizing human pancreas as substrate. The difficulties of measuring ICA using the immunofluorescent method led to efforts to harmonize the assay across laboratories, including periodic workshops. While helpful [35], the use of ICA as predictive marker has intrinsic challenges, the most important being the use of human pancreas which introduces a large amount of substrate heterogeneity. Although the advantage of ICA as a sensitive marker is well known, some difficulties arising from observer variability are recognized. Autoantibodies against two enzymes are now considered to provide a significant proportion of the humoral anti-islet cell immunity (and in some instances, ICA reactivity), these being autoantibodies against glutamic acid decarboxylase (GAD) [36] and those against a protein tyrosine phosphatase termed either ICA512 [37] or IA–2 [38].

The identification and cloning of GAD and IA–2 opened the door to the use of recombinant proteins and thereby to a better standardization of these immunoassays. These advantages notwithstanding, assays using recombinant technology are not devoid of challenges. Workshops have validated the fluid phase assays as doing better than solid phase assays, implying that conformational epitopes are important in the detection of these autoantibodies.

INSULIN AUTOANTIBODIES (IAA)

It has been known since 1983 that a considerable fraction of patients with new-onset type 1A diabetes present anti-insulin antibodies as detected by a radioligand binding assay -RBA-[39]. Initially, two different assay formats were used to detect such antibodies: ELISA format with insulin immobilized on plates, and RBA (fluid phase radioassays). The two different formats gave very different results regarding the positive predictive value for the development of type 1A diabetes. Finally it was concluded that the two formats measure different antibodies, and that only the antibodies detected with the radioassay formats are associated with the risk for type 1A diabetes [40]. The IAAs associated with diabetes risk are all of extremely high affinity and, most important, of very low concentration (10^{-12} M), making their detection with plate-binding asays problematic [40].

Different studies demonstrated that the IAA prevalence in patients with new-onset of type 1A diabetes fluctuates between 30-70% [41-53]. On the other hand, the frequency of IAA in first degree relatives of patients with type 1A diabetes is 0.7-12% [41, 54-58]. In epidemiological surveys and multicenter studies an inverse correlation between IAA levels and age at onset of type 1A diabetes was observed. Levels greater than 2000 nU/ml are found almost exclusively in patients who progress to type 1A diabetes prior to age 5. Therefore, differences between studies in the reported prevalences may be due to the different age distribution of the populations studied [59]. In addition, differences between assays regarding sensitivity and design may also influence on IAA detection.

On the other hand, individuals treated with insulin (even recombinant human insulin of the best quality and purity) produce antibodies denominated insulin antibodies (IA) [60]. The development of IA following subcutaneous therapy with human recombinant insulin has been ascribed to the possible presence of denatured insulin molecules. Nevertheless, IA are likely induced by the subcutaneous administration of insulin, especially in a depot form. However, IAA are still useful for diagnostic purposes, especially if a sample is taken prior to insulin treatment.

GLUTAMIC ACID DECARBOXYLASE AUTOANTIBODIES (GADA)

In 1982, using an immunoprecipitation assay of metabolically labeled islet cell, Baekkeskov and coworkers found autoantibodies directed to a 64 kDa autoantigen of beta cells [61]. In 1990, this 64 kDa antigen was identified as GAD [36]. This discovery was possible because of the high incidence of type 1A diabetes in patients with Stiff-man syndrome (SMS), a rare neurological disorder associated to high titers of GADA. GAD is the enzyme responsible for GABA synthesis within the nervous system and islet cells. Two forms of GAD with molecular mass of 65 kDA and 67 kDa (termed GAD65 and GAD67, respectively) are known [62]. In humans GAD65 is mainly expressed in the nervous system and in pancreatic beta cells, while GAD67 is synthesized only in the nervous system. Thus, the antigen recognized by GADA in type 1A diabetes is mainly the GAD65 form [63-65]. Most sera from type 1A diabetes patients only target conformational epitopes of GAD65, and neither bind GAD65 fragments nor react with denatured GAD65 in immunoblotting [66, 67]. In contrast, SMS sera have antibodies that bind GAD65 fragments, react with denatured GAD65, and inhibit the enzymatic activity of GAD65 [68].

GADA are present for years before the development of type 1A diabetes [69]. Moreover, GADA do not correlate inversely with the age at onset of type 1A diabetes [70, 71]. GADA prevalence in new-onset type 1A diabetes patients is 70-80% [48, 50-53, 65, 72-74], and the frequency of GADA in first degree relatives of patients with type 1A diabetes is 6-17% [75-77].

AUTOANTIBODIES TO PROTEIN TYROSINE PHOSPHATASE IA-2 (IA-2A) AND TO IA-2BETA (IA-2 BETA A)

ICA512 was initially cloned by Rabin *et al*. [37] from an islet expression library screened with islet cell autoantibody–positive sera. A longer clone of ICA512, termed IA-2, was obtained independently by Lan *et al*. [78]. Wasmeier and Hutton [79] identified IA-2beta or phogrin as a novel insulin granule membrane protein from a rat insulinoma expression library screened with anti-insulin granule membrane sera. Sequences identical to human IA-2beta [80] have been independently cloned from human fetal brain, termed ICAAR [81], and human colon carcinoma cells, termed IAR [82]. ICA512 and IA-2beta are type 1 transmembrane glycoproteins localized in dense cored secretory granules of peptide-secreting endocrine cells and neurons [79-83]. They are both members of the protein tyrosine phosphatase (PTP) superfamily and exhibit 30% sequence identity overall and 80% identity within the PTP domain.

