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Humoral Autoimmunity in Type 1 Diabetes: Prediction, Significance, and Detection of Distinct Disease Subtypes

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Type 1 diabetes mellitus (T1D) is an autoimmune disease encompassing the T-cell-mediated destruction of pancreatic β cells and the production of autoantibodies against islet proteins. In humoral autoimmunity in T1D, the detection of islet autoantibodies and the examination of their associations with genetic factors and cellular autoimmunity constitute major areas in both basic research and clinical practice. Although insulin is a key autoantigen and may be *primus inter pares* in importance among T1D autoantigens, an abundant body of research has also revealed other autoantigens associated with the disease process. Solid evidence indicates that autoantibodies against islet targets serve as key markers to enroll newly diagnosed T1D patients and their family members in intervention trials aimed at preventing or halting the disease process. The next challenge is perfecting mechanistic bioassays to be used as end points for disease amelioration following immunomodulatory therapies aimed at blocking immune-mediated β -cell injury and, in turn, preserving β -cell function in type 1 diabetes mellitus.

A famous influential scientist of the past millennium, the Renaissance polymath Leonardo da Vinci (1452–1519), wrote: “The supreme misfortune is when theory outstrips performance.” This is a situation that perhaps shares some similarities with our knowledge on the pathoetiology of autoimmune diabetes. The discovery of islet autoantigens and the identification of their immunodominant epitopes has shifted emphasis from epidemiological to mechanistic and exploratory intervention studies using these antigens, such as insulin, to prevent

T1D. An incredibly large number of immunomodulatory strategies were and are currently applied to prevent diabetes in animal models of the disease, such as the NOD mouse (Shoda et al. 2005). Many therapeutic strategies may delay or prevent diabetes in NOD mice, and the most promising ones are currently being tested in humans (Skyler 2011).

Type 1 diabetes mellitus was not always considered the classical autoimmune disease it is now known to be. For instance, insulin-dependent diabetes was known to occur occasionally

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in the Autoimmune Polyendocrine Syndrome I (APS I), a classic autoimmune syndrome with T cell and B-cell antibody abnormalities directed at adrenal, parathyroid, gonadal, thyroid, and other tissues. However, diabetes mellitus was not a constant, necessary, or sufficient feature of APS I. This condition is now known to be caused by mutations in the autoimmune regulator gene (AIRE) (Husebye and Anderson 2010). In 1974, Bottazzo et al. (1974) reported that sections of human pancreas treated with sera of diabetic patients who also had Addison's disease and myxedema showed cytoplasmic fluorescence over islets of Langerhans. This response was termed cytoplasmic islet cell antibodies (ICA). Furthermore, the existence of insulin autoantibodies and other autoantibodies against various islet proteins was not uncovered until years later. It was in 1983 that insulin autoantibodies were reported in sera of newly diagnosed patients with T1D, before any treatment with exogenous insulin (Palmer et al. 1983). In this finding, improvements of the sensitivity of the insulin antibody assay were instrumental for the determination that about one-half of newly diagnosed patients had autoantibodies that bound ^{125}I -labeled insulin.

Following the early discoveries on humoral autoimmunity in T1D, there has been a remarkable expansion in the detection of T1D-associated autoantibodies (Table 1) as well as in the characterization of the molecular basis of the antigenicity of their target proteins (Atkinson and Eisenbarth 2001; Pietropaolo and Eisenbarth 2001). This expansion has led to the uncovering of specific antigenic determinants, the development of biochemically defined immunoassays, and also to coordinated efforts to standardize assays across laboratories (Bonifacio et al. 2010b). However, it should be emphasized that T1D is primarily a T-cell-mediated disease. In humans, this conclusion was supported by a report of X-linked agammaglobulinemia in whom typical T1D developed at the age of 14 yr (Martin et al. 2001). This report shows that T1D can occur in the complete absence of B cells and autoantibodies. This observation led to the conclusion that B cells are not an essential requirement for the development of this disease

and that the principal effector mechanisms are mediated by T cells. Thus, although the presence of islet autoantibodies may not be a *sine qua non* feature of autoimmune diabetes, advances in detection of humoral autoimmunity have had critical implications in the identification of at-risk subjects that can become participants in clinical trials to assess immunomodulatory strategies to prevent and treat T1D.

ASSAY STANDARDIZATION AND HARMONIZATION

Currently, the consensus on methodological standardization encompasses assays to detect autoantibodies against four major islet autoantigens, namely, insulin, glutamic acid decarboxylase (GAD), the neuroendocrine antigen ICA512/IA-2, I-A2 β (phogrin), and the zinc transporter ZnT8. Although there is an overall agreement regarding the methodologies to assess humoral autoimmunity in T1D, the ability to detect T1D-related autoantibodies and to accurately measure their titer has also clear organizational implications because of the need to interpret values across laboratories, populations, and countries and to promote the development of assay systems that can improve the comparative assessment of results.

These strategies have included the adoption of a serum reference standard for GAD and IA-2 antibodies by the World Health Organization (WHO) and the creation of the Diabetes Antibody Standardization Program (DASP), which was established in 2000 by the Immunology of Diabetes Society (IDS) and the Centers for Disease Control and Prevention (CDC) (Verge et al. 1998; Bonifacio et al. 2002). It has been DASP activities that have led to the recognition of insulin, GAD, IA-2, and ZnT8 as the main targets for the validation and standardization of assays. The need to provide a broader basis for comparison of results has also evolved into harmonization efforts to produce unified protocols that can take into account differences in standards such as the WHO standard, relative to the use of common standards used by the National Institute of diabetes and Digestive and Kidney

