Diabetes/Metabolism Reviews
Diabetes Metab. Rev. **14**, 3–29 (1998)

Immunology in Diabetes: An Update

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Summary. Type 1 (insulin-dependent) diabetes mellitus is strongly associated with autoimmune phenomena connected to the loss of β-cells in the pancreatic islets. Despite considerable progress in our understanding of genetic susceptibility factors and islet autoimmunity preceding the clinical onset of Type 1 diabetes there are considerable gaps in our knowledge. First, the *etiology* is unclear. It is speculated that multiple etiological factors may initiate a common pathogenic pathway which results in immune-mediated β-cell destruction. In 1998 we will need to learn more about the possible importance of gestational infections, as well as isolation of viral DNA or RNA from the blood of new-onset patients or marker-positive individuals. The scan of the whole genome has provided a smorgasbord of *genetic regions* which confer diabetes risk either alone or in combination. HLA remains the major genetic risk factor, and while HLA peptide binding information is considerable, we understand less of autoantigen processing and presentation. Cloned autoantigens and their use in standardized autoantibody assays have improved our ability to identify individuals at risk for diabetes. The diagnostic sensitivity and specificity of autoantibody markers for Type 1 diabetes are high as are their predictive values. We need methods to combine autoantibodies with genetic risk factors. The identification of individuals in different stages of their *pathogenesis*, including patients with so-called slowly progressive Type 1 diabetes (SPIDDM, LADA etc.), allow approaches to novel therapeutic interventions. Insulin is currently the therapeutic agent of choice and although spontaneous insulin-dependent diabetes in the NOD mouse and the BB rat can be prevented by immune suppression or modulation, this has not yet been possible in humans. The 1998 research on the interaction between environmental factors and susceptibility genes to initiate β-cell specific autoreactivity should allow the development of therapies for prevention, and perhaps a cure, of insulin-dependent (Type 1) diabetes. © 1998 John Wiley & Sons, Ltd.

1. INTRODUCTION

Type 1 (insulin-dependent) diabetes mellitus (IDDM) is the most common chronic disease in children and young adults. The disease may develop at any age but is most frequent before the age of 20. The incidence varies between countries and ethnic groups. The genetic susceptibility is similar since HLA DQ2, DQ8 or both factors are necessary but not sufficient for disease development.[1,2] The HLA susceptibility may not, however, be the same in all countries. Environmental factors are important but it is unclear how: are they initiators or accelerators? Epidemiological studies are increasing our knowledge about incidence rates in relation to both genetic and environmental factors.[3] In countries with well-

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Contract Grant Sponsors: National Institutes of Health, DK26190, DK42654, DK53004; Juvenile Diabetes Foundation International; University of Washington Diabetes Endocrinology Research Centre, DK17047
Key Words: HLA; Type 1 diabetes, autoimmunity; autoantibodies; autoantigens; prediction
Received: 29 November 1997, accepted (revised): 30 January 1998

CCC 0742–4221/98/010003–27$17.50

developed registries it has been reported that the incidence of IDDM has increased by about 2% per year during the last 10 years.[4] The so-called north–south gradient in incidence rate as observed for example within Sweden[5] does not seem to be true for Europe as a whole since Sardinia has the second highest incidence after Finland[6] and Iceland has a rate lower than both Norway and Denmark.[7] There is no explanation for the increased incidence rate and a trend towards an earlier age at onset in some countries.

