Autoantibodies in Diabetes

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Islet cell autoantibodies are strongly associated with the development of type 1 diabetes. The appearance of autoantibodies to one or several of the autoantigens—GAD65, IA-2, or insulin—signals an autoimmune pathogenesis of β-cell killing. A β-cell attack may be best reflected by the emergence of autoantibodies dependent on the genotype risk factors, isotype, and subtype of the autoantibodies as well as their epitope specificity. It is speculated that progression to β-cell loss and clinical onset of type 1 diabetes is reflected in a developing pattern of epitope-specific autoantibodies. Although the appearance of autoantibodies does not follow a distinct pattern, the presence of multiple autoantibodies has the highest positive predictive value for type 1 diabetes. In the absence of reliable T-cell tests, dissection of autoantibody responses in subjects of genetic risk should prove useful in identifying triggers of islet autoimmunity by examining seroconversion and maturation of the autoantibody response that may mark time to onset of type 1 diabetes. The complexity of the disease process is exemplified by multiple clinical phenotypes, including autoimmune diabetes masquerading as type 2 diabetes in youth and adults. Autoantibodies may also provide prognostic information in clinically heterogeneous patient populations when examined longitudinally.
***Diabetes* 54 (Suppl. 2):S52–S61, 2005**

ISLET CELL AUTOANTIBODIES

Autoantibodies are created by the immune system when it fails to distinguish between "self" and "nonself." It is normally trained to recognize and ignore the body's own cells and to not overreact to nonthreatening substances in the environment. At the same time, the immune system must be able to create antibodies that target and fight specific foreign substances that do pose a threat. The bad news is, when this highly regulated and efficient system is turned onto self-antigens, target tissue damage ensues. Autoantibodies that bind to specific proteins found in the pancreatic islet cells were first described >30 years ago (1). The initial studies of islet cell antibodies (ICAs) were based on descriptive morphological tests aiming to localize the site of antibody binding. The indirect immunofluorescence test proved to be cumbersome and difficult to standardize. Because autoantibodies recognize unique autoantigens, it was reasoned that standard immunoprecipitation tests followed by a biochemical analysis of the purified immune complexes should make it possible to identify islet autoantigens recognized by immunoglobulin in sera from newly diagnosed diabetic children. Early studies showed the presence of a 64K protein, later shown to have GAD activity and found to represent a hitherto unknown isoform, GAD65. Subsequently, it was demonstrated that many new-onset type 1 diabetic patients had insulin autoantibodies (IAAs), and further analysis of islet autoantigens resulted in the discovery of the insulinoma-antigen 2 (IA-2), which was co-precipitated with GAD65 in many $64K^+$ patient sera. All three autoantigens are available as recombinant proteins that can be radioactively labeled either by in vitro transcription and translation or by iodination. In this way, it has been possible to develop reproducible and precise autoantibody assays to detect what are sometimes referred to as "biochemical antibodies," referred to henceforth in this article as "diabetes autoantibodies" (DAAs). These DAAs have been or are in the process of being standardized worldwide through serum exchange workshop exercises and proficiency testing, using a World Health Organization standard as reference (rev. in 2).

The ability to measure autoantibodies in type 1 diabetes using recombinant autoantigens has paved the way for the identification of several different autoantigens detected by autoantibodies in a large number of other autoimmune disorders. The utility of these autoantigen-specific—although yet to be standardized—autoantibody assays has led to the notion that many autoimmune diseases can be detected before the clinical diagnosis. The finding that ICA reactivity does not always correlate to reactivity toward defined autoantigens suggests that additional specific autoantigens remain to be identified (3). However, the current tests for autoantibodies to these three autoantigens are highly predictive of type 1 diabetes (rev. in 4). In the present article, we will discuss the mechanisms by which the autoantibodies to GAD65 (GAD65Ab), IA-2 (IA-2Ab), and insulin (IAA) appear, pathways of formation, shift between isotypes and subtypes, epitope recognition, and detection, as well as the potential usefulness of epitope-specific autoantibody tests to improve prediction and classification of autoimmune diabetes.

The human immune response to foreign antigens is often studied after vaccination or monitored using the bacteriophage oX174 (5). The latter antigen is potent and causes no recognized toxic effects in humans. It is used to monitor antigen clearance as well as primary and secondary antibody responses, including the sequence of antibody class. In parallel with antigen clearance, IgM antibodies are produced and within a couple of days, followed by low-level IgG. A second injection of oX174 is followed by a marked IgG response and only a limited IgM response. The series of events associated with the formation of autoantibodies to islet autoantigens is not known. It is assumed that there is shedding of the autoantigen within the pancreatic islet, that the autoantigen is taken up

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Received for publication 4 March 2005 and accepted in revised form 4 May 2005.

This article is based on a presentation at a symposium. The symposium and the publication of this article were made possible by an unrestricted educational grant from Servier.