Sensitive radioassays for autoantibodies to these proteins [80-85] have been developed using *in vitro* transcribed and translated ICA512 and IA-2beta. The presence of IA-2beta A correlates with ICA512A, but the quantitative relationship is not strong (r = 0.82), suggesting that multiple epitopes within the two molecules are recognized to different extents by sera from different patients [86].

Autoantibodies reacting with ICA512 and IA-2beta share several overlapping epitopes. With *in vitro* translated ^{35}S-labeled ICA512 and IA-2beta, 98% (88 of 90) of patients with new-onset type 1A diabetes and prediabetic relatives who were positive for IA-2betaA had ICA512A.

Only the cytoplasmic domain of human IA-2beta was used in the assay of IA-2betaA because previous work showed that all sera positive for full-length IA-2betaA reacted with the cytoplasmic domain of IA-2beta [80]. This region shares 80% amino acid sequence identity to ICA512 and is the site for autoantibody binding in both molecules. Nevertheless, 10% of sera from patients with new-onset type 1 diabetes and prediabetic relatives reacted with ICA512 but not IA-2beta. Competition studies using affinity-purified recombinant ICA512 revealed that reactivity to IA-2beta was completely blocked by preincubation with recombinant ICA512 in all ICA512A–positive/ IA-2betaA –positive sera. In contrast, the binding to ICA512 in most doubly positive sera was only partially blocked by preincubation with excess recombinant IA-2beta. This suggests that autoantibodies in patients with type 1A diabetes may develop primarily to ICA512 rather than to IA-2beta, and that the antigens have a relationship similar to that between GAD65 and GAD67 in this respect [63].

AUTOANTIBODIES TO ZINC CATION EFFLUX TRANSPORTER ZNT8 (ZNT8A)

The islet beta-cell zinc cation efflux transporter ZnT8 (Scl30A8) is a major, newly defined autoantigen [20]. This transporter was discovered as an autoantigen because it is specifically expressed in islet beta cells, where it is associated with the regulated pathway of insulin secretion. ZnT8 facilitates the transport of Zn^{2+} from the cytoplasm into the insulin secretory granule and the concentration of Zn^{2+} within the granule lumen, where the zinc cation binds to insulin hexamers. Fluid phase radioassays for autoantibodies to this autoantigen have been validated in the most recent CDC affiliated DASP workshop, and have shown that approximately 60% of new-onset patients have autoantibodies against the zinc transporter.

When assay thresholds for insulin, GAD, IA-2 and ZnT8 autoantibodies are set at the 99th percentile of controls, approximately 98% of children with new-onset diabetes are found to express at least one of these autoantibodies [87].

AFFINITY AND ISOTYPES OF AUTOANTIBODIES

Family history of type 1A diabetes and autoantibodies to the islet antigens are strong predictors of type 1A diabetes, but the rate of progression to diabetes in relatives which are positive for multiple islet autoantibodies varies widely. Achenbach *et al*. demonstrated a strong association between risk and broad, high titer antibody responses to IA-2 and insulin. The highest risks were associated with high titers of IA-2A and IAA, the prevalence of IgG2, IgG3, and/or IgG4 subclasses of such autoantibodies, and the presence of IA-2betaA. It has been shown that the combination of data on autoantibody titer, subclass, and/or epitope reactivity may improve type 1A diabetes risk stratification, which could be accomplished by measuring these parameters on a single sample [88].

In another work, Achenbach *et al*. demonstrated that IAA affinity, measured by competitive radiobinding assay, ranged from less than 10^6 l/mol to more than 10^{11} l/mol. High affinity was associated with HLA DRB1 *04, young age of IAA appearance, and subsequent progression to positivity for multiple islet autoantibodies or to type 1A diabetes. IAA affinity in multiple antibody-positive children was on average 100-fold higher than in children who remained only IAA positive or became autoantibody negative [89].

METHODS FOR MARKER ASSESSMENT

Immunohistochemical Tests

Indirect immunofluorescence (IIF) is one of most frequently used tests to detect organ- or tissue-specific autoantibodies. The procedure is essentially the same for all the autoantibodies associated with the respective autoimmune diseases, except for the selection of the appropriate tissue that contains the antigen (or antigens) of interest. The first useful test to detect islet cell autoantibodies, the ICA assay, was an IIF with unfixed frozen sections of human pancreas (from individuals with blood group O) as substrate [33]. The procedure is performed with appropriate dilutions of the sera to be tested (unknown and positive and negative controls), or with sera titrated in serial twofold dilutions until the endpoint is reached. The diluted sera are incubated with cryostat-cut sections of tissue, and after a washing step the sections are incubated with a commercial rabbit or goat antiserum to human immunoglobulins, conjugated with fluorescein isothiocyanate (FITC conjugate). After a further washing step the coverslips are mounted with buffered glycerol for reading the test using a fluorescence microscope with epi-illumination. The FITC conjugate bound to islet cell antibodies stains the cytoplasm of islet cells of the tissue sections in brilliant yellow over a yellow-green background corresponding to exocrine acinar cells.

It was demonstrated that the major autoantibodies contributing to ICA reactivity are GADA, IA-2A, but not IAA. On the other hand GADA may be present in a significant proportion of ICA-positive relatives of type 1A diabetic or polyendocrine disease patients followed for more than 5 years without developing diabetes. Such ICA antibodies have been termed “restricted” or “selective” in that they fail to react with mouse islets and give a beta cell-specific pattern of staining. These ICA antibodies can be adsorbed with GAD, and are similar to the ICA antibodies found in patients with SMS [90-92].

