[Frontiers in Bioscience 12, 1889-1898, January 1, 2007]

Role of Autoantibodies in Type 1 Diabetes

Dongmei Miao[1], Liping Yu[1], George S. Eisenbarth[1]

[1]Barbara Davis Center for Childhood Diabetes, University of Colorado Health Sciences Center, 1775 N. Ursula Street, Aurora, Colorado, 80010

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1. ABSTRACT

Type 1A, the immune mediated form of diabetes, is a relatively common disorder that develops in genetically susceptible individuals. The disease is associated with a series of anti-islet autoantibodies and the autoantibodies can be present for years prior to the onset of hyperglycemia. In general it is thought that type 1A diabetes is T cell mediated, but there is evidence from studies in the NOD mouse model that antibodies and B-lymphocytes contribute to pathogenesis. In man evidence is lacking that transplacental passage of anti-islet antibodies increases disease risk. Well characterized, high throughput autoantibody assays (tested in a series of international workshops) are now available, and are the mainstays of prediction of type 1A diabetes, diagnosis of the immune mediated form of diabetes, and are important for the design of trials for the prevention of type 1A diabetes. In addition to anti-islet autoantibodies, patients with type 1A diabetes develop a series of associated autoimmune disorders that are usually detected with screening for additional autoantibodies (e.g. anti-thyroid, anti-transglutaminase, anti-21 hydroxylase, anti-parietal cell). At present it is possible to predict the development of type 1A diabetes and prevent the disorder in animal models, but we lack proven therapies for disease prevention in man. The ability to detect specific anti-islet autoantibodies provides the foundation for developing and testing these preventive therapies.

2. INTRODUCTION

Type 1A diabetes has become one of the most extensively studied autoimmune disorders with multiple excellent spontaneous, antigen-induced, and genetically created animal models, and a wealth of immunogenetic and prospective immunologic analysis (1-3). The initial detection of anti-islet autoantibodies on sections of frozen pancreatic tissue greatly spurred the study of the immunologic pathogenesis of the disease (4). Cytoplasmic islet cell autoantibodies as they are called are composed of GAD (Glutamic Acid Decarboxylase) autoantibodies, autoantibodies reacting with the molecule IA-2 (also termed ICA512) and antigens awaiting characterization (see the Figure 1). Insulin autoantibodies do not react with frozen non-fixed sections of pancreas, and thus the ICA test does not detect insulin autoantibodies. Over the past two decades, tests for islet autoantibodies have improved dramatically and for most purposes, "biochemical" autoantibodies are measured, while the cytoplasmic ICA test remains important for specialized studies and for specific research groups.

3. METHODOLOGY FOR DETERMINING DIABETES RELATED AUTOANTIBODIES

The first useful assay for islet autoantibodies was the ICA test utilizing indirect immunofluorescence with frozen sections of human pancreas as substrate. To date, the

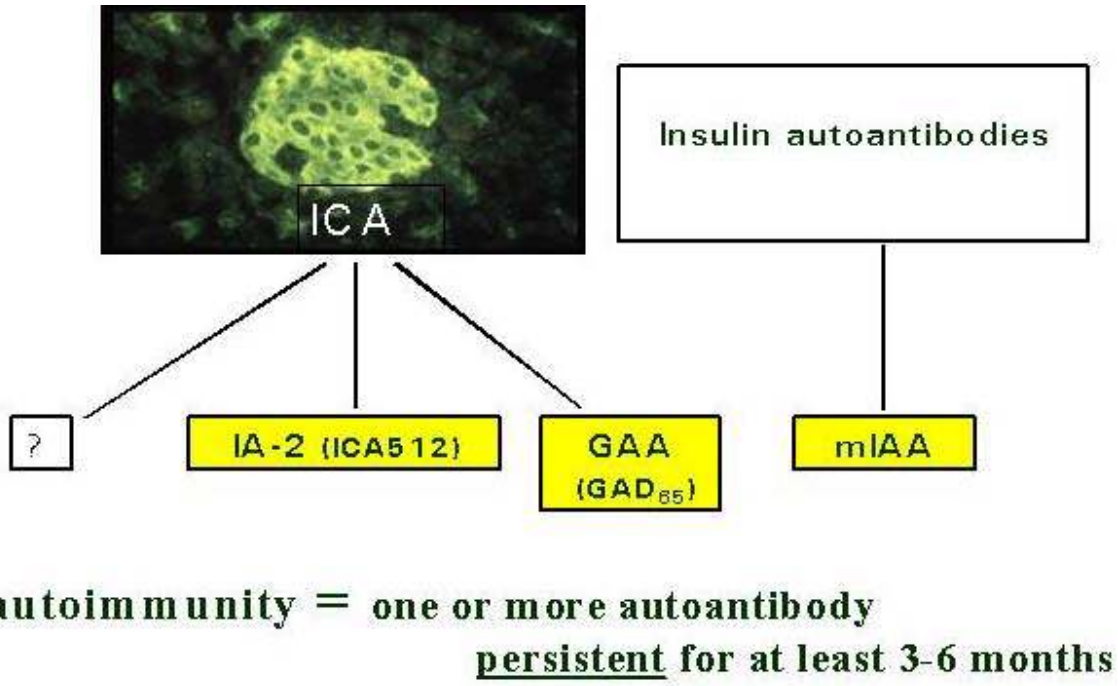

Figure 1. Cytoplasmic Islet Cell Autoantibodies are composed of IA-2 and GAD autoantibodies, and unknown specificities, but not Insulin autoantibodies.