DAA, diabetes autoantibody; DAISY, Diabetes Autoimmunity Study in the Young; DASP, Diabetes Autoantibody Standardization Program; DPT-1, Diabetes Prevention Trial; GAD65Ab, GAD65 autoantibody; IA-2, insulinoma-antigen 2; IA-2Ab, IA-2 autoantibody; IAA, insulin autoantibody; ICA, islet cell antibody; LADA, latent autoimmune diabetes in the adult.

by resident or circulating macrophages or dendritic cells, and that these cells then migrate to lymph nodes draining the pancreas where the antigen presentation takes place (6). This series of events is poorly documented in spontaneously diabetic laboratory rodents, such as the NOD mouse or the BB rat, because neither animal has an autoantibody response of the character and magnitude that is observed in human type 1 diabetes. In humans, limited information about GAD65Ab isotypes at onset and in the prodromal period is available (7,8). The common assay used for the detection of autoantibodies is based on immunoprecipitation using Protein A Sepharose. While Protein A binds efficiently to the majority of human IgG, it binds only poorly to IgA, IgM, and the IgG3 subtype. These isotypes or subtypes are therefore underrepresented in the current detection method. Our present understanding is limited to the early detection of islet autoantibodies to insulin, GAD65, and IA-2 in children with genetic susceptibility to type 1 diabetes (9–11). However, in none of these studies has the blood sampling occurred frequently enough such that the IgM to IgG isotype shift has been documented. Further studies are needed to carefully monitor the IgM, IgA, and IgG responses to autoantigen presentation in humans, and it will also be important to monitor circulating autoantigens to relate autoantigen clearance to antibody formation.

AUTOANTIBODY EFFECTS ON ANTIGEN PRESENTATION

One potential important role played by autoantibodies in the type 1 diabetes disease process is their effect on autoantigen processing and presentation by class II major histocompatibility complexes. Antigen-presenting cells can capture antigen for presentation to the immune system via both specific and nonspecific mechanisms. Antigen-specific B-cell receptors and Fc receptors on monocytes, macrophages, and dendritic cells increase the efficiency of antigen capture by the antigen-presenting cells and thus lower the threshold for a T-cell response (rev. in 12). Several sets of experiments have demonstrated that, in the presence of autoantibodies, the T-cell response to autoantigen is either enhanced or shifted in its focus (rev. in 2). This has led to the hypothesis that the process of antibody-mediated antigen internalization alters postendocytic transport and processing events, resulting in the presentation of different T-cell epitopes and potentially unmasking "cryptic" self-determinants, thus manipulating the T-cell response. Thus, in the case of type 1 diabetes, modulation of GAD65 presentation to the T-cells by disease-associated GAD65Abs may be a possible mechanism for the breakdown of immunological tolerance to the pancreatic β-cells. In this scenario, a particular GAD65Ab specificity present exclusively in pre-diabetic patients (not in GAD65Ab$^+$ nondiabetic patients with other autoimmune diseases or in GAD65Ab$^+$ nondiabetic control subjects) may therefore contribute to the initiation and/or perpetuation of the autoimmune process by altering the spectrum of T-cell determinants expressed by antigen-presenting cells and thus altering the focus of the T-cell response. Thus, depending on the presence or absence of autoantibodies, the presentation of an antigen or antigen-antibody complex may affect the generation of a pathogenic T-cell response and determine the consequences of the autoimmune process.

STANDARDIZATION OF DAAs TO IMPROVE UTILITY IN CLINICAL STUDIES

In addition to the important information DAAs provide to further our understanding of the autoimmune disease process in type 1 diabetes, DAAs have great value in studies of disease prediction. The usefulness of a test for prediction or diagnosis is evaluated by its sensitivity (number of subjects with +DAAs who develop type 1 diabetes/number of subjects who develop diabetes), specificity (number of subjects with −DAA who do not develop type 1 diabetes/number of subjects who do not develop diabetes), and positive predictive value. Because of the relatively long prodrome between initial antibody detection and clinical symptoms in type 1 diabetes, as well as their high positive predictive value, DAAs are a useful marker for prediction of disease. ICAs, and more recently GAD65Abs, IA-2Abs, and IAAs, have been studied extensively in both the general population and in populations at increased risk, namely first-degree relatives of probands with type 1 diabetes. In fact, diabetes has been the disorder in which the largest number of studies have been conducted in the predictive value of antibodies for autoimmune diseases (4). Several international workshops (Immunology and Diabetes Workshop, Immunology of Diabetes Society Workshop, and the first and second Diabetes Autoantibody Standardization Program [DASP]) have been conducted to develop thresholds for sensitivity and specificity and standardization of reference reagents, controls, and units of DAA tests (rev. in 2).

The performance of the ICA assay in different laboratories when evaluated in the Immunology of Diabetes Society Workshop combined islet autoantibody workshop and demonstrated a median sensitivity of 81% (44–100%), and the median specificity was 96% (64–100%). However, there was poor reproducibility with borderline positive sera. In the DASP 2000 workshop, both GAD65Ab and IA-2Ab radioimmunoassays were found to have high sensitivity (80 and 58%) and specificities (90 and 100%, respectively). The enzyme-linked immunosorbent assay performed less well. In DASP 2000, the IAA assay ranged in sensitivity from 4 to 42% for all 23 laboratories reporting results on this assay. Among those labs meeting DASP sensitivity and specificity criteria for designation, the median sensitivity was 32% and the median specificity was 100%. Overall, standardization of IAA and ICA assay continues to be more challenging than GAD65Ab or IA-2Ab, and radioimmunoassays continue to have a higher positive predictive value than enzyme-linked immunosorbent assays (rev. in 2). It is thought that enzyme-linked immunosorbent assays based on the absorption of GAD65 or IA-2 on plastic will destroy epitopes that are required for the autoantibody binding to the antigen. This phenomenon has therefore resulted in the notion of conformation-dependent autoantibodies. This means that the autoantibodies are unable to bind to GAD65, IA-2, or insulin if their physicochemical structure has been changed. This notion is supported by the finding that GAD65Abs in sera from type 1 diabetic patients do not recognize GAD65 in Western blot analysis. The phenomenon of conformation-dependent autoantibodies raises important questions as to the ability of the autoantibodies to recognize the autoantigen and to the generation of the autoantibodies.