Table 1. Most characterized islet autoantigens associated with type 1 diabetes

	Localization	Humoral response	Cellular response
Insulin ^a	Secretory granules of pancreatic β cells Human thymus and PAE cells (peripheral antigen expressing cells)	Insulin autoantibodies are found in virtually 100% of young children (<5 yr of age) before the onset of Type 1 diabetes Correlation with younger age and fast rate of progression to insulin requirement in first-degree relatives of IDDM patients Prophylactic subcutaneous injection of insulin, oral and intranasal administration prevents type 1 diabetes in NOD mice	PBLs from humans and NOD mice react with insulin β -chain
GAD65 ^a and GAD67	Synaptic-like microvesicles of neuroendocrine cells Present in testis and ovary Human thymus and PAE cells	A subset of 64-kDa autoantibodies recognize GAD Autoantibodies to GAD65 are present in 70%–80% of pre-diabetic subjects or newly diagnosed diabetic patients GAD antibodies are also detected in patients with stiff man syndrome, and with autoimmune thyroid disease Radioimmunoassay of in vitro transcribed/translated GAD65 is useful for large-scale screening	PBL responses to GAD65 in newly diagnosed diabetic patients and in NOD mice
ICA512(IA-2) ^a and phogrin (IA-2 β)	Neurosecretory granules (pancreatic β cells, CNS, pituitary, adrenal) Human thymus and PAE cells	Autoantibodies to ICA512 (IA-2) are present in ~60% of pre-diabetics or newly diagnosed IDDM patients Relationship between 37,000- and 40,000-Da tryptic fragments and ICA512(IA-2) Radioimmunoassay of in vitro transcribed/translated ICA512(IA-2) is useful for large-scale screening	PBL responses in newly diagnosed diabetic patients
ZnT8 (Slc30A8) ^a	Zn transporter, a member of the cation diffusion facilitator family showing abundant expression in β cells Expressed also extra-pancreatically	Targeted by autoantibodies in 60%–80% of newly diagnosed T1D patients and in ~26% of patients negative for other islet autoantibodies Relevant polymorphic variants are Trp-325 and Arg-325	Autoreactive T cells to ZnT8 found in human T1D

Continued

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Table 1. *Continued*

	Localization	Humoral response	Cellular response
Islet cell autoantigen 69 kDa (ICA69)	Predominantly neuroendocrine tissues Human and mouse thymus	Autoantibodies to ICA69 can be detected in 43% of pre- diabetic subjects by Western blotting	Association between HLA-DR3 and PBL responses in newly diagnosed type 1 diabetics
Chromogranin A	Neurosecretory granules Neuroendocrine tissues	Circulating ChgA found in NOD mice	Autoreactive T cells to ChgA found in NOD mice
Carboxypeptidase H	Neurosecretory granules	Autoantibodies to carboxypeptidase H found in ~20% of pre-diabetics	Present
Ganglioside GM2-1	Pancreatic islet cells	Autoantibodies to GM2-1 detected in ~80% of pre- diabetic subjects and NOD mice	?
Imogen 38 (38 kDa)	Mitochondria widely distributed with variable levels of expression	Presence of circulating antibodies to 38-kDa proteins Possible presence of antibodies to imogen 38	PBLs from newly diagnosed diabetics proliferate to imogen 38
Glima 38	Amphiphilic N-Asp glycated β -cell membrane protein that is expressed in islets and neuronal cell lines	Autoantibodies to Glima 38 can be detected in 14%–22.7% of newly diagnosed diabetics and pre-diabetics. The majority of these patients are negative for GAD65 and/or ICA512(IA- 2) autoantibodies	?
Peripherin	Neuronal cells	Autoantibody response against peripherin in NOD mice and in patients with type 1 diabetes and other autoimmune disorders	T-cell responses against peripherin in NOD mice
Heat-shock protein (Hsp60)	Ubiquitously inducible	Antibodies to Hsp60 in pre- diabetic NOD mice	Hsp60-reactive T cells can accelerate disease in pre-diabetic NOD mice

^aBiochemical autoantibody assays readily available for large screening programs.

diseases (NIDDK) consortia laboratories (Bonifacio et al. 2010b).

A study by Bonifacio et al. (2010a) on the harmonization of GAD and IA-2 autoantibody assays by NIDDK consortia laboratories, using large-volume calibrators and common protocols, indicated that common protocols and use of large-volume working calibrators are effective measures to ensure consistency in autoantibody measurements, although the results were com-

parable but not identical. However, other studies indicate that complete harmonization and cross-validation and interpretation of results will require additional effort.

Even though there has been considerable progress in standardizing GAD65 and IA-2 autoantibody assays, the insulin autoantibody assay is least standardized, with most laboratories in DASP workshops having less than acceptable sensitivity and/or specificity. This probably



relates to the very low capacity of T1D autoantibodies and a limited conformational epitope recognized by these antibodies that is obscured if insulin is bound to solid phase. We have recently developed an electrochemiluminescent insulin autoantibody assay using pro-insulin as target antigen that appears to enhance sensitivity and specificity (Yu et al. 2012).

The advent of biochemically defined assays has permitted the development of chimeric and hybrid antigen constructs that can simultaneously assess the presence of autoantibodies against more than one antigen. This strategy is illustrated in a recent study with a triple chimeric islet autoantigen containing key antigenic determinants to IA-2 and key variants of the Zn transporter (ZnT8WR) as an accurate and relevant T1D antigen (Yu et al. 2010). The use of these chimeric assays holds promise to save labor and resources to more efficiently screen at-risk populations.

DISCOVERY OF AUTOANTIGENS AND RELEVANT EPITOPES BASED ON AUTOANTIBODIES, MAJOR T1D-ASSOCIATED ISLET AUTOANTIBODIES CURRENTLY ASSAYED

Initial studies by Atkinson et al. (1993) identified a subset of islet cell antibodies (ICA) associated with a more clinically significant pancreatic β -cell injury in a subgroup of first-degree relatives of T1D probands. This subset of ICA was termed “non-GAD reactive” because ICA reactivity could only be partially blocked by GAD65 (Atkinson et al. 1993). This observation implied that multiple islet autoantigens are recognized by T1D-specific humoral responses. We subsequently found that a subset of cytoplasmic ICA is associated to a more rapid progression to insulin-requiring diabetes in GAD65 and IA2 antibody-positive relatives as compared with relatives with GAD65 and IA2 antibodies without ICA. This ICA reactivity more than likely is caused by a subset of ICA-recognizing unidentified islet autoantigen(s) (Pietropaolo et al. 2005).

As autoimmunity in T1D progresses from initial activation to a chronic state, there is often

a higher number of islet autoantigens reacting with T cells and autoantibodies. This condition is termed “epitope spreading.” Compelling evidence indicates that islet autoantibody responses against multiple islet autoantigens are associated with progression to overt disease (Verge et al. 1996). Several additional T1D-related autoantigens have been identified, which include islet cell autoantigen 69 kDa (ICA69), the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP), chromogranin A (ChgA), the insulin receptor, heat shock proteins, and the antigens jun-B,16, CD38 (Pietropaolo and Eisenbarth 2001), peripherin, and glial fibrillary acidic protein (GFAP), and the like (Table 1) (Winer et al. 2003).