ICA test remains cumbersome and the Immunology of Diabetes Society (IDS) workshops demonstrated that there existed large variation between laboratories in terms of sera called ICA positive (5).

Currently, many sequenced autoantigens with recombinant autoantibody assays are available and three major autoantibody assays are at present being used in many laboratories across the world: assays for GAD65 (GAA-Glutamic Acid Decarboxylase), ICA512 or IA-2 (IA-2AA-Insulinoma Antigen) and insulin (IAA) autoantibodies (6). The methodology for these assays depends on the reaction of the antigen with the antibody in fluid phase and is illustrated in the Figure 2. Such fluid phase assays detect high affinity anti-islet autoantibodies that are disease-related. A series of antigens can be *in vitro* transcribed and translated to produce labeled autoantigen, and this labeled antigen is then reacted with patient sera. Finally antibody bound antigen is separated from free antigen with specific reagents such as Protein A/G. Assays can be performed in 96-well plates and filtration used to separate bound from free label. In general ELISA assays where the antigen is bound to plate have lacked sensitivity and specificity comparatively to these fluid phase assays (7;8).

The IDS and CDC (the Centers for Disease Control) have initiated the Diabetes Autoantibody Standardization Program (DASP) and organized multiple autoantibody workshops (4 DASP workshops with patient sera to date and 2 with sera from animal models). The animal model workshops analyzing sera from NOD mice and control strains concluded that only IAA measured by the fluid phase radioassay are strongly associated with diabetes autoimmunity while neither specific association with GAA nor IA-2AA were demonstrated (8). For patient sera, the majority of laboratories have excellent assays for GAA and IA-2AA. A standard calibration sample (World Health Organization) was distributed and used in these workshops and results for GAA and IA-2AA can be reported in WHO units. The assays for IAA still have poor correlations between laboratories in the most recent 2005 DASP workshop, although it has been improved compared with previous workshops. The sensitivity of the IAA assay was relatively poor for many laboratories. However as illustrated by the receiver operating characteristic (ROC) curve in the Figure 3, the signal to noise ratio for IAA of DASP workshop samples is very small compared to the curves of GAA and IA-2AA. All attempts with standard ELISA assays (e.g. antigen bound to plates) to measure insulin autoantibodies have failed to achieve the sensitivity and specificity exhibited by the fluid phase radioassay. Recently (2005 DASP workshop), a modified ELISA assay with fluid phase reaction of antibody and antigen with capture of the complex on a plate gave results equivalent to the radioassay for both GAA and IA-2AA assays. This assay depends upon binding one chain of the autoantibody to plate bound antigen with the other chain free to react in the fluid phase with labeled antigen.

4. ANIMAL MODELS OF TYPE 1A DIABETES

The non obese diabetic (NOD) mouse is the most-studied spontaneous type 1A diabetes-susceptible animal model and it shows islet lymphocyte infiltration at 5-7 weeks of age followed by overt diabetes with specific islet beta cell destruction. Similar to man, NOD mice develop IAA in their peripheral blood prior to diabetes onset and the

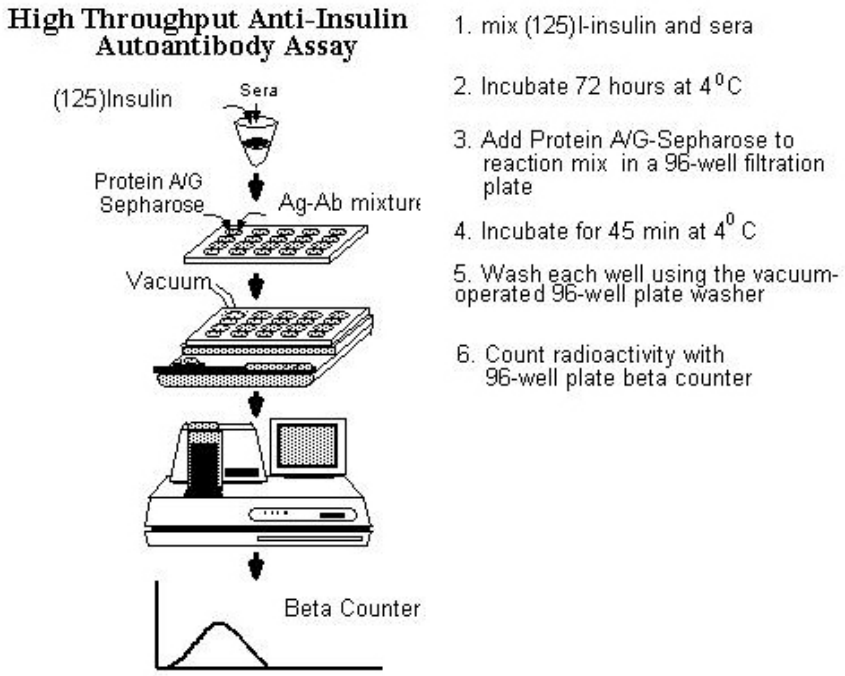

Figure 2. High throughput filtration assay for insulin autoantibodies.