The last 10 years of IDDM research has further supported the strong association between Type 1 diabetes development and islet autoimmunity. Although it is common to assert that Type 1 diabetes is a T-cell-mediated disease (which seems self-evident since neither cytotoxic T-cells nor high-affinity IgG autoantibodies are formed without T-cells), reproducible, precise and standardized methods to study β-cell autoreactive T-cells in Type 1 diabetes have yet to be developed. Such methods are badly needed. Non-invasive analysis of insulitis is not yet possible. The exception is a recent biopsy investigation in a limited number of Japanese IDDM patients.[8] While we need to develop T-cell and antigen-presenting cell (APC) assays, the molecular cloning of islet autoantigens resulted in a rapid development of autoantibody radioimmunoassays. These assays have been disseminated to the diabetes research community following international standardization workshops supported by the Juvenile Diabetes Foundation International and organized by the Immunology of Diabetes Workshops committee.[9,10] Human insulin has been available since the description of insulin autoantibodies[11] and assays for conformation-dependent insulin autoantibodies (enzyme-linked immunosorbent assay (ELISA) does not work) have been subject to standardization workshops.[12] The 64K autoantigen in Type 1 diabetes was described in 1981[13,14] but it was not until the identification of the 64K protein as glutamic acid decarboxylase (GAD) in 1990[15] and the cloning of a novel GAD isoform (GAD65) in human islets in 1991[16] that an autoantibody assay could be developed and subjected to standardization workshops.[9,10,17–19] Similarly, the successful identification of the 64K protein tryptic fragments 40K and 37K[20] as the IA-2 (ICA512)[21–23] and IA-2β[24–26] proteins, respectively, was rapidly used in autoantibody radioautoantigen binding assays. Several studies now suggest that a combination of the three autoantibody assays, IAA, GAD65Ab and IA-2Ab, can replace the old ICA workhorse.[27,28]

The autoantibody radioautoantigen binding assays have increased our understanding of immunological abnomalities associated with diabetes. In particular, the use of the precise and reproducible autoantibody assays with recombinant autoantigens has generated novel information not only in new-onset patients and their first-degree relatives but also in studies of populations of newborn children, schoolchildren and adult populations. In the absence of reliable T-cell and APC tests, high-quality antibody assays against recombinant autoantigens offer a non-invasive way to study the anti-β-cell immune response by the determination of autoantibody subtypes, isotypes and epitope specificity as immune markers. The current simplified model of Th1 and Th2 immune responses may be conveniently studied by isotype and subtype analysis as the readout since we do not have reliable and reproducible T-cell assays. Taken together, the new information on islet cell autoantibodies as markers for a disease process associated with β-cell autoimmunity supports the view that "Immunology in Diabetes" is truly important to the β-cell specific killing and life-long dependency on insulin. In the present review we will therefore discuss recent data on the etiology, pathogenesis and therapy of Type 1 diabetes. Readers are referred to several comprehensive reviews which will also include data from animals with spontaneous diabetes.[29–32]

2. ETIOLOGICAL FACTORS

It has long been held that Type 1 diabetes results from the influence of the environment and that certain viruses are diabetogenic. Case–control investigations have shown an increased risk for Type 1 diabetes by blood group incompatibility,[33] dietary factors and length of breast-feeding,[34] vaccinations,[35,36] as well as numerous investigations on infections Similarly, based on animal results it is speculated that environmental factors may both accelerate and decelerate the Type 1 diabetes process. Among virus can-

didates the best evidence seems to be Coxsackie virus[37,38] and congenital rubella.[39,40] Several other viruses have been implicated.[38,41] Studies involve the isolation of Coxsackie virus from the pancreas of children with new-onset Type 1 diabetes,[38] the demonstration of virus DNA in blood[42] as well as of the subsequent IgM[43,44] and IgG[45] antibody responses. There is no conclusive evidence that Type 1 diabetes can be caused by a Coxsackie virus infection and the conflicting reports are many.[44,46] The interest in Coxsackie as a possible etiological agent was, however, further sparked by the observation that the PEVKEK GAD65 sequence is also present in the Coxsackie PC-2 antigen.[47] Current studies on T-cells recognizing this sequence have, however, given conflicting results[48–50] and Type 1 diabetes GAD65 autoantibodies do not seem to be able to recognize this part of the GAD65 autoantigen.[51–53] Other infectious agents such as rubella have also been implicated in a possible scheme of molecular mimicry.[54]