The existence of IgG immunoglobulins directed to epitopes of islet antigens strongly implies the influence of T-cell participation in the autoimmune response. Naturally processed epitopes of islet autoantigens represent the targets of effector and regulatory T cells in controlling β -cell-specific autoimmune responses (Di Lorenzo et al. 2007). In particular, naturally processed HLA class II allele-specific epitopes recognized by CD4⁺ T cells (corresponding to the intracellular domain of IA2) were identified after native IA2 antigen was delivered to EBV-transformed B cells and peptides were eluted and analyzed by mass spectrometry (Peakman et al. 1999). Additionally, evidence suggests a synergistic pro-inflammatory role for plasmacytoid dendritic cells and IA-2 autoantibodies in T1D (Allen et al. 2009). These studies may lead to the identification of novel naturally processed epitopes recognized by CD4⁺ T cells, which, in turn, may represent potential therapeutic agents for T1D, either in native form or as antagonistic altered peptide ligands.

Insulin Autoantibodies

Insulin is a hormone produced by the pancreatic β cells, which is not only central to regulating carbohydrate and fat metabolism, but is also central in its pathological role as a T1D autoantigen. Insulin is the predominant secretory product of pancreatic β cells whose autoimmune destruction leads to insulin deficiency

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and consequent metabolic decompensation of glucose homeostasis (Nakayama et al. 2005). Investigations of the immunologically relevant regions of the insulin molecule conducted in NOD mice revealed that the 9–23 amino acid sequence of the insulin B chain (termed B9-23) and the effect of intracellular processing of molecules, such as insulin, within the β cell can lead to formation of immunogenic epitopes (Fig. 1) (Crawford et al. 2011).

A high titer of insulin autoantibodies (IAA) at younger ages is consistent with the concept that these patients develop a more aggressive dis-

ease course. In particular, insulin autoantibodies levels >2000 nU/mL are almost exclusively found in patients who progress to T1D before age 5 yr, and less than half of individuals developing T1D after age 15 yr carry detectable levels of IAA.

A study from the Finnish Type 1 Diabetes Prediction and Prevention Study, comprising a large population of 2448 genetically at-risk children (Kimpimaki et al. 2001), showed that IAAs are usually the first islet autoantibody to appear in the natural history of T1D and a powerful identifier of disease progression in children followed from birth (Steck et al. 2011).

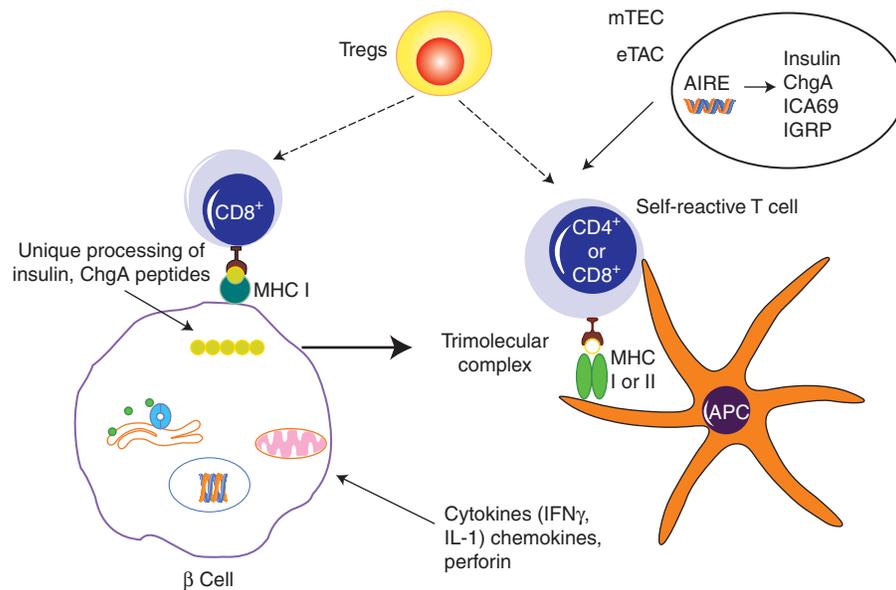


Figure 1. Unique processing of β -cell proteins may lead to antigenicity. The β cell itself not only produces target antigens, but also it modifies molecules, such as insulin and ChgA, by cleavage at critical sites creating peptides recognized by pathogenic T-lymphocytes (Stadinski et al. 2010; Crawford et al. 2011). Processing of molecules such as insulin within the β cell generates peptides that are then taken up by antigen-presenting cells either as whole dead β cells or specifically granules of β cells for eventual further processing/presentation of islet peptides to self-reactive T cells (Crawford et al. 2011). The trimolecular complex, involving an (MHC-presenting molecule)/(peptide in appropriate “register”)/(T-cell receptor recognizing both), like a lock and key is an essential recognition unit for adaptive organ-specific autoimmunity. The fact that in the thymus the absence of this type of processing combined with the low affinity of B:9-23 binding to IA⁸⁷ in register 3 may explain the escape of insulin-specific CD4⁺ T cells from the mechanisms that usually eliminate self-reactive T cells. Both regulatory (Treg) and effector T-lymphocytes are produced, and their balance is crucial for maintaining tolerance (Bluestone and Boehmer 2006). Evidence suggests that the inappropriate MHC class I expression detected in pancreatic islets in T1D may not be solely due to the effect of cytokines such as IFN γ (Bottazzo et al. 1985). It is conceivable that this molecule may function as an indicator of cell stress, likely present in residual pancreatic islets from T1D patients, and may be recognized by a subset of T cells in an unusual interaction.



Glutamic Acid Decarboxylase (GAD) Autoantibodies

In an earlier seminal report, incubation of rat islets with radioactively labeled [³⁵S]-methionine and subsequent immunoprecipitation of solubilized membranes with serum from newly diagnosed patients with T1D or controls showed that an antigen with a molecular weight of 64 kDa was precipitated by sera from T1D patients (Baekkeskov et al. 1989). The antibodies to this 64-kDa antigen were present in ~80% of new onset patients and in pre-diabetics before the appearance of clinical disease. The nature of the 64-kDa antigen remained unknown until the report by Solimena et al. (1988) showing autoantibodies to GABA-ergic neurons and pancreatic β cells in an unusual condition termed Stiff Man syndrome. Glutamic acid decarboxylase is the enzyme that catalyzes the conversion of glutamic acid to gamma amino butyric acid (GABA), a potent inhibitory neurotransmitter. This led Baekkeskov et al. (1990) to rapidly identify GAD as the 64-kDa autoantigen in T1D (Karlsen et al. 1991; Atkinson et al. 1993). Other molecular-related forms of GAD, such as the 67-kDa isoform, have subsequently been identified (Hagopian et al. 1993). Autoantibodies against GAD are a predictor of progression to overt diabetes. When coupled with insulin autoantibodies and islet cell antibodies (ICA), their ability to predict the likelihood of developing T1D in asymptomatic first-degree relatives of T1D patients is quite high.