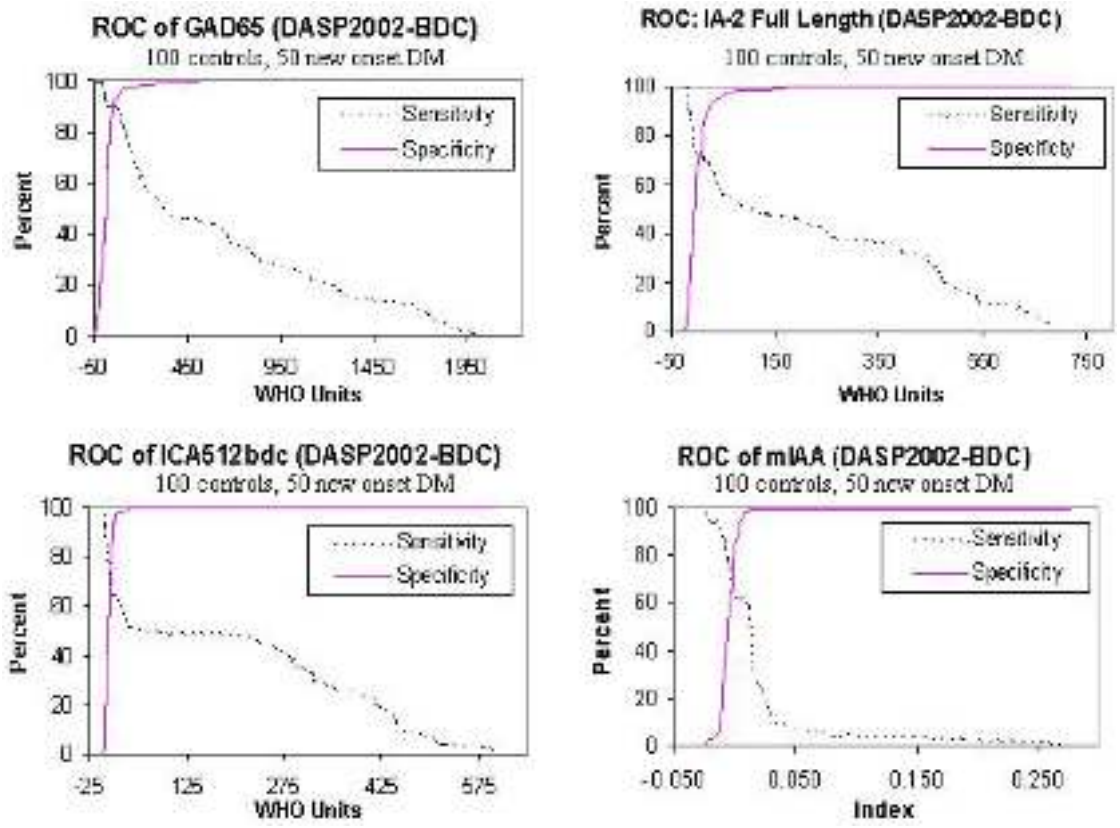

Figure 3. ROC (receiver operating characteristic) curves for autoantibodies measured by the Barbara Davis Center laboratory in the 2002 DASP workshop. Note the strong signal for GAD65, IA-2, and ICA512bdc (alternative splice variant of IA-2) constructs, while the signal for insulin autoantibodies is minimally greater than highest sample from normal controls.