Since the discovery of ICA it was rapidly demonstrated that the ability to detect ICA positive sera varied widely between laboratories. Hence, a standard for ICA was established utilizing dilutions of sera, and the units assigned

were termed JDF (Juvenile Diabetes Foundation) units [93]. The use of that standard allowed the comparison of assay sensitivity, and it became apparent that greater than 10-20 JDF units of ICA were highly predictive of diabetes among first-degree relatives. Subsequently other workshops have been held, and a Proficiency Testing Service has been established for laboratories measuring such autoantibodies [35].

During several years ICA served as the principal serological test for definition of active autoimmunity against the beta cells. However its disadvantages were apparent early on, including the need for fresh human pancreatic tissue and the difficulty for obtaining true quantitative results. Further, biochemically-defined beta cell antigens were described, leading to the development of alternative high sensitive and specific autoantibody assays to detect the specific humoral autoimmunity.

Fluid Phase Radioassays

The general assay format for measuring islet autoantibodies is a variation of the radioimmunoassay (RIA) for insulin assessment described by Berson and Yalow [94], adapted to determine insulin antibodies, and then termed the radioligand binding assay (RBA). This test was originally used by Kurtz *et al.* [95] to detect insulin antibodies produced by patients treated with insulin and later applied with some modifications by Palmer *et al.* [39] for the discovery of insulin autoantibodies in patients with recent-onset type 1 diabetes. The general procedure of RBA carried out by the above mentioned authors was essentially as follows: Duplicate serum aliquots were incubated for a week at 4 °C with a buffer containing ^{125}I-human insulin as a tracer (in the method described by Palmer *et al*. the tracer was homogeneous A14 mono ^{125}I-human insulin). The bound insulin fraction was then precipitated and separated using polyethyleneglycol 6000 (PEG). The use of relatively low final serum dilutions in the assay and of very low antigen concentrations to increase sensitivity are critical conditions of RBA. In fact, the A 14 mono ^{125}I-human insulin tracer with high specific activity (~380 μCi/μg, corresponding to 1 atom of ^{125}I per insulin molecule) added with an activity ~ 20,000 cpm, fulfils the theoretical condition of antigen added in infinitesimal concentration (lower than the inverse of K, the affinity equilibrium association constant of the involved antibodies) to obtain maximum signal levels.

Since these initial developments there were a number of modifications proposed and implemented for the determination of markers by using RBA. For example, some assays included parallel incubations with excess of cold antigen (i.e. insulin) subtracting unspecific binding to calculate the total antigen binding in the test [43]. For assessing samples with high levels of IA (sera from insulin-treated patients with insulin resistance associated with extremely high levels of insulin antibodies), the assays were performed in a format based on dose-response curves until complete displacement was achieved, and data were processed to obtain the binding capacity in absolute units (molar antibody concentration or the equivalent amount expressed in units of insulin/L serum) [60].

Several alternative RBA variants included minor changes in the labeling procedure (protocols differing in the ^{125}I: oxidant: protein ratio), in the bound to free separation step (protein A and/or protein G coupled to sepharose instead of PEG) or in the general format of the test (samples placed in 96-well filtration plates instead of conventional tubes, and measured in counters able to handle the plates).

A major advance in radioassays was the production of endogenously labeled proteins, as ^{35}S-methionine-GAD, by *in vitro* transcription and translation of cDNAs to produce appropriate tracers [64, 65]. The conventional radioassay for autoantibodies to IA-2 or its fragments also use ^{35}S-methionine based tracers [96]. Assays for ZnT8A are also performed using ^{35}S-methionine labeled protein, although the presence of a SLC30A8 polymorphism (Arg325Trp) located at the most distal loop in the ZnT8 protein has moved researchers to measure such autoantibodies by RBA using ZnT8 carboxy-terminal constructs (amino acids 268-369) carrying 325Trp(CW) and 325Arg(CR) and also a hybrid construct (CW-CR) [97].

Synthesis of ^{35}S based tracers exhibited some advantages as compared to tracers labelled with ^{125}I. Since iodine is a bulky substituent of tyrosine (or histidine) residues and is introduced by chemical modification, it has the potential to alter epitopes and to produce oxidative damage to the labeled protein. In this regard, an antigen carrying no extra atoms could be expected to improve the interaction with the respective antibodies and hence to improve the assay. In other cases, however, this labeling procedure is not feasible. For example, since insulin and proinsulin have not methionine residues, ^{35}S-cysteine was chosen by our laboratory to carry out the *in vitro* tracer synthesis for an IAA/PAA radioassay [98].

Recombinant Chimeric and Truncated Autoantigens in GADA Assays

As an alternative to the conventional assays, chimerical autoantigens were also used in designing marker assays specific for type 1 diabetes. Daw and Powers pioneered the use of GAD65/GAD67 chimerical proteins [99]. Since it has been shown that only a minority of GADA recognizes the GAD67 isoform, these authors used such chimeras to demonstrate that the GADA profile in diabetes and the SMS sera was quite complex and includes antibodies that recognized continuous and discontinuous epitopes between three majors regions. Subsequently Falorni *et al.* [100] further narrowed the main epitope regions of GAD65 using new GAD65/67 chimeras applied to the study of GADA profile in type 1 diabetes.