ISLET CELL AUTOANTIBODIES PREDICT AUTOIMMUNE DIABETES

Early studies primarily of first-degree relatives followed over time demonstrate that islet cell autoantibodies may predict type 1 diabetes (13). After the development of robust autoantibody assays that are high capacity, precise, and reproducible, considerable data have accumulated to demonstrate that autoantigen-specific antibodies predict type 1 diabetes (14,15). The quest to identify one type of autoantibody as a better predictor than another has failed, because no clear order of appearance has been detected. Rather, several studies taken together suggest that the number of autoantibodies is predictive rather than the order of their appearance (16). This is particularly true for young children, since the age (17,18) as well as sex (19) affect the expression of both insulin and IA-2 autoantibodies (rev. in 2). The diagnostic sensitivity of these two autoantibodies decreases with increasing age. While IAAs have their highest diagnostic sensitivity (~50–60%) below the age of 10 years (15,18), autoantibodies to GAD65 remain at 70–80% regardless of age (17).

In the large U.S. study, the Diabetes Prevention Trial (DPT-1), four autoantibodies (ICA, IAA, GAD65Ab, and IA-2Ab) were analyzed to assess the risk for developing diabetes; 98% of first-degree relatives who went on to develop type 1 diabetes had one or more autoantibodies, and 80% had two or more autoantibodies. Individuals with two or more positive biochemical autoantibodies had a 68% 5-year risk for developing type 1 diabetes, and those with all three biochemical antibodies had an estimated 100% 5-year risk (14). Several studies have confirmed that the risk for developing diabetes increases significantly with each additional positive antibody in first-degree relatives of individuals with type 1 diabetes (rev. in 4). At this point, it is not clear whether specific combinations of autoantibodies confer different degrees of risk (20) or whether the quantity of different autoantibodies present in an individual is more important than the specific combination (16). In DPT-1, it was shown that GAD65Ab positivity is the most sensitive marker for detecting multiple antibody positivity, in that 91% of individuals who were found to be GAD65Ab positive were also positive for other antibodies, compared with 82% of ICA^+ individuals (21). Based on extrapolated risk from DPT-1, as well as actual risk measured in earlier studies, the TrialNet oversight committee has proposed an antibody screening paradigm in which GAD65Abs and IA-2Abs, in addition to IAAs in younger subjects, are all measured on initial screening. If one or more of these markers is positive, then ICAs should be measured, as ICA positivity appears to confer a higher risk, particularly in individuals with single autoantibody positivity on initial screening.

Once islet autoantibodies (one or several) have developed, the next question is what factors determine β-cell killing to the extent that diabetes develops. These investigations have so far been dependent on longitudinal investigations of primarily first-degree relatives either in controlled clinical trials (European Nicotinamide Diabetes Intervention Trial [ENDIT], DPT-1) or as cohort studies (Washington State Diabetes Prediction Study, Karlsburg Schoolchildren Study, the BOX study, and others). The DPT-1 ascertained participating first-degree relatives based on ICA positivity and partial loss of β-cell function. Although the intervention with parenteral and subcutaneous insulin had no effect, the study accurately predicted the onset of diabetes (22). Similar observations were made in ENDIT, where nicotinamide had no effect on progression to type 1 diabetes (23). It has been suggested that the failure to prevent or delay the onset of diabetes in these trials may in part be due to inefficient timing of the intervention (rev. in 24). The conclusion from both studies was that it is possible to predict type 1 diabetes in first-degree relatives selected based on islet autoantibodies alone or combined with a measure of β-cell function (22,23). To date, there has been no effective treatment initiated before the clinical onset of type 1 diabetes. The use of quantitative methods to detect DAAs, isotypes, and subtypes as well as autoantibody epitopes should prove useful in the attempts to better understand progression to diabetes and when a treatment approach may be most effective.

First-degree relatives of type 1 diabetic patients have provided an excellent study population for defining risk factors for the progression to type 1 diabetes because of the increased disease incidence in this group; however, this population represents only 10–15% of the incident cases diagnosed annually. Thus, screening for high-risk individuals only among first-degree relatives will miss the majority of future cases of type 1 diabetes, which occur in individuals with no family history of type 1 diabetes. With the development of more efficient screening assays, researchers have begun to evaluate the risk of developing type 1 diabetes among individuals in the general population. This raises the question of whether autoantibody positivity, used as a marker for screening, confers the same risk for individuals from the general population as it does for individuals who have a family member with type 1 diabetes and therefore share other genetic factors (primarily HLA) that may be important in the disease process. Although population screening approaches have varied, it is clear that higher levels of autoantibodies as well as multiple autoantibody positivity confer a higher predictive value for type 1 diabetes in the general population, just as they do in first-degree relatives (15,25). The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) project used a screening strategy in which genetic susceptibility of newborns is evaluated by first genotyping for HLA markers known to confer increased or decreased risk for type 1 diabetes, followed by repeated measurements for autoantibody positivity in genetically susceptible individuals (26). This approach identifies ~75% of infants with no family history who subsequently develop type 1 diabetes, and this approach has proven to be more cost-effective than repeated measurements of autoantibodies in a large study cohort. Additional findings from the Diabetes Prediction and Prevention study include that DAAs appear in random order at ~2–3 years of age and that a child may be double or triple antibody positive for up to 7 years before the onset of type 1 diabetes (27). The presence of DAAs also predicts the loss of first-phase insulin (28). Once a child has developed one or more persistent DAAs including ICAs, an intervention with nasal insulin is offered (26). This clinical trial, initiated a decade ago, is still ongoing.