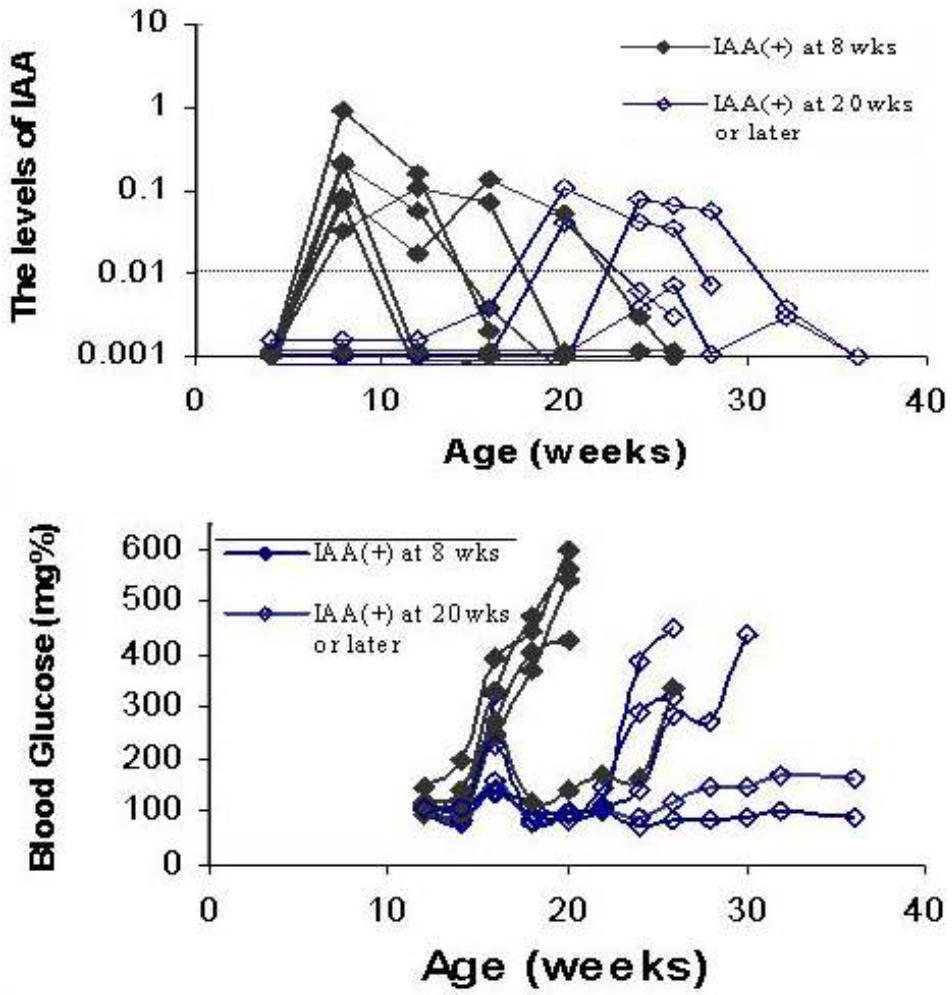

Figure 4. Development of insulin autoantibodies (IAA) in individual NOD mice. Upper panel shows serum IAA levels (upper limit of normals: 0.010) and low panel is levels of blood glucose. Mice that expressed insulin autoantibodies (above upper limit of normals) at 8 weeks of age developed diabetes prior to 16 weeks, while mice expressing insulin autoantibodies after 20 weeks developed diabetes later.

early development of IAA of NOD mice correlates with early development of type 1 diabetes. Nearly 90% of NOD mice expressing IAA by 8 weeks of age develop diabetes by 16 weeks of age (Figure 4). NOR mice, a strain closely related to NOD with the I-A^{g7} MHC allele, develop IAA but have limited or no progression to type 1 diabetes. NOR mice do have insulitis and the presence of IAA correlates primarily with insulitis and not simply with progression to hyperglycemia. Recent studies of NOD mice indicate that both B-lymphocytes and transplacental autoantibodies increase the development of diabetes. As illustrated in the Figure 5, the development of diabetes in NOD offspring was greatly decreased if maternal sera had no autoantibodies and thus transplacental passage of autoantibodies was abrogated (9).

The BB-DP rat model of type 1 diabetes requires a unique lymphopenia (lyp) gene for spontaneous disease as well as a specific MHC class II allele and develops severe insulitis and beta cell destruction(10;11). Unlike man and the NOD mouse, islet autoantibodies are not detected during, or post disease onset.

5. TYPE 1A DIABETES OF MAN

The term type 1A diabetes has been adopted to reflect disease etiology (the immune mediated form of diabetes with specific islet beta cell destruction). Over 90% of non-Hispanic Caucasian and near 50% of Hispanic and African American children with diabetes have the type 1A form. In addition between 5 and 15% of adults presenting with diabetes express anti-islet autoantibodies and have type 1A diabetes. There are no tests that can absolutely exclude a diagnosis of type 1A diabetes and it is possible for individuals to have mixed forms of type 1A (e.g. presence of anti-islet autoantibodies) and type 2 diabetes (e.g. severe insulin resistance). In general, presence of anti-islet autoantibodies (with high specificity assays) in a

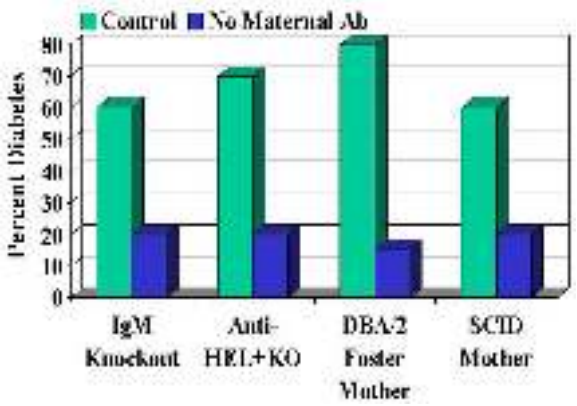

Figure 5. Percent developing diabetes in offspring is plotted. Control= offspring from wild type NOD mother containing anti-islet autoantibodies. HEL= Foster mothers with transgene producing antibodies to Hen Egg Lysozyme; KO=Foster mothers with IgM knockout; DBA/2 strain of mouse used as foster mother; SCID=Severe Combined Immunodeficient foster mother. In all four experimental groups, mothers either had no antibodies (KO and SCID mice) or had non-diabetes related antibodies (Anti-HEL and DBA/2 mice). Development of diabetes is greatly reduced if maternal anti-islet autoantibodies are not present during pregnancy, presumably due to a lack of transplacental autoantibodies.