IA-2 (ICA512) Autoantibodies

The neuroendocrine antigen IA-2 (ICA512) is a major autoantigen in T1D (Lan et al. 1994). It is an enzymatically inactive member of the tyrosine phosphatase family, involved in regulating insulin secretion. Assessment of the presence of IA-2 autoantibodies contributes to the predictability of the likelihood of developing T1D. As shown by Verge et al. (1996) and others (Pietropaolo et al. 2002; Achenbach et al. 2004), ICA512 (IA-2) and its homolog IA-2 β (phogrin) are both neuroendocrine molecules. The deduced ICA512(IA-2) cDNA sequence reveals a 979-

amino-acid protein with a single transmembrane region and with significant homology to the receptor-type PTP (RT-PTPase). A PTP homolog, termed phogrin, was subsequently identified. Subcellular fractionation of insulinoma tissue showed that both IA-2 and phogrin had a very similar cellular distribution to that of insulin and carboxypeptidase H, and these two molecules are predominantly localized in the secretory granules of neuroendocrine cells (Wasmeier and Hutton 1996; Mziaut et al. 2006).

Although the main immune reactive region of the IA-2 molecule was thought to reside within its intracellular domain (amino acids 601–979), the inclusion of this region together with other reactive epitope regions of the molecule (encompassing amino acid residues 256–979) has permitted the development of highly accurate constructs to assess the presence of antibodies against IA-2 (Kawasaki et al. 1997). However, other research has also suggested that humoral autoimmunity against the intracellular domain of the molecule is related to a high risk of faster T1D development (Fig. 2) (Morran et al. 2010).

Zinc Transporter Family Member 8 (ZnT8) Autoantibodies

ZnT8 is a member of the cation diffusion facilitator family, with abundant expression in pancreatic β cells, although it is also expressed in extra-pancreatic tissues (Chimienti et al. 2004; Wijesekara et al. 2009). In the β cell, it plays an important physiological role because Zn, which is highly concentrated in β cells, is needed for normal insulin storage. β -Cell-specific deletion of ZnT8 in mice results in glucose intolerance, reduced β -cell zinc accumulation, and anomalous insulin granules, as well as blunted first-phase glucose-stimulated insulin secretion, reduced insulin-processing enzyme transcripts, and increased pro-insulin levels (Wijesekara et al. 2010). Its relevance as an important T1D autoantigen was first described by Wenzlau et al. (2007), following an evaluation of 68 candidate islet autoantigens compiled from multidimensional analyses of microarray mRNA expression profiling. The assessment of the zinc transporter ZnT8 (Slc30A8 encodes ZnT8) indicated that

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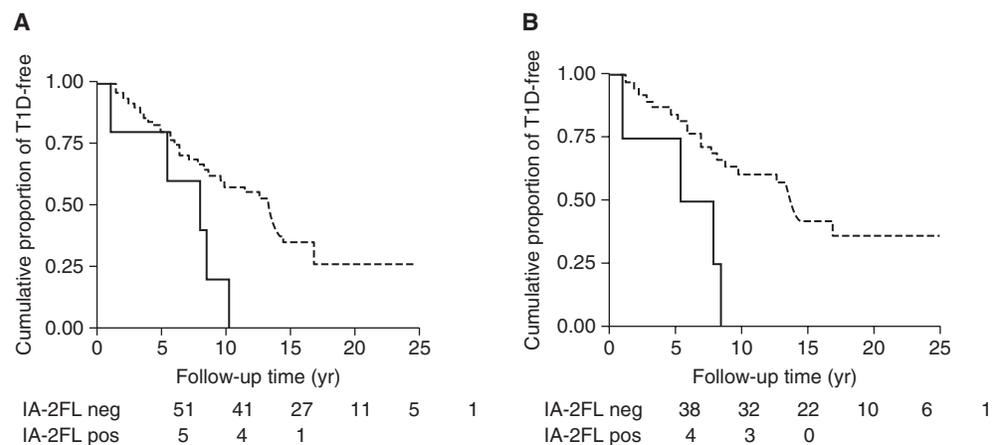


Figure 2. (A) The rate of progression to T1D development in relatives negative for ICA512bdc carrying GAD65 in the absence (dashed line) or presence (solid line) of autoantibodies reacting with IA-2 full-length (IA-2FL) (amino acids 1–979) (log rank, $P = 0.016$). In this subgroup of relatives, the cumulative risk of developing insulin-requiring diabetes in IA-2 full-length antibody-positive FDR was strikingly high, being 100% by 11 yr of follow-up in GAD65 antibody positives (log rank, $P = 0.026$). (B) This effect was also observed in FDR selected for being negative for ICA512bdc having at least two islet autoantibodies in the absence (dashed line) or presence (solid line) of autoantibodies reacting with IA-2 full length (IA-2FL) (amino acids 1–979) (log rank, $P = 0.003$). In these relatives, the cumulative risk of progressing to overt diabetes was 100% by 10 yr of follow-up (log rank, $P = 0.022$). (Figure is from Morran et al. 2010; reprinted, with permission, from The Endocrine Society © 2010.)

it was targeted by autoantibodies in 60%–80% of new-onset T1D compared with <2% of healthy controls, <3% type 2 diabetic patients, and in up to 30% of patients with other T1D-associated autoimmune pathologies. Interestingly, ZnT8 antibodies were found in 26% of T1D subjects who had not shown antibody positivity to other commonly measured autoantigens such as glutamate decarboxylase (GAD), protein tyrosine phosphatase IA-2 (IA2), insulin (I), or in the assay for cytoplasmic islet cell antibodies (ICA). Further research has revealed polymorphisms in ZnT8 that are relevant to its role as a major T1D autoantigen. There are three polymorphic variants located in the intracellular (carboxyl terminus) domain of the transporter protein, namely, Arg-325, Trp-325, and Gln-325. Of these variants, Trp-325 (W) and Arg-325 (R) have been shown to be the major autoantigenic polymorphisms in T1D, and use of a construct containing the W and R variants (ZnT8WR) (Wenzlau et al. 2008) has proven its efficacy as a screen for T1D-associated humoral autoimmunity. More recently, a chimeric

construct containing amino acid residues 609–979 of the intracellular domain of IA2, linked to peptides containing both ZnT8 W and R polymorphisms, has been successfully developed and tested as a broader and more economical screen to detect patients showing humoral autoimmunity against IA-2 and/or ZnT8 (Yu et al. 2010).