In the BABYDIAB study, children of parents with type 1 diabetes are followed prospectively (11). This group of children represents ~5% of all children developing diabetes and are of particular interest, since a father with type 1 diabetes is thought to yield a higher risk for the offspring compared with a diabetic mother (29). The BABYDIAB study has revealed not only the order of islet autoantibody appearance (11), but also their predictive value and iso-

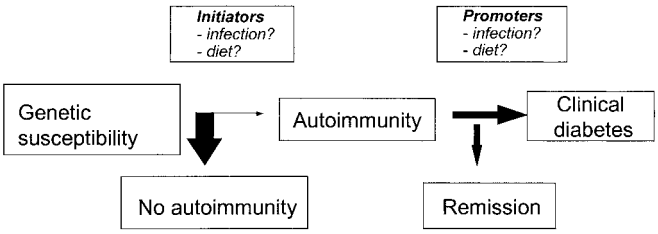

FIG. 1. Two-stage development of type 1 diabetes. Step one is the development of persistent islet autoimmunity measured by the presence of GAD65, IA-2, or insulin autoantibodies, alone or in combination. The second step is the progression from islet autoimmunity to type 1 diabetes.

type and subtype relationship (8). Hence, insulin-IgG1 predominates depending on the HLA genotype of the child (DQ8 children are more frequently affected). The offspring to type 1 diabetic mothers are also unique, since many of these mothers are positive for GAD65Abs and sometimes IA-2Abs, as well as positive for insulin antibodies due to years of insulin injections. It was discovered that children born to mothers with positive GAD65Abs, as evidenced by the presence of GAD65Abs in the cord blood, were less likely to develop GAD65Ab positivity later in life (30). This suggests that these neonates with in utero exposure to GAD65Abs develop immunological tolerance to GAD65, associated with a reduced GAD65 immunity later in life. It was also observed that tissue transglutaminase IgG or IgA antibodies tended to appear in BABYDIAB children in relation to gluten exposure and that these autoantibodies were also associated with an increased risk for developing both GAD65Abs and IA-2Abs (31). This would raise the question that islet autoimmunity might be related to food exposures (rev. in 32).

The Diabetes Autoimmunity Study in the Young (DAISY) study is a combined study of general population and first-degree relatives. In contrast to findings in the BABYDIAB study, early gluten exposure in the DAISY study was associated with transglutaminase IgG and IgA, but not GAD65Abs (33). In the most recent DAISY report, a total number of 112 islet autoantibody–positive children are followed, and of these, 24 have developed type 1 diabetes (34). Despite the fact that the IAA assay is not fully standardized to the same inter-laboratory precision as the assays for GAD65Abs and IA-2Abs (35), the DAISY authors suggest that insulin is the primary autoantigen and trigger of all subsequent islet autoimmunity (36). Other studies of newborn children include the PANDA (Prospective Assessment in Newborns for Diabetes Autoimmunity) (37), Diabetes Evaluation in Washington (DEW-IT) (38), All Babies in Southeast Sweden (ABIS) (39), and Diabetes Prediction in Skane (DiPiS) (40) studies.

Studies in monozygotic twins have demonstrated a concordance rate of ~50%, suggesting that environmental factors play a role in the development of diabetes. Epidemiologic studies suggest that viruses, nutrition, toxic agents, or psychosocial factors may contribute to the etiology alone or in combination (rev. in 41). As indicated in Fig. 1, the long interval between exposure and clinical diagnosis, as well as the interaction of multiple genes, insults, or both, complicates the identification of triggers. Numerous studies have investigated environmental influences but have yielded conflicting results. There are several possible explanations, including failure to account for genetic susceptibility, time of study onset, and the time and frequency at which measures are collected.

The Environmental Determinants in Diabetes of the Young (TEDDY) study will investigate genetic and genetic-environmental interactions, including gestational infection or other gestational events, childhood infections, or other environmental factors after birth, in relation to the development of pre-diabetic autoimmunity and type 1 diabetes. The consortium of six centers has been assembled to participate in the development and implementation of studies to identify environmental factors that trigger the development of type 1 diabetes in genetically susceptible individuals.

HLA ASSOCIATION WITH DAAs

Whereas detailed understanding of the pathogenesis of type 1 diabetes remains to be elucidated, it is apparent that there is a genetic predisposition on which environmental triggers are superimposed. The major contributor to genetic risk is HLA. The HLA complex on chromosome 6 is the strongest genetic risk determinant for type 1 diabetes, conferring up to 40–50% of the inheritable diabetes risk (rev. in 42). In addition to DAAs, HLA genotype has proven to be a significant contributor to the prediction of type 1 diabetes (rev. in 2). By combining data from several population studies, investigators have shown that the DQB1*0302-A1*0301 (DQ8) haplotype confers the greatest risk, with an additive diabetogenic effect if the DRB1*0401 allele is inherited as part of the haplotype. The highest genetic risk is conferred by the DQB1*0302-A1*0301/DQB1*0201-A1*0501 (DQ8/DQ2) genotype. More than 40% of children with new-onset type 1 diabetes have this genotype, compared with 3% of healthy children. Other alleles have been shown to have a negative association with risk for diabetes (a protective effect), most notably HLA DQB1*0602-A1*0102 (DQ6).