patient with diabetes is characteristic of type 1A diabetes. Though T-lymphocytes are pathogenic, there is at present a lack of assays for anti-islet T cells with sufficient specificity and sensitivity to aid diagnosis.

A recent study by Ziegler and coworkers (12) suggests that the presence of anti-islet autoantibodies (GAA and ICA512AA, not IAA) at birth in offspring of mothers with type 1A diabetes was associated with a decrease of developing anti-islet autoantibodies in the BabyDiab study. The five-year risk of developing anti-islet autoantibodies and diabetes was 1.3% and 1.1% in offspring from mothers with anti-islet autoantibodies at birth versus 5.3% and 3% respectively in children of mothers who were negative for anti-islet autoantibodies. Of note, a child with genetic B cell deficiency who had no antibody production was found to progress to type 1A diabetes (13). In addition, in infants born to anti-islet autoantibody positive mothers who developed type 1A diabetes during pregnancy there is lack of evidence of direct beta cell damage by trans-placental anti-islet autoantibodies (GAA and IAA), which are readily detected in the infants.

5.1. Insulin Autoantibodies

Insulin and its precursor, proinsulin, are the major beta cell specific autoantigen within human islets (GAD65 and IA-2 autoantigens are also present in glucagon and somatostatin-producing islet cells of man). In the 1950s, Berson and Yalow developed the first radioimmunoassay utilizing sera with anti-insulin antibodies from patients treated with bovine insulin (14). In 1983, Palmer and coworkers discovered the presence of insulin autoantibodies (IAA) in patients with newly diagnosed type 1A diabetes prior to the administration of exogenous insulin (15). Subsequent studies have demonstrated that IAA are present for years before the clinical onset of overt diabetes (16). The presence of anti-insulin antibodies, either induced by exogenous insulin injection or naturally occurring, does not usually interfere with insulin therapy. A rare subset of insulin-treated patients with extremely high levels of insulin antibodies (binding capacity >30 mU/ml serum) is insulin resistant. Patients with a very rare syndrome termed Insulin Autoimmune Syndrome, also termed Hirata syndrome, express extremely high levels of insulin autoantibodies, often with episodes of severe hypoglycemia (17).

Type 1A diabetes associated IAA reacts with a conformational epitope and does not react with either isolated insulin A or B chain. The levels and presence of IAA are dramatically age-dependent. More than 90% of young children who progress to type 1A diabetes prior to age 5 are IAA positive and levels greater than 2000 nU/ml are almost exclusively found in these young children, while less than half of individuals developing type 1A diabetes after age 15 have IAA (18). IAA are usually the first autoantibody to appear in young children developing type 1A diabetes. This has been observed in studies of children followed from birth in both the US Daisy study and German BabyDiab study (19;20).

Achenbach and coworkers (20) have analyzed the affinity of IAA in children followed prospectively in the BabyDiab study. The children who went on to develop multiple anti-islet autoantibodies (very high risk to develop diabetes) or to progress to diabetes express high affinity IAA ($>10^9$), while the children who failed to develop additional anti-islet autoantibodies (remained IAA only) or had transient IAA, have low affinity IAA. It is likely that most of the low affinity IAA are "false positive" autoantibodies relative to diabetes risk.

Williams and coworkers modified the IAA assay using protein A Sepharose to precipitate antibodies rather than polyethylene glycol in the conventional IAA assay (21). The assay (termed micro-IAA [mIAA] assay) uses a small volume (25 μl) of serum rather than 600 μl in the conventional IAA assay and eliminated two artifacts apparently related to the use of polyethylene glycol. Both cord blood and hemolyzed sera cause false positive results with polyethylene glycol precipitation, but this does not occur with protein A Sepharose precipitation. We have further modified the Williams assay by utilizing a 96-well filtration plate and direct counting on a 96-well beta counter, which allows semi-automated determination of IAA (22). Standard ELISA assays for insulin autoantibodies fail to detect type 1 diabetes associated insulin autoantibodies though such assays can detect insulin autoantibodies following insulin injection (7). In general, the IAA associated with diabetes risk have an extremely high affinity, but very low capacity, which makes their detection with plate-binding assays problematic. At present, mIAA assays have relatively poor correlations between laboratories participating in DASP workshops compared with GAA and IA-2AA assays and

approximately half of the laboratories have either poor sensitivity or specificity, or both (23).