Similar to other human diseases of autoimmune character, it has also been suggested that superantigens are of etiological importance in Type 1 diabetes. Superantigens are protein products of either bacteria or viruses which show specificity to the β-chain of the T-cell receptor (TCR).[55] Multiple T-cells expressing a certain TCR type are stimulated when superantigens bind to the major histocompatibility complex (MHC). It has also been tested if superantigens have a role in Type 1 diabetes.[56,57] A highly selective expansion (over 30% of total) of a TCR variable segment of the β-chain, Vβ7, was reported in two newly diagnosed Type 1 diabetic patients. The expressed Vβ7 segments were not clonal, which suggested a superantigen as a cause of the expansion. Furthermore, islet cell membrane preparations from those patients but not from MHC-matched healthy controls were able to selectively expand Vβ7-bearing T-cells from healthy donors.[56] Reverse transcriptase activity in supernatant of cultured leukocytes from islets of Type 1 diabetic patients also suggested the presence of an active retrovirus.[56] A retrovirus, designated $IDDMK_{1,2}22$, was identified following a complex set of PCR amplification indicating the possible presence of a novel retrovirus related to a family of human endogenous retrovirus elements. The $IDDMK_{1,2}22$ sequence was cloned and the virus was found to encode Vβ7-specific superantigen. The authors propose a model in which superantigen activated autoreactive T-cells can lead to autoimmunity in susceptible patients. However, further studies on large patient populations are necessary to elucidate whether the presence of $IDDMK_{1,2}22$ viral integrant is widespread in Type 1 diabetic patients (and of potential major importance in etiology) or merely coincidental with the disease in highly selected, small groups of patients. The possible role of a superantigen is also difficult to understand since the risk for Type 1 diabetes is highly restricted to a limited number of HLA DR and DQ alleles.

3. GENETIC SUSCEPTIBILITY

Twenty-five years ago it was first reported that HLA is associated with Type 1 diabetes. Among genes associated with Type 1 diabetes mellitus (IDDM), the HLA on chromosome 6p21 is still the genetic factor with the strongest association. The *IDDM2* gene located on chromosome 11p15 in the upstream region of the insulin gene also has a significant association with the disease. Additionally, 18 other chromosome regions were tentatively linked to Type 1 diabetes. However, only 10 of them show statistically significant evidence for linkage for the disease[58] (Table I).

The strongest susceptibility to Type 1 diabetes is with HLA-DQ alleles. Caucasian susceptibility is more strongly associated with DQA1*0501–DQB1*0201/DQA1*0301–DQB1*0302 than with DRB1*03/DRB1*04.[59,60] DRB1*03 and DRB1*04 are in strong linkage disequilibrium with class I molecules B8 and B15, respectively. Conversely, the DRB1*04 allele is in linkage disequilibrium with the DQB1*0302 allele.[60–62] Negative association with Type 1 diabetes was observed for DQA1*0102–DQB1*0602–DRB1*1501 genotype. The DQB1*0602 allele is probably immunodominant over susceptibility DQB1 alleles and may be protective even among islet cell autoantibody positive first-degree relatives to patients with Type 1 diabetes.[63,64] Among people with the DQA1*0501–DQB1*0201/DQA1*0301–DQB1*0302 genotype protection may be associated with the DRB1*0403 allele, while the DRB1*0401 allele is

Table I. Type 1 diabetes genes and the highest reported maximal LOD score (MLS) for the linked genetic marker

Gene	Chromosome	Locus (most susceptible allele)	Highest reported MLS (ref.)
IDDM1	6p21	HLA (DRB1, DQB1)	7.3 (58)
IDDM2	11p15.5	TH/VNTR/INS (class III)	2.1 (58)
IDDM3	15q26	D15S107 (103)	0.2 (83)
IDDM4	11q13	FGF3, D11S1337	3.9 (83),
IDDM5	6q25	ESR	1.8 (84, 248)
IDDM6	18q21	D18S487 (4)/A181,2 (2)	4.5 (83)
IDDM7	2q31	D2S152	1.6 (87)
IDDM8	6q27	D6S281	1.3 (93)
IDDM9	3q21–q25	D3S1303	3.6 (83)
IDDM10	10p11	D10S193 (7)	2.1 (90)
IDDM11	14q24.3–q31	D14S67	4.6 (92)
IDDM12	2q33	*CTLA-4* (G)	3.2 (94)
IDDM13	2q34	D2S164	3.3 (100)