Some years later our laboratory used different engineered variants of human GAD including full-length GAD 65, GAD65/67 hybrids displaying different GAD65 residues (1-95, GAD-N; 96-444, GAD-M; 445-585, GAD-C), a truncated GAD65 spanning residues 69-585, and full-length GAD 67 (Fig. **1**) for autoantibody epitope recognition in patients with type 1 diabetes, adult-onset diabetes mellitus (AODM, usually designed as LADA) or autoimmune thyroid disease (AITD) [101]. Most patients in that study recognized both middle and C-terminal epitopes and had low reactivity against N-terminal epitopes, and a few patients displayed

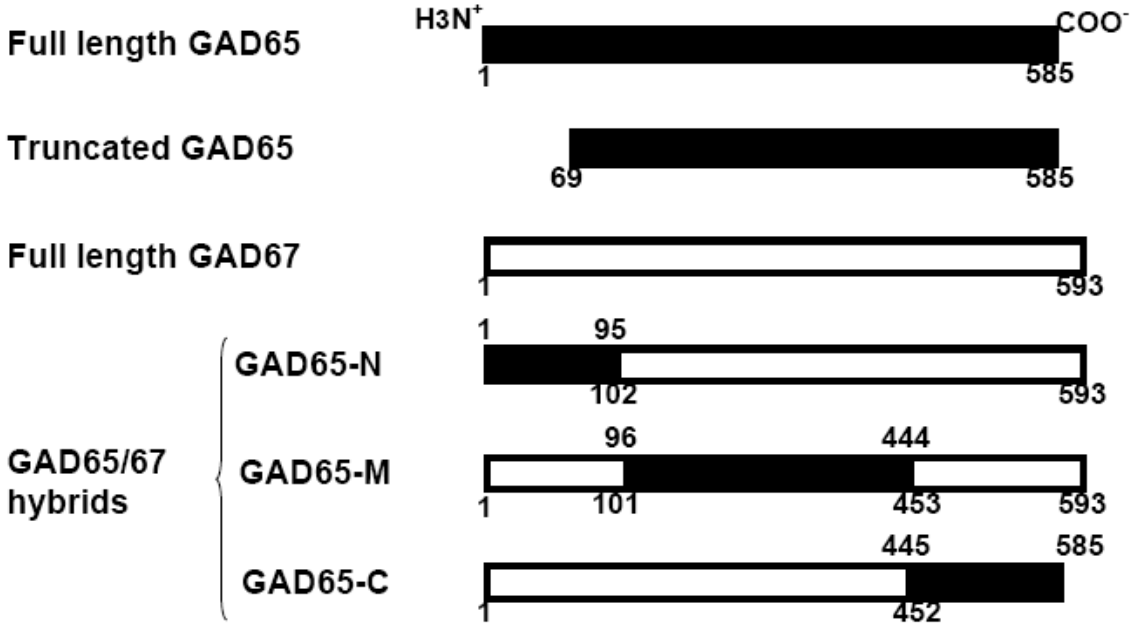

Fig. (1). GAD variants used for GADA assessment as indicated in the work by Primo *et al*. [101]. For details see the text.

reactivity limited to the N or C terminus. Compared with type 1 and AODM, AITD patients showed a greater prevalence of multiple reactivity and higher incidence of GAD67 positivity.

Full-Length *vs* Selected Fragments in IA-2A Assays

To date IA-2A are detected using sensitive and specific RBAs, differing in terms of the radiolabeled IA-2 construct used, usually chosen from one of the following proteins (Fig. **2**): the full-length IA-2 (residues 1-979), the truncated N-terminally spliced IA-2 variant lacking exon 13, IA-2 bdc (bdc for Barbara Davis Center) (256-556:630-979), and the intracytoplasmic IA-2ic (604/605-979) construct [37, 102-104]. Cytoplasmic IA-2ic (605-979) is the construct which detects IA-2A with the highest sensitivity in both newly diagnosed type 1 diabetic patients and pre-diabetic individuals, thus suggesting that such construct should be used in type 1 diabetes–related autoantibody screening studies [105]. On the other hand it has been suggested that IA-2A detection by using IA-2 middle domains (256-760) is the best choice in LADA patients [106].

We recently solved the x-ray structure of the mature ectodomain of IA-2 (meIA-2) to 130 Å resolution and demonstrated that the folding of meIA-2 is related to the SEA (sea urchin sperm protein, enterokinase, agrin) domains of mucins. This suggests a role of meIA-2 in adhesive contacts to the extracellular matrix and provides clues on how this kind of molecule may associate and form homo- and heterodimers [107]. Moreover, we discovered in the same work that meIA-2 is self-cleaved *in vitro* by reactive oxygen species, suggesting the possibility of a new shedding mechanism that might be significant in normal or pathological processes. We speculated that these mechanisms may include the exposition of previously cryptic epitopes in the mature ectodomain of IA-2. Preliminary unpublished results from our laboratory have shown that, when ^{35}S-methionine meIA-2 (449-576) is used as a tracer, approximately 10% of LADA patients sera but none from type 1 diabetic patients yield a positive result.

Combined Assays

A useful procedure for marker screening would involve combined or single tube assay for GADA, IA-2A, and IAA. Although such combined assay cannot discriminate which particular marker is being disclosed, it may readily identify individuals at risk and serve as a guide for further detailed analysis on a more restricted cohort of samples. This approach, most likely time and money saving, was evaluated, at least for GADA and IA-2A, in several laboratories [84, 108, 109]. The convenience of combining the measurement of GADA, IA-2A and IAA -or GADA and PAA [110] in diabetic patients was assessed in our laboratory several years ago. We concluded that a combined double-antigen test for GADA and IA-2A is a useful strategy for prospective screening of type 1A diabetes. However, in adults, the profile of individual markers discloses the course to insulin dependence. Therefore, it seems advisable to measure the markers separately, to allow a better classification of these patients and help define their best treatment [53].