CONNECTING GENETICS WITH AUTOANTIBODIES

Type 1 diabetes is a complex polygenic disease for which there is a small number of genes with large effects (i.e., HLA) and a large number of genes with small effects (Todd et al. 2007; Barrett et al. 2009). Risk of T1D progression is conferred by specific HLA DR/DQ alleles (e.g., DRB1*03-DQB1*0201 [DR3] or DRB1*04-DQB1*0302 [DR4]) (Eisenbarth 2007). In addition, HLA alleles such as DQB1*0602 are associated with dominant protection from T1D in multiple populations (Pugliese et al. 1999).

It is commonly accepted that HLA typing is not the optimal primary screening tool for T1D, and it is not sufficient alone to predict the disease onset. However, HLA genotyping in first-degree relatives of T1D probands can be useful. Aly et al. (2006) reported that risk for islet autoimmunity drastically increased in DR3/4-DQ2/DQ8 siblings who shared both HLA haplotypes identical by descent with their diabetic proband sibling (63% by age 7, and 85% by age 15) as compared with siblings who did not share both HLA haplotypes with their diabetic proband siblings (Fig. 3). These data indicate that HLA genotyping at birth may identify individuals at very high risk of developing T1D, before the occurrence of clear signs of humoral autoimmunity and eventually overt disease. In addition, we previously identified a phenotype in subjects with high-risk HLADQ haplotypes who remained seronegative for islet autoantibodies even after significant length of follow-up during progression to the insulin-requiring T1D (Pietropaolo et al. 2002). Finally, in a more recent study, Howson et al. (2011) examined the relationships between the presence of GAD and IA-2 autoantibodies with HLA genes and 3779

single-nucleotide polymorphisms (SNPs) in 2531 subjects with childhood-onset T1D. The results of this comprehensive assessment indicated that GAD autoantibodies were associated primarily with HLA-DQB1. For IA-2 autoantibodies, the strongest association was with HLA-DRB1. However, there was no association between the presence of antibodies against either IA-2 or GAD and the T1D high-risk genotype, HLA-DRB1*03/04, although surprisingly, there was an association of IA-2 autoantibody positivity with the T1D-protective allele HLA-DQB1*030. These results overall suggest that the presence of high-genetic-risk HLA haplotypes and the presence of islet autoantibodies do not necessarily follow an obligate correlation in the development of autoimmune T1D.

CLINICAL APPLICATIONS

Relevance as Predictors of Risk for T1D, Role of Age and Specificity

Combining both immunological and metabolic strategies (e.g., oral glucose tolerance test or the

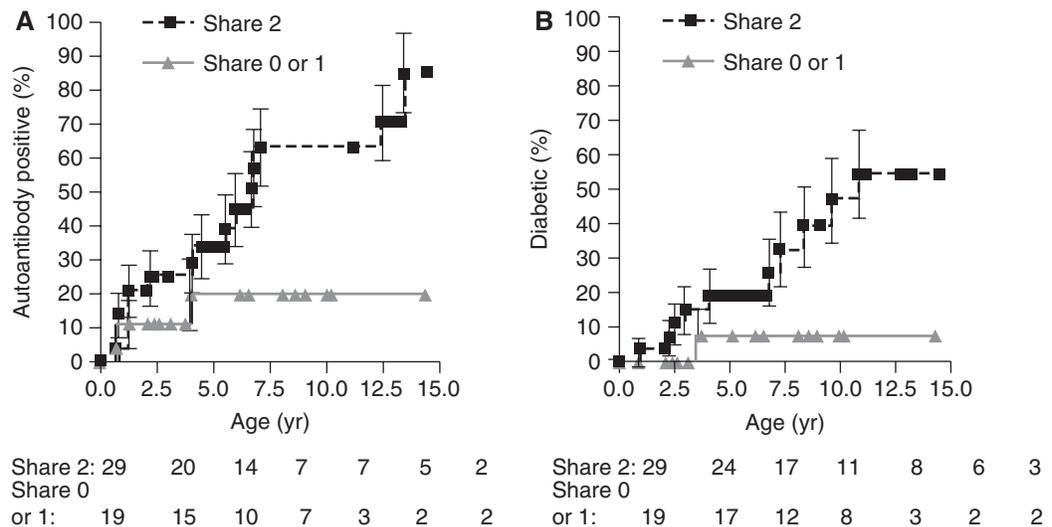


Figure 3. Haplotype sharing survival curves. Shown is progression to anti-islet autoimmunity (A) and type 1A diabetes (B) in DR3/4-DQ8 siblings stratified by the number of HLA haplotypes shared with their proband siblings. $n = 48$ for A and B; error bars for all panels represent the SEM. (Figure from Aly et al. 2006; reprinted, with permission, from National Academy of Sciences © 2006.)

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first-phase [1 + 3 min] insulin response of an intravenous glucose tolerance) is helpful, the current opinion is that type 1 diabetes progression can be predicted with 80%–100% accuracy within 5- and 10-yr follow-up, respectively (Xu et al. 2010).

A study on the Diabetes Autoimmunity Study in the Young (DAISY) cohort showed that 89% of children who progressed to T1D had two or more islet-related autoantibodies (Steck et al. 2011). Importantly, age of diagnosis of diabetes was strongly correlated with age of

appearance of first autoantibody and IAA levels. By life-table analysis (Fig. 4A), children exhibiting two or more autoantibodies showed a nearly linear progression to diabetes ($P < 0.0001$). Children with persistently positive IAA levels had a higher progression rate to overt T1D (100% by 5.6 yr) as compared with children with fluctuating IAA levels (63% by the 10-yr follow-up; $P < 0.0001$) (Fig. 4B). Finally, in children enrolled in the DAISY study followed to the development of diabetes onset, only high IAA titer correlated with rapid progression to T1D