susceptible.[62,65] This negative association may, however, not be detected in all populations.[61] Also, the DQA1*0301–DQB1*0301 haplotype may be protective, in spite of the small sequence differences between this molecule and the susceptible DQA1*0301–DQB1*0302 molecule.[66] The association of the DQ alleles with diabetes is correlated with amino acid residue 57 on the β-chain[67] and amino acid residue 52 on the α-chain.[68] However, these individual amino acids do not solely explain susceptibility or protection. Many individuals develop Type 1 diabetes despite the presence of Asp-57 on the DQ β-chain. The presence of negatively charged aspartic acid at position 57 of DQ β is associated with unique peptide binding capacity[69] which may influence disease etiology, pathogenesis or both processes.

Analysis of the HLA class II region has led to the identification of additional genes, such as a transporter-associated with antigen processing (TAP1 and TAP2) genes located between the DQ and DP regions,[70] as well as large multifunctional protease (LMP) genes.[71] The comparison of the genetic susceptibility linked to TAP in Type 1 diabetes without or with another autoimmune endocrinopathy indicated that DRB1*03 and DRB1*04 differed in those two clinical forms of IDDM. The TAP1-C allele occurred more often among DRB1*04 patients. However, the TAP genes are in linkage disequilibrium with HLA DQ-DR[72] and their association may be secondary to a primary effect of DQ-DR.

The *IDDM2* gene was initially detected in a case–control study.[73] It was mapped to the insulin (*INS*) locus, located on chromosome 11p15.5, downstream to the gene encoding tyrosine hydrolase (*TH*) and upstream to the insulin and the insulin-like growth factor II genes (*IGF2*).[74] The *IDDM2* locus seems restricted to the variable number of tandem repeat (VNTR) microsatellite, located 596 bp upstreams of the insulin gene.[75–77] Among three VNTR classes, different in the number of repeats of the variable oligonucleotide, the short class I (26–63 repeats) predisposes to Type 1 diabetes while the longest class III (140–210 repeats) is dominantly protective.[75] Among diabetic offspring of class I/III heterozygous parents, class III VNTR alleles occur less frequently.[76] A possible mechanism is that VNTR alleles regulate Type 1 diabetes susceptibility by transcriptional effects on adjacent genes. In pancreatic islet cells, class III VNTR correlated with approximately 30% lower levels of insulin mRNA expression.[78] It was further suggested that lower expression of this gene can reduce the Type 1 diabetes risk by a decrease in the levels of circulating autoantigen (i.e. insulin). Alternatively, as a response to insulin deficiency, the expression of *IGF2* would increase, together with expression of IGF receptors. This would mediate mitogenic and anti-apoptotic effects, which would neutralize destruction of pancreatic β-cells.[78] However, the proposed mechanisms did not provide sufficient explanation for the dominant protection of class III allele.

Moreover, it cannot be excluded that, due to described heterogeneity within VNTR class III allele, reduction of insulin mRNA expression in pancreas can be regulated by the VNTR class III subtype which does not protect, but in fact increases the risk of IDDM (see below).

A more persuasive hypothesis to explain the dominant protective effect of VNTR class III was suggested in recent studies of the expression of both insulin and proinsulin mRNA in human fetal and post-natal thymus.[78,79] The level of *INS* mRNA expression, as well as the level of insulin, were associated with the VNTR class III allele. The amounts of *INS* mRNA expressed in thymus, compared to pancreas, were very low. The expression level of *INS* mRNA in VNTR I/III heterozygotes was approximately 2.5-fold higher than in VNTR class I homozygotes. It was speculated that this increase in expression may be sufficient to induce negative selection in thymus, resulting in deletion of autoreactive T-lymphocytes. Since proinsulin in the thymus was expressed in levels higher than insulin,[79] it is likely that epitopes shared by proinsulin and insulin are important in tolerance induction. Similar to pancreas, however, some thymic glands showed VNTR class III alleles which correlated to a lower level of *INS* mRNA expression. In those patients, class III was in linkage disequilibrium with the Z allele of a microsatellite neighboring the tyrosine hydrolase locus, *HUMTH01*. This haplotype may represent a less protective subtype of the VNTR class III allele.