5.2. Glutamic Acid Decarboxylase Autoantibodies

Glutamic acid decarboxylase (GAD) is an important component of the 64-KD islet autoantigen. GAD is present in all human islet cells (e.g. α, β, δ) and there are two forms of GAD, termed GAD65 and GAD67 that are 76% homologous in amino acid sequence. GAD is also synthesized in testes, ovary, and neurons in addition to islets, though only pancreatic beta cells are destroyed in type 1A diabetes. Of note in mouse islets, GAD is usually not detectable, and in the rat GAD is only expressed in beta cells (24). Given the broad tissue expression of GAD, it is possible that the autoimmune response to GAD is of secondary rather than primary pathogenic importance, though autoantibodies to GAD for man are important for diagnosis and diabetes prediction.

The GAD autoantibodies (GAA) from patients with stiff man syndrome (SMS) are unusual in that they react with fixed sections of the brain and react with denatured GAD molecules on Western blots and with GAD protein fragments (25). In contrast, GAA from diabetic patients only target conformational epitopes of the GAD protein and do not bind to GAD fragments or react with denatured GAD protein. In 1991, it was discovered that some high titer GAA from patients with polyendocrine failure and relatives of patients with type 1A diabetes reacted in a pattern similar to the autoantibodies from SMS patients. The sera from these individuals failed to stain mouse islets and stained only beta cells on rat islets, which therefore were termed “restricted” ICA or “selective” ICA. These ICA can be completely absorbed by GAD (24).

The levels of GAA are remarkably constant. Harrison and coworkers reported that there is an inverse correlation between levels of GAA and T cell proliferation to GAD (26). This observation has not been confirmed given perhaps the difficulties of current T cell assays. The DPT-1 (Diabetes Prevention Trial – type 1) prospective study indicates that the levels of each autoantibody including GAA are positively correlated with disease risk among relatives.

The majority of laboratories are currently using fluid phase GAD65 radioassays, which showed excellent sensitivity and specificity in DASP workshops(27;28). The results of the assays are well correlated between laboratories and a standard WHO unit has been introduced. As demonstrated in the most recent DASP workshop, a modified ELISA assay correlated well with the radioassays. This ELISA was based upon the binding of one chain of the autoantibody to GAD fixed on the ELISA plate, and having the second chain react in the fluid phase with labeled GAD. Thus this ELISA assay was similar in principle to fluid phase radioassays but with the advantage that no radioactivity was utilized.

5.3. IA-2 and IA-2β Autoantibodies

The insulinoma antigen-2 (IA-2 or ICA512) was the second identified ICA antigen component, a protein tyrosine phosphatase (29;30). IA2β, also called phogrin is a related molecule with regions of identical sequence. IA-2 and IA2β are components of the 40 and 37 (or 38) KD trypsinized membrane islet autoantigen protein fragments (31;32). Almost all autoantibodies to IA2β also react with IA-2, while approximately 10% of diabetic patients have autoantibodies only reacting with IA-2 but not with IA2β. In addition, two differentially spliced IA-2 messenger RNAs are expressed in islets with one form lacking exon-13 (termed ICA512bdc), which includes the transmembrane region of the molecule. Approximately 5-10% of patients with newly diagnosed type 1A diabetes have autoantibodies reacting with only one of the two forms of alternatively spliced IA-2 molecules. Very few patients with type 1A diabetes develop autoantibodies only to a single epitope of IA-2 and also lack autoantibodies reacting with GAD65 and insulin. Thus determination of autoantibodies reacting with one of the IA-2 constructs is usually sufficient and IA-2ic (intracytoplasmic portion of IA-2) is the most commonly utilized construct.

The major epitopes of IA-2 antigen are in the C-terminal portion of the molecule, or PTP domain of the intracellular region, and very few patients developing type 1A diabetes have IA-2AA only to the juxtamembrane region. Bonifacio and coworkers reported that autoantibodies to the juxtamembrane epitope are among the first to appear in individuals developing type 1A diabetes (33).

IA-2AA usually develop after GAA and IAA in studies of young Infants, that are either relatives of patients with type 1 diabetes or sampled from the general population (6). Thus it is very likely that the autoimmune response to IA-2 occurs late and closer to the time of diabetes onset. In multiple studies IA-2AA have the highest positive predictive value compared to other autoantibodies including GAA, IAA, and ICA.

Like the GAA assay, IA-2 assays have an excellent sensitivity and specificity in DASP workshops for the majority of laboratories. A modified ELISA for measuring IA-2 autoantibodies is available and gave results almost as sensitive as the radioassays in the last DASP workshop.