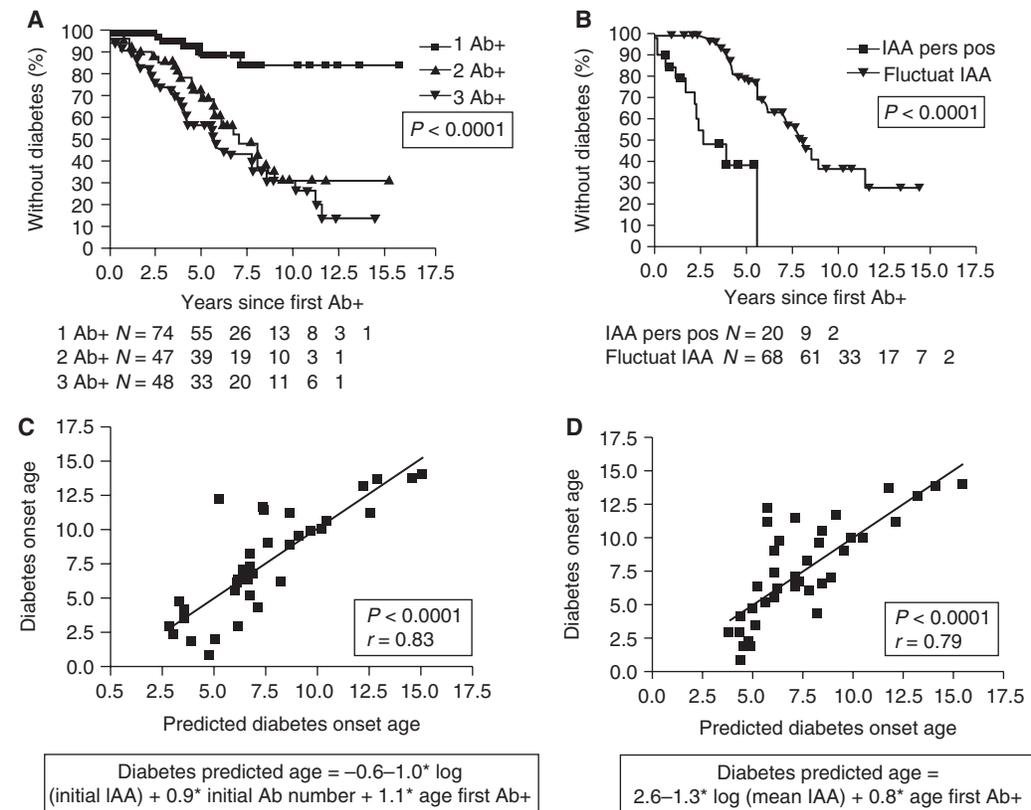


Figure 4. (A) Progression to diabetes in children positive for anti-islet autoantibodies ($n = 169$). There was no significant difference in the progression rate between subjects with two or three positive antibodies. (B) Progression to diabetes in children with persistently positive IAA levels and fluctuating IAA levels ($n = 88$). (IAA pers pos) Persistently positive IAA levels; (Fluctuat IAA) fluctuating IAA levels. (C) Predicted age of diagnosis of diabetes (initial IAA, GAD, and IA-2 levels; $n = 38$). Analyses performed in all subjects who had their first antibody measurement before 1.5 yr and progressed to diabetes. (D) Predicted age of diagnosis of diabetes (mean IAA, GAD, and IA-2 levels; $n = 38$). Analyses performed in all subjects who had their first antibody measurement before 1.5 yr and progressed to diabetes. (Figure from Steck et al. 2011; reprinted, with permission, from the American Diabetes Association © 2011.)



onset (Fig. 5) ($P < 0.0001$). As a matter of fact, this effect was not evident with respect to the presence of high GAD65 or IA-2/ICA512 autoantibody titer (Fig. 6). Therefore, insulin autoantibody levels at the time of diagnosis are inversely related to the age of the patient, being highest in those < 5 yr of age, and hence, IAAs appear to be an early marker of β -cell destruction. The titer of insulin autoantibodies along with the insulin secretory response judged by the first-phase insulin levels at 1 and 3 min after an intravenous glucose challenge, has also been successfully used to construct mathematical models to predict the likelihood of clinical diabetes in asymptomatic first-degree relatives of patients (Eisenbarth 1986). Investigators from the DAISY study reported that five children were found to have persistent IAAs before 1 yr of age, and four of them went on to develop the clinical onset of T1D (all before 3.5 yr of age). In contrast, children not exhibiting persistent IAAs before the age of 1 yr rarely rapidly developed an insulin requirement. When analyzing only children followed from birth who progressed to diabetes, the two major predictors of age of diabetes diagnosis were the age at which autoantibodies first appeared and the mean level of insulin autoantibodies (Fig. 4A,B). These observations emphasize the utility of IAAs, particular-

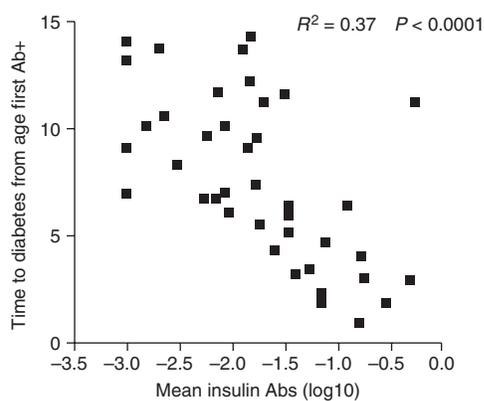


Figure 5. Mean log IAA versus time to diabetes progression in first-degree relatives of T1D probands. In these children from the DAISY study followed to the development of diabetes onset, high IAA levels correlated with rapid progression to T1D onset.

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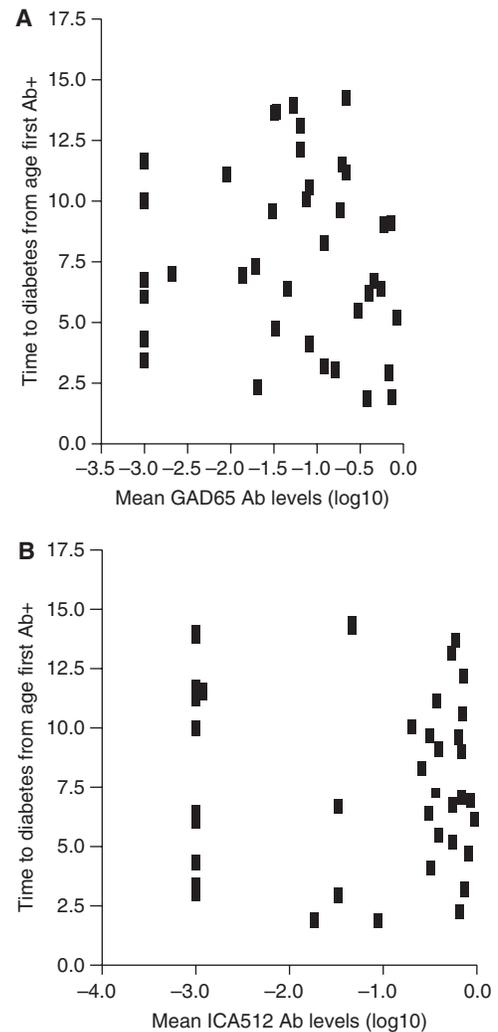


Figure 6. (A) GAD65, and (B) IA-2/ICA12 antibody titers in children from the DAISY study followed to the development of diabetes onset. There is no correlation with high GAD65 or IA-2/ICA512 titers and rapid progression to diabetes.

ly in younger populations, and justify the need to design trials focused on such a young group. However, the success of these strategies depends on the safety and effectiveness of therapeutic regimens. Indeed, this was the strategy in the trial to prevent development of T1D (DPT-1) that successfully predicted the development of diabetes in first-degree relatives of T1D patients (Skylar et al. 2001).