Although no exclusive associations between certain HLA alleles and autoimmunity to a particular autoantigen have been demonstrated, several studies have shown correlations between HLA alleles and DAAs, suggesting that the HLA genotype may have a modifying effect on the generation of autoantibodies targeting a specific autoantigen. For example, several studies have demonstrated an increased frequency of GAD65Ab positivity in type 1 diabetic patients with DR3 and/or DQB1*0201 haplotypes (17,25). IA-2Ab positivity, on the other hand, has been correlated with DQ8 (17,25,43) and/or DR4 positivity (44) and negatively associated with DR3/DQB1*0201 (17). Similarly, IAAs and ICAs are found at higher frequency in individuals positive for DR4 (45) and DQ8 (17). Because the DR4/DQ8 allele confers the highest risk for type 1 diabetes and the DR3/DQ2 allele confers a more broad-based risk for a spectrum of autoimmune diseases, including type 1 diabetes, it has been suggested that the DR3-associated anti-GAD65 response is a marker of general autoimmunity, while the DR4-associated anti–IA-2 response is a more specific marker of β-cell destruction (44). Thus, HLA genotypes appear to have a modifying influence on the expression of diabetes-associated autoantibodies, both of which are important predictors of disease risk, particularly at young ages.

DIABETES CLASSIFICATION AND DAAs

Autoantibodies, reflecting activation of the immune system and β-cell response, are used diagnostically in a variety of diseases to establish whether or not the disease

is autoimmune in nature. This is the case with diabetes and other conditions such as adrenal insufficiency, hypogonadism, and thyroid disorders. Current classification of diabetes endorsed by both the American Diabetes Association and the World Health Organization is based on etiopathogenesis. The two major classifications of diabetes are type 1 diabetes, characterized by a state of β-cell destruction, and type 2 diabetes, characterized by a combination of resistance to insulin action and an inadequate compensatory response in insulin secretion (46). The majority of cases of type 1 diabetes are attributable to an autoimmune process and are termed T1A. Evidence that type 1 diabetes is an autoimmune process is most commonly based on the presence of DAAs (ICAs, GAD65Abs, IA-2Abs, or IAAs).

The diagnostic sensitivity of GAD65, IA-2, and insulin autoantibodies varies with age at onset and sex. GAD65Abs are present in ~70–80% of Caucasian subjects newly diagnosed with type 1 diabetes (rev. in 4,47). GAD65Abs are less frequent among boys developing diabetes before the age of 10 years, but in older children, teenagers, and young adults, the diagnostic sensitivity is ~80% in both males and females. GAD65Ab titers are higher and more prevalent in patients with other associated autoimmune diseases, such as thyroiditis (48). IA-2Abs have been reported in 32–75% of subjects with newly diagnosed type 1 diabetes (4). This wide variation in frequency may be attributed in part to the age range of the study population, since IA-2Abs decrease in frequency with increasing age at onset (17). Diagnostic sensitivity varies most with age in IAAs, decreasing from 50–60% in the very young (below age 10 years) to ~10% among those diagnosed before 30 years of age (17,18,49). In addition, IAAs often may precede other autoimmune markers (9,18,50), which has led to the hypothesis that insulin may be an autoantigen in type 1 diabetes that plays a role early in the pathogenic process.

The mechanisms by which these islet autoantigen–specific autoantibodies show an age-dependent effect are not understood. Type 1 diabetes is twice as common among men in subjects >20 years of age (51); however, this difference between sexes is not easily explained by the diagnostic sensitivity of the DAAs. The fact that the diagnostic sensitivity varies with age and sometimes with sex has important consequences when using DAAs to predict type 1 diabetes.

Although work continues on understanding the progression to type 1 diabetes, current trends in diabetes incidence have posed new questions about using DAAs in diabetes classification. There have been numerous reports of a rise in incidence of type 1 diabetes, particularly in the youngest age-groups. The EURODIAB collaborative group reported an annual increased incidence of type 1 diabetes of 3.2% in youth <15 years of age, with the highest increase in incidence observed in children <4 years of age (52). In a compilation of published studies from 27 countries of the incidence of type 1 diabetes from 1960 to 1996, a similar increase of 3.0% was reported (53).

The DAA profile in very young children, where type 1 diabetes is increasing most rapidly, differs from that in older children. In children <2 years of age, ICA and IAA titers are higher than in older children and IA-2Abs are lower (49). In a study of 40 children in Los Angeles, diagnosed before 5 years of age, the frequency of positive IA-2Ab sera was also observed to be lower than in older children (28.6 vs. 77% in 0- to 5-year-old children vs. children 6 years or older) (54). Whether or not these age-dependent DAA profiles reflect different disease mechanisms is not fully understood.