Solid Phase Assays

The "classic" Enzyme-Linked Immunosorbent Assay (ELISA) for autoantibody assessment is a solid phase procedure essentially performed as follows: polystyrene microplates are coated with a solution containing the specific autoantigen. After several washing steps and treatment with an unrelated protein to saturate the remaining polystyrene reactive sites, duplicates of the appropriate serum sample dilution are transferred onto the coated plates. To measure non-specific signals, the same duplicate serum sample dilution is preincubated with an excess of the autoantigen and thereafter similarly processed. After several washes an

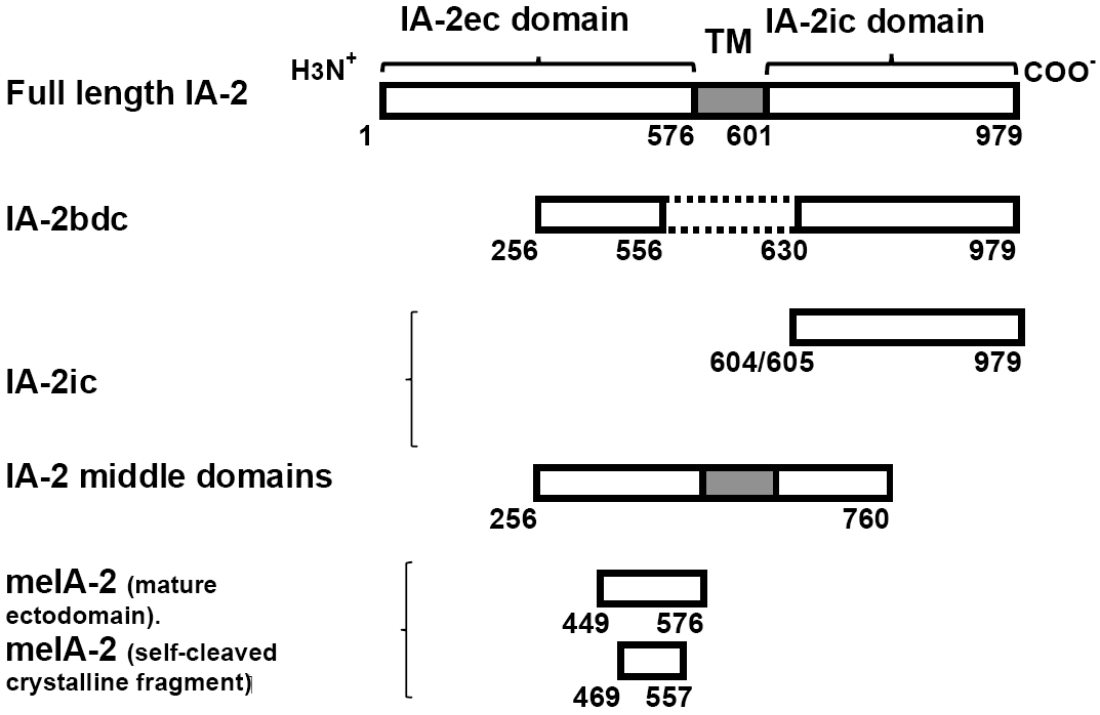

Fig. (2). Schematic representation of IA-2 constructs used for IA-2A determinations. TM, transmembrane fragment. Dashed lines in IA-2bdc fragment represent residues missing in the construct (corresponding to a splicing product lacking exon 13). For details see the text.

anti-immunoglobulin serum conjugated with an enzyme (peroxidase) is added. After reaction and washing steps, the substrate (o-phenylenediamine) is added and about 15 min later the reaction is stopped by adding a solution of H_2SO_4. Oxidized chromogen is measured using a microplate reader. Blank values are obtained from wells which received all reagents except for human serum. Results are expressed as specific optical density (OD) minus the OD from the wells preincubated with antigen excess. Variations of the procedure include the use of a mouse monoclonal anti-human IgG as first antibody followed by a rabbit anti-mouse immunoglobulin serum conjugated with the enzyme as second antibody (amplified ELISA) [111].

Other ELISA formats have been also used for marker measurement. For example, GADA assessment was carried out in a 2-step procedure in which serum GADA were first allowed to react with a fixed dose of GAD65-biotin in solution and the residual free antigen was later assayed by a conventional ELISA [112].

Native recombinant GAD65 for immunochemical tests can be obtained from eukaryotic cell cultures but initial attempts to produce native GAD65 with high yield in bacteria, as it is required for ELISA were unsuccessful [66]. In 1997 we described the production of recombinant human GAD65 from *E. coli* as a soluble and properly-folded fusion protein with thioredoxin (Trx-GAD). This chimera retains full enzymatic and immunochemical reactivity [113] and can be easily produced and purified. Having a handy source of native GAD65 allowed us to design different ELISA formats for GADA detection and to compare them with RBA. In such assays either Trx-GAD65 or biotinylated Trx-GAD65 were used as antigens. Since direct adsorption of the antigen to plastic, as in the conventional ELISA, may cause disruption of the native structure of GAD [114], we developed different ELISA formats, including a capture ELISA, with the antigen indirectly immobilized by a non-adsorptive process, and an ELISA with antigen–antibody preincubation (p-ELISA), where GADA were allowed to react with the free antigen in solution, and the residual free antigen was assayed by conventional ELISA [115].