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Detection of Novel Forms and Subgroups of Diabetes, the LADA Example

Although the detection of islet autoantibodies is overall the major diagnostic hallmark of autoimmune T1D risk assessment and progression, the ability to detect these autoantibodies has also allowed the characterization of unique subpopulations of the disease, with evident implications for therapeutic strategies. The specific characterization of the diverse subtypes of diabetes has been a dynamic field of research and an active area of discussion. A well-documented example is the case of patients who are generally adults, who present a type 2 diabetes phenotype as well as circulating islet autoantibodies (Zimmet et al. 1994). These characteristics are defined as Latent Autoimmune Diabetes of Adults (LADA) and sometimes are termed type 1.5 diabetes (Palmer and Hirsch 2003). The immunological diagnosis of LADA relies primarily on the detection of autoantibodies against GAD65 in the serum of clinically diagnosed T2D patients who also show impaired insulin secretion and a high frequency of being on insulin treatment. Additionally, it has been previously reported that LADA patients possess T cells reactive to islet proteins as is also the case in “canonical” type 1 diabetes, in contrast to classic autoantibody-negative T2D patients (Brooks-Worrell et al. 2011).

The presence of GAD65 and/or IA-2 autoantibodies in T2D patients diagnosed by conventional criteria (ADA or WHO) is not uncommon among older diabetics, being 5%–10% or higher, especially in those on insulin therapy (Barinas-Mitchell et al. 2004, 2006; Leslie et al. 2006; Pietropaolo et al. 2007). Thus, there may be as many GAD65 antibody-positive older diabetics as there are children affected by T1D. This is not a trivial issue. Moreover, additional immunological markers of autoimmune diabetes might identify even a larger sample of clinically diagnosed T2DM patients. Given its relative simplicity, testing for GAD65 and IA-2 autoantibodies should be part of the diagnostic assessment for clinically diagnosed T2D because it might predict the rate of progression to insulin requirement in older populations. Those found to be GAD65 antibody-positive are prob-

ably candidates for early insulin therapy or more aggressive oral therapies to lower their glycohemoglobin levels. Clinical trials and epidemiological observational studies should clearly separate GAD65 antibody-positive older diabetics from those who are GAD65 antibody-negative.

Relationship with Other Autoimmune Diseases (Altered Antigens)

During immune system development, lymphocytes that react to self-antigens in the thymus and bone marrow are deleted. However, host molecules, in particular proteins and nucleic acids, are constantly being modified in the course of normal physiological events. A key posttranslational modification in autoimmunity appears to be the citrullination of arginine amino acid residues, by the enzymatic deimination of arginine to citrulline (Eggleton et al. 2008; Doyle and Mamula 2012). This reaction is catalyzed by the enzyme peptidyl arginine deiminase (PAD) (Soderlin et al. 2004). In multiple sclerosis and RA, citrullinated isoforms of myelin basic protein (Moscarello et al. 2007) and fibrin (Masson-Bessiere et al. 2001) have been found in the brain and synovia, respectively. It must be pointed out that the detection of anti-citrullinated protein antibodies (ACPA) has proven extremely useful in the early diagnosis and assessment of prognosis in rheumatoid arthritis (RA) and has also led to insights into gene environment effects in autoimmune disease (Kastbom et al. 2004).

With regard to T1D, processing of molecules such as insulin within the β cell generates peptides that are then taken up by APCs either as whole dead β cells or specifically granules of β cells for eventual further processing/presentation of islet peptides to self-reactive T cells (Fig. 1) (Crawford et al. 2011). Furthermore, Stadinski et al. (2010) have shown that chromogranin A (ChgA) is an autoantigen in T1D (Table 1), and that the peptide WE14 from ChgA stimulates diabetogenic CD4⁺ T-cell clones. The natural form of the antigen in β -cell extracts is far more potent than an unmodified synthetic WE14 peptide, suggesting that this peptide may be posttranslationally modified with a carbonyl group in murine pancreatic islets.

It is quite possible that the number of reported altered neo-antigens will increase in T1D, because the attendant hyperglycemic and pro-oxidative metabolic milieu includes abnormal glycosylations and oxidative damage to proteins. The latter are processes that affect autoantigenic proteins in other diseases such as RA and SLE, and thus provide a conceptual relationship of diabetes with other autoimmune diseases with respect to autoantigenesis.

How Do Autoantibody Specificities Relate to T-Cell Response and T-Cell Specificity?

A widening body of both basic and clinical investigations has tried to address the relationship between islet autoantibodies and the activation of T cells by cognate autoantigens in T1D. An early report was a study by Keller (1990), who described proliferative responses to human insulin in T-lymphocytes during the peri-diabetic period, in a significant proportion (67%) of ICA-positive, first-degree relatives of T1D patients who had never been treated with insulin, whereas in contrast, none of the controls showed the proliferative effect. Interestingly, the T-cell responses were not correlated with levels of insulin autoantibodies. Similarly, studies addressing other islet autoantigens by Atkinson et al. (1992) reported that there was a higher likelihood of a proliferative response to GAD in peripheral-blood mononuclear cells from patients with T1D and in relatives positive for ICA, compared with that seen in healthy controls and ICA-negative relatives of the patients. Other T1D-associated autoantigens targeted by humoral autoimmunity have also been shown to stimulate T cells, such as the cases of T-cell proliferation in response to IA-2 (Durinovic-Bellò et al. 1996; Roep et al. 1996; Reijonen et al. 2004). More recently, cellular immunoblot, U.K.-ELISPOT, and T-cell proliferation assays seemed to distinguish responses from patients with type 1 diabetes and healthy control subjects (Herold et al. 2009). Albeit many attempts have been made, the demonstration in the clinical setting of the relationship between T cell and autoantibody responses is difficult for at least the following reasons: (1) The frequency

of antigen-specific autoreactive CD4⁺ T cells is very low in peripheral blood. (2) There might be variability in their appearance in peripheral blood. (3) The methodologies to detect these T cells can be laborious (i.e., some methodologies require expansion of CD4⁺ T cells with antigen for up to 10 d). (4) There is very a high degree of variability inherent to T-cell proliferation assays.

Does Ig Isotype Matter?