Another observation related to diabetes incidence is the marked increase in incidence of clinical type 2 diabetes in children and adolescents (55,56). Whereas many studies have relied on clinical findings alone to distinguish between type 1 diabetes and type 2 diabetes, examining autoimmune measures in these youth reveals interesting findings. Hathout et al. (54) reported that 30.3% of subjects with a clinical diagnosis of type 2 diabetes had positive GAD65Abs and 34.8% had positive IAAs, out of 48 youth followed in that center with clinical type 2 diabetes. DAAs and T-cell reactivity were compared in children and adolescents who presented with findings of type 1 diabetes, type 2 diabetes, or an admixture of clinical findings. The frequency of positive DAAs and T-cell reactivity in individuals with clinical features of type 2 diabetes or an admixture were 71 and 34%, respectively (57).

Autoimmune diabetes may masquerade as type 2 diabetes in adults as well. It has been known since the early days of ICA measurements that diabetic patients who failed oral hypoglycemic agents often had ICAs (13). These early observations were confirmed and extended when standardized methods to measure GAD65Abs became available (58). The most common DAA found in this group is GAD65Ab, which is found in ~5–10% of subjects; ~2–4% of adults have IA-2Ab and <1% have IAA. When this subgroup is compared with DAA^- counterparts, it is apparent that the DAA^+ group progresses more quickly to insulin dependence (rev. in 2). In a study of Japanese adults with type 2 diabetes, 6.6% were found to have GAD65Abs. Those with higher GAD65Ab titers (≥20 units/ml) were more like classic type 1 diabetic patients, in that they more frequently had HLA DRB1*0405, had lower urinary C-peptide concentrations, had associated autoimmune thyroid disease, and were more quickly treated with insulin after diagnosis than those who had lower GAD65Ab titer or were GAD65Ab negative (59). These patients are referred to as having latent autoimmune diabetes in the adult (LADA), type 1½ diabetes, or slowly progressive insulin-dependent diabetes mellitus (SPIDDM) (rev. in 60). It is much debated to what extent LADA is a disease entity by itself. Many features of insulin release and metabolic phenotypes in LADA patients distinguish them from both type 1 diabetic and type 2 diabetic patients. On the other hand, the autoimmune character of the LADA condition is indisputably similar to type 1 diabetes in that HLA DQ8, DQ2, or both are the predominating allele(s) (61).

Several longitudinal studies of patients classified with type 1 or type 2 diabetes indicate that GAD65Abs or IA-2Abs remain positive for up to 12 years (62). While the autoantibodies against IA-2 decrease rapidly with increasing duration of disease, this is not the case for GAD65Abs. These autoantibodies tend to remain at high titers despite documentation that the patient is no longer producing C-peptide (62). This observation is puzzling, since it is often hypothesized that antibody levels are maintained only if there is repeated antigen stimulation. Currently, IAAs cannot be studied longitudinally because antibodies to the injected insulin develop as early as 7–10 days after initiation of insulin therapy.

DO AUTOANTIBODIES PREDICT CLINICAL COURSE?

The relationship between DAAs at diagnosis and C-peptide disappearance or clinical course has been examined in children and adults. Örtqvist et al. (63) demonstrated independent correlations between age, sex, and ICA positivity with duration of partial remission. In a study of DAAs in children with clinical diagnosis of type 2 diabetes, all of the children who were ICA positive were on insulin therapy 1 year after diagnosis (64). In addition, in DAA^+ adult diabetic patients, ICAs predict a decline in C-peptide (R.A. Jensen, L.K.G., C. Törn, M. Landin-Olsson, F.A. Karlsson, J.P. Palmer, I. Kockum, K. Åkesson, B. Lernmark, K. Lynch, N. Breslow, Å.L., unpublished data) as well as future insulin dependence in subjects not requiring insulin at diagnosis (rev. in 2). Moreover, in DAA^+ young adult diabetic subjects, GAD65Abs were an important predictor for loss of C-peptide over a 6-year period after diagnosis (R.A. Jensen, L.K.G., C. Törn, M. Landin-Olsson, F.A. Karlsson, J.P. Palmer, I. Kockum, K. Åkesson, B. Lernmark, K. Lynch, N. Breslow, Å.L., unpublished data).

At the time of clinical diagnosis, it would be important to know whether a child positive for three islet autoantibodies has a more rapid loss of C-peptide than subjects positive for only one or two. Alternatively, it would be important to know if loss of C-peptide is associated with one particular autoantibody or autoantibody in combination with genetic propensity. Furthermore, it cannot be excluded that islet cell autoantibody isotype, IgG subtype, or epitope specificity are important and perhaps predict the rate of C-peptide loss. Careful prospective analyses will be required to clarify these issues that may be of importance to the strategy by which to most effectively treat new-onset, preferably young type 1 diabetic patients. Also for patients with an atypical clinical phenotype, these measures may be valuable in predicting clinical course. The relationship of DAA at diagnosis and subsequent clinical course is currently being examined in the large-scale population-based study, SEARCH for Diabetes in Youth (65).

DAA EPITOPES

While DAAs are useful tools in prediction, classification, and prognosis, they provide limited information about the disease process at the cellular level. Studies have shown that the presence of all three autoantibodies provides the highest predictive value for type 1 diabetes (66–68). However, because autoantibodies appear successively (9,11,69), the time period required to account for all three may be counteractive in the aim of prevention; the suppression of insulitis as early as possible is a key for success. A prediction system based on one autoantibody alone would therefore be beneficial. Changes in autoantibody isotypes, affinity, and epitopes could serve as a reflection of the maturation of the autoimmune response leading to the development of diabetes. Therefore, GAD65Abs and IA-2Abs have been studied for possible changes in epitope recognition as an early marker for disease progression.