GADA has been also measured in an ELISA that depends on the ability of divalent autoantibodies to form a bridge between immobilized GAD65 and biotinylated GAD65 present in the fluid phase [116]. Our laboratory developed a similar method based on the use of immobilized Trx-GAD65 and soluble biotinylated Trx-GAD65 [117].

Solid- *vs* Fluid-Phase Assays

Multiple studies and workshops utilizing sera from patients with type 1 diabetes have demonstrated that the above mentioned standard ELISA formats lack both sensitivity and specificity compared to the fluid-phase RBA. Hence, in the past, solid-phase assays have proved less useful than fluid-phase assays in identifying diabetes-associated antibodies [72, 103]. The lack of correlation between RBA and ELISA in marker assessment may be explained in several ways. On the one hand, both techniques detect antibodies under different thermodynamic conditions [118, 119]. In RBA the antigen-antibody interaction follows the mass action law and the concentration of ligand is extremely low. Thus, this assay is greatly dependent on antibody affinity and preferentially detects small amounts of high affinity antibodies. In contrast, in ELISA, the excess of antigen attached to the plastic matrix promotes "monogamous" antibody binding which can make the apparent affinity reach effective irreversibility, thereby minimizing the role of antibody affinity on signal

development. Moreover, another fact that increases the effective affinity in ELISA is the enormously high effective local concentration of binding sites at the surface [120]. Therefore, ELISA detects antibodies over a wide range of affinities, behaving as a capacity assay and proving sensitive for low affinity antibodies. On the other hand, it has been speculated that differences in detection frequency between RBA and ELISA may be due to the orientation of antigen molecules on the solid phase (particularly in the case of insulin) which results in the masking of certain epitopes [118]. Although in most cases the immobilization has not involved a specific orientation of the antigen onto polymeric surfaces, different approaches were used to selectively attach a protein. Recently we used arsenical grafted membranes for immobilization of Trx-GAD65 [121]. This study demonstrated that the fusion protein immobilized onto the modified material maintains the enzymatic activity (determined measuring the formation of $^{14}CO_2$ from L-[U-^{14}C] glutamic acid) for a longer time as compared to the free protein under the same storage conditions. Thus the modified polymer has shown a satisfactory performance for its future development as a solid support for marker detection kits.

Other hypotheses to explain the low correlation between results from fluid and solid phase methods propose that the site of iodination may alter the RBA behavior of the tracer molecule. However our laboratory demonstrated that in most cases the variability in RBA-ELISA signal ratios are best explained in terms of differences in the basic principles operating in both methods instead of artifacts due to tracer preparation [111].

Nowadays new ELISA formats achieve high levels of sensitivity and specificity both in the hands of the manufacturer and in a number of laboratories using such kits or in-house procedures. It should be noted, however, that the performances of different ELISAs fluctuate widely and that the improvement is related to kit design. It is therefore important that potential users seek details of the performance of any kit in the context of a blinded sample exchange under control programs.

Other Methods for Marker Assessment

Some questions about the structure and folding of the autoantigens in fluid phase assays resembling the "true" *in vivo* molecule involved in specific antibody induction have not been completely resolved. For example, since GAD65 undergoes post-translational processing and targeting to subcellular compartments and membranes, it may exhibit different immunochemical properties in the cell context compared with the soluble protein expressed in the cell-free eukaryotic system used in the RBA for GADA assessment. We recently detected and characterized GADA from different sera using analytical systems in which GAD was expressed in a cellular context compared with methods based on immunoprecipitation of metabolically labeled GAD65 [122]. In that work, we detected and characterized GADA in 72 sera from patients with type 1 diabetes mellitus and 14 sera from adult-onset diabetes patients (LADA) using analytical systems in which GAD65 was expressed in a cellular context: confocal indirect immunofluorescence (IIF) and electron microscopy after immunogold labeling on monolayers of transfected Chinese hamster ovary (CHO) cells, and immunoprecipitation (IP) of metabolically-labeled GAD65. Eighteen serum samples, 16 from type 1 diabetes patients and two from adult-onset diabetes patients, were positive by confocal IIF but scored negative by RBA. All of these 18 sera immunoprecipitated a 65 kDa protein, supporting the existence of the GADA marker in such patients (Fig. **3**). It was concluded that GADA negativity by the conventional RBA method using the soluble antigen, as well as negativity for other common markers measured by similar methods, is not enough to rule out the existence of the specific autoimmune component in childhood or adult-onset diabetes. Other analytical methods based on a more physiological presentation of the autoantigen structure, as confocal IIF and IP, bring an extra support to assess the complete repertoire of specific autoantibodies to native-like and membrane-bound, or denatured, beta-cell antigens.

Standardization Programs to Improve Measurement of Markers

The Diabetes Autoantibody Standardization Program (DASP), a collaboration of the Immunology of Diabetes Society (IDS) and the US Centers for Disease Control and Prevention, was established on the basis of a series of IDS workshops to improve and standardize the measurement of the markers predictive of type 1 diabetes [93, 102, 123, 124].

The aims of the first DASP evaluation were to assess and improve the comparability of islet autoantibody assessment among laboratories and to undertake extended evaluation of the new WHO international reference reagent for the principal markers, that is autoantibodies to GAD and IA-2 [103].

To achieve those aims, the format for subsequent workshops was established, based on the circulation of aliquots of coded sera from relatively large numbers of unselected patients with new-onset diagnosed type 1 diabetes and healthy controls, and differing from other autoantibody programs that generally rely on pre-selection of autoantibody positive and negative sera.

The results on GADA and IA-2A determinations from the workshops in 2002-2005 compared with those of DASP 2000 were reported in 2008 [125].