IDDM1 and *IDDM2* are estimated to contribute to about 40% and 10%, respectively, of familial clustering of Type 1 diabetes. Other contributing genes are therefore suspected especially to explain the predominating number of patients who developed diabetes without having a first-degree relative with the disease. The combination of HLA with contributing genes may be viewed as a punitive model for the disease. HLA is necessary but not sufficient. HLA in combination with one or several contributing genes will contribute to the risk. It is important that genetic polymorphisms rather than genetic defects caused by mutations are likely to be important to this punitive model of diabetes risk. Linkage analysis of affected sibling pairs from Canadian, British and American families allowed the identification of several Type 1 diabetes contributing genetic factors.[80] Some of these contributing factors have been confirmed in the same or in other data sets, others have not. We will shortly review the regions reported so far whether replicated or not.

A locus located near the D15S107 marker (*IDDM3*) on chromosome 15q26[81] was also found in Danish families,[82] using case–control and intrafamilial association studies, as well as the transmission disequilibrium test (TDT), which allowed it to be established that the allele *D15S107*130* was the most significantly associated with Type 1 diabetes. However, significance of linkage for *IDDM3* remains questionable, since additional data sets obtained from affected sibling pairs from American and Italian families did not confirm linkage.[83]

In the same affected sib pairs, linkage was also observed for markers on chromosome 11q13 (*IDDM4*) near the fibroblast growth factor 3, *FGF3*.[81,84] The addition of additional markers centromeric to the *FGF3* region (near *D11S1337*) improved linkage.[83] Although positional cloning is required for a final answer, it is tantalizing that the Fas-associated death domain protein (FADD) maps to 11q13.3.[85] FADD is a 23 kDa cytoplasmic protein which interacts with Fas intracellular domain and transduces an apoptosis signal which is leading to T-cell destruction mediated by IL-1 β-converting enzyme-like proteases. It cannot be excluded that regulation of T-lymphocytes apoptosis may be important to Type 1 diabetes pathogenesis.[85]

IDDM5 was localized to chromosome 6q25 using a marker in the estrogen receptor locus (*ESR*).[58] The linkage was first merely suggestive,[58,84] but was lately confirmed.[83,86] Another contributing factor, *IDDM8*, was mapped 28 cM more telomeric than *ESR*, near *D6S264–D6S446* on chromosome 6q27.[83,84] The *D6S281* (*IDDM8*) marker region contributed significantly to familial IDDM clustering by multipoint linkage mapping.[86]

The linkage analysis of affected sib pairs to identify yet other contributing genetic factors are complemented by analysis of transmission disequilibrium tests (TDT). TDT detected a significant contribution to a marker on chromosome 18q12–q21 (*IDDM6*).[87] This factor was reported 15 years earlier using classical analysis of protein polymorphisms.[88] Analysis of 12 markers in the region spanning the Kidd blood

cell surface antigen (*Jk*) confirmed positive linkage to allele 4 of *D18S487*. An additional marker located 60 kb from *D18S487*, allele 2 of *A181,2*, proved stronger linkage in one group of 1067 families but not in second group of 390 families.[87] It is therefore questionable how much *Jk* on chromosome 18q12 contribute to Type 1 diabetes. There was no bias in the transmission of the Jk^b allele to diabetic offspring along with no linkage of the Jk^a/Jk^b polymorphism to Type 1 diabetes.[89]

Another region detected in the first genome-wide search for Type 1 diabetes genes was *IDDM10*. The region of markers tested is spanning between *D10S197* to *D10S220* on chromosome 10p11–q11. Multipoint affected sib pairs and TDT linkage analysis revealed evidence for linkage of allele 7 of *D10S193* and weak linkage of allele 4 of *D10S588*. However, this allele was more frequently transmitted to non-diabetic siblings. There was no linkage to a chromosome 10 *GAD65* microsatellite,[90] which is consistent with a previous study.[91]

An extensive search for contributing genes on chromosome 14q resulted in strong disease linkage to *D14S67* on 14q24.3–q31, located between the *CCC1* and *D14S128* microsatellite markers.[92] Among affected siblings with no HLA sharing, the reported linkage was stronger than any other non-HLA genes. The same analysis in a different set of Canadian families, however, did not confirm linkage.[92] These results from the same authors illustrate that there are problems in the current approach to identify Type 1 diabetes-contributing genes. It is as yet unclear how these problems of lack of replication will be resolved, except that many more sib pairs may need to be analyzed.