IA-2Ab epitopes have been mapped using different approaches including the closely related IA-2β (70–73), fusion proteins of IA-2 and IA-2β (74,75), and human monoclonal antibodies specific to IA-2 (76,77). IA-2 appears to be the primary autoantigen in type 1 diabetes, whereas antibodies to IA-2β are considered to be the result of subsequent epitope spreading (8,70,75,78). The cytoplasmic portions of IA-2 and IA-2β were identified to carry the major antibody epitope regions (70,79). Several epitopes are located within this region, including two linear epitopes in the juxtamembrane domain, and conformational epitopes in the middle and COOH-terminal region of the protein tyrosine phosphatase domain (72,80–82). The presence of multiple epitopes is associated with the development of diabetes, while IA-2Abs in individuals who are only transiently positive for IA-2Abs usually recognize few epitopes and do not react with the protein tyrosine phosphatase domain (83). Antibodies in the early disease process are predominantly directed toward IA-2 and recognize epitopes in the juxtamembrane domain (78), while at the time of diagnosis, antibodies to epitopes in the protein tyrosine phosphatase regions of both IA-2 and IA-2β dominate (70,71,75,78,80–82). High susceptibility HLA genotypes were associated with the presence of multiple epitopes to both IA-2 and IA-2β, but not with IA-2Ab specific to the juxtamembrane region (78,80). In an effort to better characterize the conformational epitopes, competition assays with human monoclonal antibodies were developed (76). A major conformational epitope located at the COOH-terminal region of the protein tyrosine phosphatase domain was identified using this approach (76,77).

Epitope spreading to multiple epitopes on IA-2/IA-2β was reported in young children, whereas the epitope reactivity in older subjects remained more stable (70). Both multiple epitopes (7,8,70,75,78) and the reaction to the juxtamembrane region have been shown to be linked with progression to diabetes (7,78). In a longitudinal study of first-degree relatives, intramolecular epitope spreading was only observed among the progressors, while some of the nonprogressors showed a decrease in the number of epitopes bound (75). However, these disease-specific epitope changes were not observed in other longitudinal studies (78,82).

GAD65Ab epitopes have been the focus of many studies. Using fusion proteins of GAD65 and the closely related but nonantigenic isoform GAD67, differences in GAD65Ab epitopes of type 1 diabetic patients and other $GAD65Ab^+$ individuals were revealed (84–86). GAD65Abs in type 1 diabetes are predominantly directed to conformational epitopes located in the middle region of the molecule, whereas GAD65Abs in other antibody-positive individuals also recognize linear epitopes and epitopes located at the NH_2-terminus (86–88).

Dynamic changes in the GAD65Ab binding pattern during the period before clinical onset were suggested by several observations. Using GAD65/67 fusion proteins, Bonifacio et al. (85) found the middle epitope to be primarily recognized in the early stages of GAD65 autoimmunity with subsequent epitope spreading to the NH_2-terminus. Using naturally occurring isoforms of GAD65, we were able to show epitope shifts in a subgroup of newly diagnosed children within the first 12 months after disease onset (89).

Considering the strong dependence on conformation for autoantibody recognition, other methods for the analysis of conformational epitopes were developed. Blocking experiments using monoclonal antibodies (90) and recombinant Fab derived from monoclonal antibodies (91,92) have been useful to study conformational GAD65Ab epitopes. When comparing the traditional epitope analysis of GAD65Abs using GAD65/67 fusion proteins with the recombinant Fab competition assay, we were able to ob-

serve significant differences between the two approaches. Whereas fusion proteins are useful in the definition of large epitope regions, important conformational epitopes—located mainly in the middle of the molecule—are destroyed or altered (93).

Using 10 human monoclonal GAD65-specific antibodies (MICA 1–10), the earlier observations of two major conformational epitope regions in the middle and the COOH-terminus were confirmed (90). A limited analysis of nine children with newly diagnosed type 1 diabetes suggested that MICA 1–6 represent a wide range of both common and unusual epitopes (94). However, no disease-specific GAD65Ab changes in the preclinical stages of type 1 diabetes were identified using these reagents (90).

In our analysis of GAD65Abs in different GAD65Ab$^+$ phenotypes, we used a set of five different recombinant Fab whose epitope binding sites were located at different sites of the molecule. We were able to identify two middle epitopes that were significantly associated with type 1 diabetes (91,92). In a recent study, we compared GAD65Ab epitope changes in a longitudinal study of children at high risk for developing type 1 diabetes with those present in matched children with low risk (95). Using recombinant Fab derived from four GAD65-specific monoclonal antibodies in competition experiments, we were able to show dynamic changes of the GAD65Ab epitopes and their pattern only in the high-risk individuals. These changes were even more profound in the individuals that eventually progressed to the disease than in the children who have not developed diabetes to date. The observed increase of GAD65Ab binding to the type 1 diabetes–associated middle epitope occurred in 72% of the high-risk children, whereas only 10% of the low-risk children showed this dynamic epitope change, resulting in a positive predictive value of 80%. Further analysis of larger longitudinal study populations will be necessary to establish the use of the dynamic changes in GAD65Ab epitope binding and patterns. However, based on these findings, a predictive system based on the analysis of only one autoantibody appears feasible.