The study design of the four workshops was as follows: Participating laboratories received uniquely coded sets of sera from 50 patients with newly diagnosed type 1 diabetes and 100 healthy control individuals. Coded sera in 100 µL aliquots were distributed to 46-50 laboratories in about 18 countries. Sera from patients were obtained within 13 days of diabetes diagnosis (median 2 days). The median patient age was 18 years (range 9–35 years). Control sera were obtained from 100 US blood donors (median age 20 years; range 18–28 years). The same 100 control sera were circulated in the 2002, 2003 and 2005 workshops. Laboratories were asked to test the sera with any assays currently in use for detection of autoantibodies.

Data analyses were processed as follows: Laboratory-defined sensitivity for each assay was calculated as the percentage of sera from patients reported as positive using the laboratory's own cutoff. Laboratory-assigned specificity was calculated as the percentage of healthy control sera

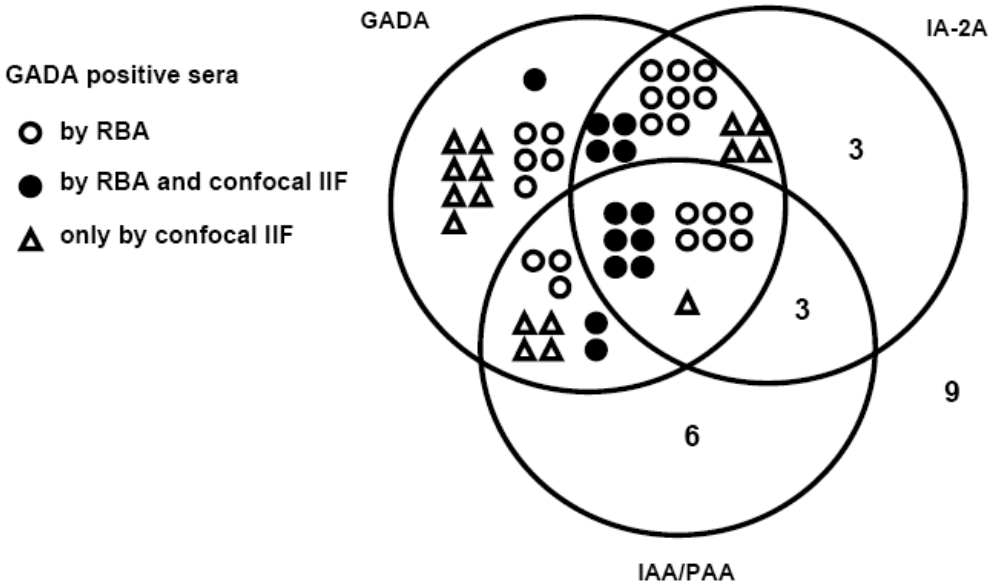

Fig. (3). Venn diagram indicating the profile of autoimmunity markers for 72 selected type 1 diabetic patients. Circles include RBA results for each marker. In addition, GADA positive results obtained by both RBA plus confocal indirect immunofluorescence (IIF) or only by confocal IIF are depicted by symbols as indicated in the insert. From the experimental evidence here shown it may be concluded that GADA negativity as assessed by the conventional RBA method using the soluble recombinant antigen does not allow to rule out the existence of the specific autoimmune component in diabetic patients, even in those negative for other common markers. The analytical methods based on a more physiological presentation of autoantigens structure, as confocal IIF, bring an extra support to assess the complete repertoire of specific autoantibodies to native-like and membrane-bound, or denaturated, beta-cell antigens. Confocal IIF allowed to detect GADA in 7 individuals who were RBA negative for GADA and/or IA-2A/IAA, which may be in fact type 1A diabetes patients and, therefore, would not be misclassified as idiopathic type 1B diabetes. Reproduced from the work of Villalba *et al*. [122].

reported as negative using the same cut-off. Receiver operator characteristic (ROC) curves were used to evaluate the performance of each assay in discriminating disease from non-disease.

The main conclusions of the workshops were as follows: Many laboratories participated using commercial assay kits, both RIA and ELISA, and some of these achieved levels of sensitivity and specificity equivalent to in-house RBA. Both GADA and IA-2A assays showed remarkable concordance among laboratories in categorization of samples and quantification of antibody levels in spite of the use of different principles and assay formats. GADA assays generally maintained high levels of sensitivity and good discrimination between disease and health, while the performance of IA-2A assays progressively improved over the three workshops. In the these workshops there has been a particular improvement in the performance of GADA by using ELISA, and in DASP 2005 a commercial ELISA performed very well in a number of laboratories. The disadvantages of current ELISA kits are that they are generally more costly than in house RIA, and they require larger serum volumes than RIA based on ^{35}S-labelled antigens.

A small number of combined assays for GADA and IA-2A were included in DASP 2005 and, as expected, some achieved high sensitivity, confirming the value of this approach as first-line screening.

Although used in fewer laboratories, the ELISA for IA-2A also improved and achieved results equivalent to those of in-house RBA. Comparison of the median levels obtained in assays using IA-2ic and IA-2bdc clearly demonstrates that there are a small number of patient sera that are identified as strongly IA-2A positive in the former but are negative or weakly positive in the latter, according to the concept that IA-2bdc lacks at least one disease-related epitope. The proportion of laboratories using the IA-2bdc clone has progressively decreased. In this respect it was suggested that this may, at least in part, explain the improvement in IA-2A sensitivity, and imply that laboratories using the IA-2bdc clone should consider switching to IA-2ic.