5.4. Other Putative Autoantibodies

In addition to the three autoantigens discussed above, many other islet related autoantigens have been described. A protein termed ICA69 was identified with ICA-positive human sera by Pietropaolo and coworkers and it was demonstrated that many diabetic patients have autoantibodies reacting with ICA69 in Western blot format (34). The assay for ICA69 could not be converted to a fluid phase radioassay and in general the Western blot format lacks sufficient specificity. ICA69 autoantibodies are also present in patients with rheumatologic disorders (35). ICA69 is identical in sequence to the cow’s milk-related protein p69 described by Dosch and coworkers (36). It was reported that patients with type 1A diabetes have antibodies to bovine serum albumin but this has not been confirmed (37). A number of studies indicate that

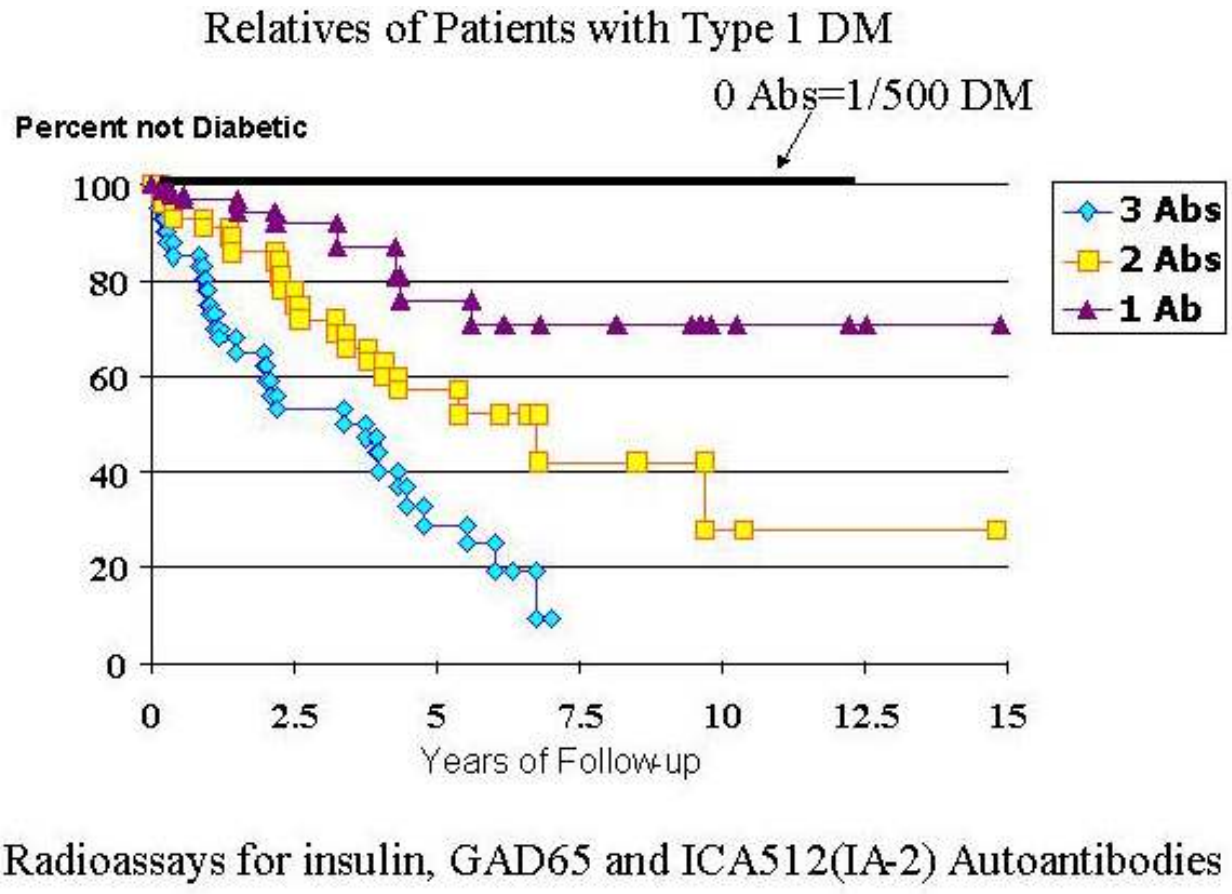

Figure 6. Combinatorial Autoantibody prediction. The risk of progression to type 1A diabetes is associated with the number of autoantibodies detected (of IAA, GAA, IA-2AA). Expression of 2 or more of these autoantibodies confers a higher risk for relatives of patients with type 1 diabetes (Modified with permission from 44).

glycolipids may be a component of ICA and sulphatides, GT3, and a GM2-1 ganglioside are three possible candidates (38). Buschard and coworkers reported that with immunochemical staining of sulphatides following thin-layer chromatography they detected anti-sulphatide autoantibodies in the majority of patients with recent-onset diabetes (39). Marcus and coworkers reported the presence of autoantibodies to GT3 in diabetic patients with a liposome-based assay (40). Finally, Dotta and coworkers isolated and partially sequenced a GM2-1 ganglioside from human pancreas and developed a TLC-based assay. They found the majority of individuals developing type 1 diabetes expressed autoantibodies to GM2-1 (38). To answer the questions of which glycolipids are autoantigens and which are the target autoantigens for ICA, it will be necessary to develop more robust assays for anti-glycolipid autoantibodies and such assays may eventually aid in the prediction of type 1A diabetes.