ANTIBODY ISOTYPES

The current understanding is that antibodies do not themselves cause β-cell destruction, but rather reflect autoimmune activity; the β-cell destruction is mediated through T-cells. Different antibody isotypes carry out different functions, i.e., IgG but not IgM antibodies can penetrate tissues where they activate complement and bind Fc receptors on macrophages and NK cells to induce antibody-dependent cellular cytotoxicity (95). Because of their ability to activate multiple immune effector systems, IgG autoantibodies pose a greater risk to the host than IgM autoantibodies (96,97). This risk is exemplified by the relatively common finding of IgM but not IgG autoantibodies in sera from healthy individuals (98).

Isotype switching is stimulated by helper T-cell–dependent signals requiring both ligation of CD40 and helper T-cell–derived cytokines. The isotype class depends on the cytokine profile provided by the helper T-cell, which is in turn regulated by multiple factors, including the cytokine milieu at the time of activation. Other regulatory factors are the antigen dose (99), the strength of T-cell receptor binding by the antigen–major histocompatibility complex (100), and the type of antigen-presenting cell interacting with a T-cell (101). IgG4 and IgE are selected in the presence of interleukin (IL)-4 and IL-13 (102,103), whereas IgG1 and IgG3 require the presence of IL-10 (104,105), and IgA needs the combined presence of IL-10 and transforming growth factor-β (106,107).

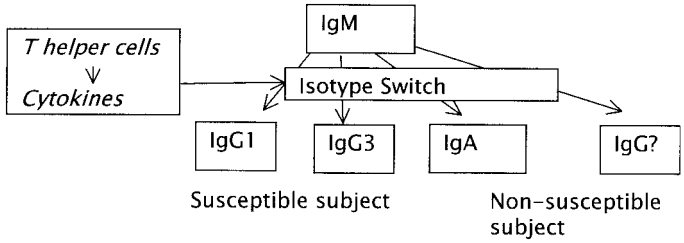

FIG. 2. Illustration of isotype switching associated with disease susceptibility. We hypothesize that while the predominant GAD65Ab isotypes in the susceptible subject will be of IgG1 and IgG3, the majority of GAD65Abs in nonsusceptible subjects will be of the IgM class and disappear during follow-up. Only a small percentage will switch to IgG.

In progression to type 1 diabetes, data describing autoantibody isotypes are highly controversial. Previous reports have indicated that the predominant diabetes-associated autoantibody isotypes in type 1 diabetes are IgG1 and IgG3 (108). Hawa et al. (109) noted no significant difference in GAD65Ab or IA-2Ab isotypes when comparing children and young adults with type 1 and adult type 2 diabetic patients. The predominant isotype was IgG1, consistent with antigen-driven B-cell activation, predominantly Th1 activity. A more recent report exploring IAA isotypes in genetically susceptible (HLA-DQB1) young children reported a higher frequency of IgG3 in progressors compared with nonprogressors and higher integrated levels of IgG1 and IgG3 IAA compared with nonprogressors (110). In LADA adults, a difference in IgG subclasses was observed, with IgG4 found more commonly in LADA patients compared with type 1 diabetic patients; however, IgG1 remained the most common subtype in both LADA and type 1 diabetic patients (96).

One study (7) in a few first-degree relatives of type 1 diabetic patients failed to identify any GAD65 epitope- or isotype-specific antibody reactivity that could be used as a marker for progression to disease. This is in contrast to another study (8), which showed that epitope- and isotype-specific autoantibodies were capable of separating infant (children born to type 1 diabetic mothers) progressors from nonprogressors.

We hypothesize that the initial autoantibody response in pre-diabetic subjects, as well as healthy subjects not at risk for type 1 diabetes, is IgM specific. After the initial period, the autoantibody response in pre–type 1 diabetic individuals switches to an IgG-dominated response. However, the majority of the autoantibodies in healthy individuals do not undergo isotype switching and disappear at follow-up (Fig. 2). It is also known that 1–2% of the healthy population produce IgG autoantibodies to GAD65. It will be of interest to learn which IgG subtype they produce and whether other isotypes are present in these individuals, indicative of other types of immune responses (such as allergic reactions). Therefore, the isotype class(es) will also provide valuable information on the nature and route of activation of the T-cells involved in disease pathogenesis.

CONCLUDING REMARKS

DAAs provide valuable information about predicting type 1 diabetes. Using biochemical DAAs, e.g., recombinant

radiolabeled antigens that provide improved efficiency and consistency over cytoplasmic ICAs, DAAs are now being used in studies of high-risk populations (first-degree relatives) as well as the general population. Following DAAs prospectively in genetically at-risk subjects affords the opportunity to identify environmental triggers and introduce preventative measures. At the patient level, DAAs are an integral part of studies to understand pathogenesis of disease, how that varies with age, and clinical features including obesity. Because autoimmune diabetes does not appear to be limited to type 1 diabetes, continued study of the relationship between DAAs and C-peptide disappearance is needed. Epitope studies have demonstrated specific dynamic changes in individuals who progress to disease and thus aid in assessing risk in DAA^+ individuals. Another approach to refine DAA information is to assess DAA isotypes. Isotype switching reflective of Th1 activity is observed in individuals who progress to type 1 diabetes more often than nonprogressors or individuals with slow progression, such as in LADA. In summary, DAAs are informative markers of humoral immunity that aid in prediction, prevention, classification, and intervention strategies. Expanded studies of DAAs, e.g., epitope and isotype studies, combining DAAs with genetic and inflammatory measures, will lead to a better understanding of diabetes pathogenesis.

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