Finally, the new ways to discriminate disease-relevant antibodies, for example, including the assessment of autoantibody affinity, were considered very important [126].

The Expression Units in Marker Determinations and the Use of Standards in Antibody Quantification

Results for the healthy subjects were routinely used to calculate their signal control mean and standard deviation [44], both in fluid and solid phase-based methods. Based upon such values, and to avoid bias and allow individual assays to be compared, data from each serum sample in workshop studies were expressed in standard deviation scores (SD scores, or precision units). This data expression is based on the displaced signals from normal sera, as a measure of the certainty with which a test serum can be deemed different from normal, hence SD score = (sB% or sOD of test serum)-(mean sB% or sOD of control sera) /SD of control sera [127]. Results expressed in these units showed a lack of RBA-ELISA agreement, as mentioned previously [111]. These considerations may be applied also

to insulin antibodies induced during insulin therapy (IA) or associated to most biopharmaceutical therapeutics that elicit some level of antibody response against the product. It was stated that in such circumstances appropriate detection, quantitation and characterization of antibody responses are necessary despite the lack of the respective standards. In this sense B% and OD in RBA or ELISA, respectively, express results in terms of the signal, in continuous units, from procedures classified as quasi-quantitative assays [128]. The methods used for marker assessment fall under the class of quasi-quantitative assays due to the lack of similarity between the standard samples and test samples. In other words, the reference standards may not accurately reflect the antibody affinities, proportions and other conditions in the test sample. Moreover, the analytes in test samples are not similar amongst themselves. Since the lack of similarity implies non-parallelism in dose-response curves, the analytical results determined from dose interpolation from the standard curve during calibration, in the absence of parallelism will represent an inexact approximation. Despite this, in DASP workshops GADA and IA-2A results were expressed in WHO units/ml derived from previously calibrated in-house standards. Hence, once local assays had been calibrated, laboratories were able to report all their results in WHO units/ml, allowing closer comparability between results obtained by different laboratories.

The WHO international reference reagent for GADA and IA-2A was defined as containing 100 WHO units/vial (250 WHO units/ml) [124]. This standard had been used in DASP 2000 and allows laboratories to express GADA and IA-2A levels in common units. Participating laboratories were asked to calibrate their local standards in DASP 2002, assigning values in WHO units/ml on the basis of the median of five assays. These calculations were checked centrally and laboratories were not asked to re-calibrate for subsequent workshops if the format of their assay was unchanged. Reference reagent was provided to laboratories participating in the program for the first time or if there had been substantial changes in the assay between workshops. Laboratories used between one and 11 standards to calculate their results. The instructions provided for the calculation of WHO units/ml were as follows: [1] single standard: units = [(cpm test sample − cpm negative control)/(cpm local WHO calibrated standard − cpm negative control)] × WHO units of local standard; and (2) multiple standards: a regression curve was constructed for cpm (or optical density) *vs* the assigned WHO units/ml for each of the standards. This regression curve was used to convert cpm (or optical density) of the proficiency samples into WHO units/ml.

APPLICATIONS

Despite uncertainty about the pathogenic importance of serum markers, the ability to biochemically detect autoantibodies to a series of autoantigens present in beta cells greatly facilitated the prediction of type 1A diabetes. At present utilizing four RBAs or ELISA for GADA, IA-2A, IAA, and ZnT8A, >95% of prediabetic or recent-onset diabetic children can be detected (expressing at least one marker, *vs* only 4% of controls). More than 80% of patients present two or more markers (*vs* none in controls), and approximately 25% of diabetic or prediabetic individuals express all of the above autoantibodies.

On the other hand, some adult patients with phenotypic type 2 diabetes also elicit antibodies directed against beta cells antigens. These patients, who slowly progress to insulin deficiency, are considered to have slow-onset or latent autoimmune diabetes (LADA), and typically are GADA positive. Although this proposed nomenclature may not be considered completely satisfactory because it is based on clinical phenotype, the assessment of markers together with other biochemical parameters and genetic typing may have important implications to our understanding of the disease process, and consequently to selection of the best therapy. The clinical nature and management of autoimmune diabetes in adults poses important questions regarding conventional treatment for hyperglycemia as well as regarding therapy aimed at protecting residual beta cell function.

It may be concluded that autoantibody measurement in autoimmune diabetes together with other biochemical and functional tests have an important role in the clinical classification of diabetes, in the prediction of the need for insulin treatment, in the identification of individuals at risk of developing type 1 diabetes, in their use as endpoints in observational studies and as one of the recruitment criteria in prediction and prevention trials.

Nowadays assessment of markers is performed in many laboratories throughout the world. Determinations of the four principal markers are no longer mere research tools, but are beginning to be used in clinical practice. These tests are increasingly run in clinical laboratories as well as in research centers, where they were first established, or in laboratories with experience in protein expression by recombinant technology and immunochemical developments. Workshops like DASP highlight systematic differences related to assay formats and allow full evaluation of novel procedures in laboratories with different levels of experience. Quality controls and comparative studies have important implications for the routine use of these tests in clinical laboratories, allowing high throughput testing using a less technically challenging format and avoiding the use of radioactivity. It should be noted that the performance of different ELISA kits included in the 2002-2005 workshops varied widely, and improvements were related to changes in kit design. It is therefore important that potential users seek details on the performance of any kits in the context of workshops such as DASP. This will facilitate a more widespread measurement of markers by allowing their determination in non-reference laboratories. This must be complemented with large studies comparing results from different laboratories, with further harmonization towards the use of ordinary antigens, standards and protocols.

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