6. NATURAL HISTORY OF TYPE 1A DIABETES

Despite uncertainty about pathogenic importance, anti-islet autoantibodies provide the best current assays for the prediction of type1A diabetes. Prediction is not absolute, but can be expressed as a percentage risk of developing diabetes within a given time period. As illustrated in the Figure 6, the risk of progression to type 1A diabetes is mainly associated with the number of autoantibodies detected (of IAA, GAA, IA-2AA) and expression of 2 or more of these autoantibodies confers a risk of >50% to develop type 1A diabetes at 5 years for relatives of patients with type 1 diabetes (41). The recent national prospective large population DPT-1 study had a similar result. In the DPT-1 analysis the presence of cytoplasmic ICA in addition to the biochemical autoantibodies increased risk, confirming the existence of important islet autoantigens that are not yet identified. Among prediabetic or recent-onset diabetic children, 90% express one or more (3% in controls) and 80% express two or more of these autoantibodies (approximately 1/300 controls express>=2 of the autoantibodies). The expression of autoantibodies assorts independently in prediabetic individuals and thus, by testing all three autoantibodies, a high sensitivity can be achieved.

From several studies where children are followed from birth for the development of anti-islet autoantibodies, anti-islet autoimmunity frequently develops in the first year of life. IAA is usually the first anti-islet autoantibody to appear in life and less often GAA can be the first to appear.

Young children with developing multiple anti-islet autoantibodies have a high risk of progression to diabetes for both relatives and the general population. Anti-islet autoantibody positivity can develop at any age, and progression to diabetes is usually less rapid in older individuals. It is noteworthy that screening of the general population for diabetes risk is likely to be a major importance as 90% of individuals who develop type 1A diabetes have no family history of type 1A diabetes.

Approximately 5 to 10% of patients with gestational diabetes and adults with a diagnosis of type 2 diabetes develop anti-islet autoantibodies and are called LADA patients (Latent Autoimmune Diabetes of Adults) (42). These patients usually more rapidly progress to insulin dependence. Assays for GAA are most useful to identify LADA patients. It has been reported that the immediate initiation of insulin therapy rather than oral hypoglycemic agents may benefit LADA patients, but this is controversial (42). A diagnosis of type 1A diabetes alerts one to the risk of accompanying autoimmune disorders (e.g. thyroid autoimmunity, Celiac disease, and Addison's disease). Thus screening of these possible accompanying autoimmune diseases is highly recommended for patients with type 1A diabetes. Approximately ¼ of patients with type 1A diabetes are positive for anti-thyroid autoantibodies, 10% positive for transglutaminase autoantibodies (Celiac disease related), and 1.5% positive for 21-hydroxylase autoantibodies (Addison's disease related) (43). An increasing number of children presenting with diabetes lack anti-islet autoantibodies and appear to have forms of type 2 diabetes. Of note anti-islet autoantibodies decrease in prevalence with diabetes duration except for insulin antibodies which are induced by subcutaneous insulin injections, even with human insulin.

7. CONCLUSION

Anti-islet autoantibodies contribute to the development of diabetes in the NOD mouse model, but to date there is no firm data that they contribute to diabetes risk in man. The current generation of fluid phase high specificity/sensitivity assays for autoantibodies reacting with multiple islet autoantigens allow prediction and diagnosis of type 1A diabetes for the great majority of individuals. At present assays for autoreactive T cells lack specificity and sensitivity, but as these are the mediators of islet beta cell destruction, development of improved T cell assays, especially for monitoring trials of immunotherapy, is an important goal. Finally important islet autoantigens remain to be identified , with evidence provided by cytoplasmic islet cell antibody staining which is not absorbed with current identified antigens of GAD65, and IA-2. As additional islet autoantigens are biochemically characterized and assays for autoantibodies developed, it is likely that using "combinatorial" antibody algorithms, the sensitivity of prediction will improve, while specificity will be maintained by relying upon expression of multiple autoantibodies (e.g. >=2 autoantibodies present).

8. ACKNOWLEDGEMENTS

This work was supported by the National Institutes of Health (DK32083, DK32493, DK057538) Autoimmunity Prevention Center (AI50964), Diabetes Endocrine Research Center (P30 DK57516), Clinical Research Centers (MO1 RR00069, MO1 RR00051), the Immune Tolerance Network (AI15416) the American Diabetes Association, the Juvenile Diabetes Research Foundation and the Children's Diabetes Foundation.

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Key Words: Type 1 Diabetes, Autoantibodies, Prediction, Autoimmune, Review

Send correspondence to: George S. Eisenbarth, MD, PhD, Barbara Davis Center, P. O. Box 6511, Mail Stop B-140, Aurora, CO 80045, Tel: 303-724-6847, Fax: 303-724-6839, E-mail: george.eisenbarth@uchsc.edu

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