It is well known that pro-inflammatory antibodies of the human IgG1 and IgG3 subclass can cause complement activation, attract lymphocytes to the target organ, and are used as surrogate markers of disease activity (Gomez et al. 2010). In T1D, a study investigating Ig isotypes against insulin, assessed insulin autoantibody (IAA) isotypes in children who were at genetic risk of T1D and encompassed IAA-positive children who progressed to T1D as well as non-progressor infants (Hopppu et al. 2004a). In this study, it was found that children who progressed rapidly to T1D showed strong IgG1 and IgG3 immunoreactivity to insulin, in contrast to a weak or absent IgG3 response, which was deemed to confer relative protection. Hopppu et al. (2004b) examined progression to onset, among non-diabetic first-degree relatives of children with T1D and found the GAD65 isotype response, and concluded that the detection of isotypes against GAD65 could not reliably discriminate progressors from non-progressors to diabetes onset among antibody-positive siblings of children with T1D.

In summary, the characterization of autoantibody isotypes against the islet autoantigens in the estimation of T1D risk has revealed a diversity of results that need a body of confirmatory reports before a consensus can be achieved on their applicability for clinical practice.

EVIDENCE FOR INVOLVEMENT OF B-LYMPHOCYTES AND AUTOANTIBODIES IN PATHOGENESIS

An accurate account of the relevance of B cells in autoimmune diabetes was shown earlier in

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seminal studies using the NOD mouse to generate B-lymphocyte-deficient mice (Hanson et al. 1996) and also through the use of monoclonal antibodies to deplete the population of B cells (Noorchashm et al. 1997). In these studies, the inhibition of B cells resulted in the concomitant inhibition of insulinitis. The physiological significance and potential clinical relevance of these early studies have now been validated and underscored by the use of an anti-CD20 antibody (rituximab) proved highly promising not only in the treatment of T1D but also in other autoimmune disorders that are oftentimes coincident with T1D, such as autoimmune thyroiditis and Stiff Person syndrome, in which high-titer GAD autoantibodies are a diagnostic identifier (Dupond et al. 2010). The use of rituximab in human clinical trials has also stimulated the interest in B-cell antigen capture and presentation in T1D, particularly following the results of the rituximab trial in newly diagnosed T1D patients showing delay in disease progression, associated with transient B-cell depletion (Pescovitz et al. 2009). These observations in humans are reinforced by studies in NOD mice using specific mAbs for CD20 (Xiu et al. 2008) and by in vivo neutralization of the B-lymphocyte stimulator (BLyS/BAFF), which in both cases prevented autoimmune diabetes progression (Zekavat et al. 2008). Interestingly, long-term in vivo BLyS neutralization also led to reduction of IAA titers. This effect in anti-BLyS-treated NOD mice also correlated with a restoration of Ig λ clonotype negative selection at the lymphopenic transitional B-cell compartment (TR) \rightarrow splenic follicle (TR \rightarrow FO) checkpoint. This is a defect, which has been delineated as an aberrant characteristic of B-lymphocyte homeostasis in NOD mice (Zekavat et al. 2008). These data collectively suggest that long-term in vivo BLyS neutralization is capable of correcting a defect in NOD B-cell tolerance by increasing the stringency of negative selection at the TR \rightarrow FO checkpoint. Two recent reports have shed additional light on the broad physiological meaning of B-cell inhibition in autoimmune diabetes by anti-CD20 therapy, because the study showed that rituximab differentially inhibited anti-islet autoanti-

bodies in T1D patients, and it blocked IAA for > 1 yr, in insulin-treated patients (Yu et al. 2011). Finally, the murine CD20-specific 18B12 antibody that, like rituximab, depletes the follicular (FO) but not marginal zone subset of B cells (MZB), efficiently inhibited diabetes development in NOD mice in a likely regulatory T-cell-dependent manner only when treatment was initiated before IAA detection. The latter observations suggest that MZBs, which are known to be potent APCs, may well play a role in the chain of events leading to overt diabetes (Serreze et al. 2011).

Potential Role of Humoral Autoimmunity in Non- β -Cell Tissues

Humoral autoimmunity in T1D has been shown to target pancreatic nervous system tissue structures, suggesting the possibility that non- β -cell elements can elicit immune responses in T1D (Rabinowe et al. 1989; Winer et al. 2003; Louvet et al. 2009). One of these molecular targets seems to be peripherin (Boitard et al. 1992). This molecule is expressed in multiple endocrine tissues, including nerve fibers surrounding islets of Langerhans in the pancreas, adrenal medulla, nerve fibers in interstitial tissue between thyroid follicles, and nerve fibers adjacent to ovarian follicles (Chamberlain et al. 2010). Serologic responses to peripherin have been found in autonomic fibers in the pancreas, thyroid, and ovary, supporting clinical observations indicating that neuronal elements may be a molecular target for immune-mediated injury in multiple forms of endocrine autoimmunity, including T1D (Chamberlain et al. 2010). However, it remains to be established as to whether or not the presence of peripherin antibodies, along with serologic responses to other putative neuronal elements, are predictive for the development of small fiber neuropathy (autonomic and/or somatic) and for the progression to overt diabetes.

Although the presence of these autoantibodies reacting with neuronal tissues is thought-provoking and may somehow play a role in the pathophysiology of a subset of diabetic neuropathy, definite data establishing their role as

causal factors remains elusive (Ejskjaer et al. 1998). The examination of inflammatory responses in peripheral and autonomic nervous structures is becoming a growing area of investigation.

CONCLUDING REMARKS

Type 1 diabetes results from autoimmune destruction/dysfunction of pancreatic β cells. In physiological conditions, there is balance between pathogenic T cells that mediate disease such as T cells with marked conservation of their TCRs (e.g., insulin), and regulatory cells that control autoimmunity (Bluestone et al. 2010). In T1D and other autoimmune disorders, there is an altered balance between pathogenic and regulatory T cells.

Autoantibodies are some of the most potent risk determinants for autoimmune diseases such as T1D with an estimated relative risk exceeding 100%. The archetypical model for the application of autoantibodies in disease prediction is T1D. Humoral bioassays have achieved a high degree of accuracy and sophistication to allow the design of algorithms for inclusion of individuals at risk of developing T1D in clinical trials around the world. In particular, seminal studies have suggested that using a combination of humoral immunological markers gives a higher predictive value for T1D progression, and greater sensitivity, without significant loss of specificity. There is a growing effort and a large opportunity for exploring novel strategies alone or in combination with immunomodulation with the ultimate goal to find the cure for T1D. Development of therapies targeting specific B-lymphocytes is underway in animal models of autoimmune diabetes and eventually may be examined in humans. Even though the possible pathogenic role of islet-associated autoantibodies has not been elucidated, the list of useful biomarkers such as autoantigen-specific CD4⁺ and CD8⁺ effectors, memory and regulatory T cells related to disease pathogenesis (Khadra et al. 2011), and their relationship with the relevance of cognate autoantibodies will continue to expand.

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