Another approach to identify diabetes-contributing genes is to test whether genetic factors in the NOD mouse or the BB rat also contribute to the human disease. This test is possible because of widespread synteny between rodent and human genomes. This approach to homology mapping has indicated the following. Chromosome 1 markers were linked to NOD mouse diabetes.[3] A syntenic region on human chromosome 2q was searched and initially two loci were detected: one near the microsatellite marker *D2S152* on chromosome 2q31, *IDDM7*,[93] and another, *IDDM12*, at the chromosome 2q33 region encoding the genes of *CTLA-4* and CD28.[94] The association between *IDDM7* and Type 1 diabetes is unclear and the distance to *IDDM12* is approximately 10.7 cM. In all analyzed data sets, the linkage between *D2S152* and Type 1 diabetes remains either weak[84,93] or show no linkage.[83]

The evidence for linkage to *CTLA-4* is also controversial. *CTLA-4*, expressed on stimulated T-lymphocytes, is a negative regulator of T-cell activation. The binding of *CTLA-4* to B7 molecules triggers T-cell apoptosis. *CTLA-4* could potentially play an important role in Type 1 diabetes susceptibility. Studies in *CTLA-4* knockout mice support this view since these mice have islet lymphocytic infiltration (insulitis) as well as to up to 100-fold increase of serum immunoglobulin level.[95,96]

In addition to linkage between *CTLA-4* and diabetes, there is also evidence for linkage to Graves' disease.[94] The contribution of *CTLA-4* for Type 1 diabetes is a biallelic variant (G to C) in exon 1 at position 49 which results in treonine to alanine substitution in the leader peptide. The G allele was more often transmitted to diabetic offspring among Caucasian Type 1 diabetic patients.[94] The G allele does not show linkage in all studied ethnic groups, and further studies are needed to confirm linkage or show association. Linkage of the G allele was verified in German and Canadian populations.[97] Stronger linkage to Graves' disease, particularly among HLA DQA1*0501 patients, was also observed.[97] In an American sib pair analysis there was no evidence for linkage of *CTLA-4* to Type 1 diabetes.[98] In the same study a few more candidate genes on chromosome 2q31–35 were tested by the TDT for their susceptibility to Type 1 diabetes. Among them were: *HOXD* gene cluster, *β2*, *CD28*, *IGFBP2* and *IGFBP5*. Similarly to *CTLA-4*, none of those genes displayed any association with Type 1 diabetes.[99]

An additional search of chromosome 2q identified linkage on chromosome 2q34 to *D2A164* (*IDDM13*).[100] Linkage was strongest in families with a preponderance of affected females, as well as among individuals who had islet cell antibodies (ICA) with no clinical symptoms of Type 1 diabetes. This is of potential interest since it indicates that autoimmune phenomena which are associated with or predict Type 1 diabetes in fact may show stronger genetic linkage compared to Type 1 diabetes as the end-point.

Taken together, in 1998 it needs to be worked out how the major genetic factor, HLA-DQ, contributes to diabetes risk. HLA is necessary but not sufficient and we still don't understand how. Are HLA-DQ 8, 2 or 2/8 presenting diabetogenic peptides? How do we explain the potentiated but age-dependent risk of HLA-DQ2/8 heterozygotes? We need to find out if DQ2-DR3 is predisposing for one subgroup of Type 1 diabetes and DQ8 for another. DQ2/8 individuals may in these model have propensity for two types of Type 1 diabetes.

We also need to understand contributing diabetes genes. Novel methods of statistical genetics which take multiple risk factors into account should be useful to generate novel hypotheses on the complex interaction between HLA and contributing genetic factors in response to environmental factors.

4. PATHOGENESIS

The pathogenesis of Type 1 diabetes is strongly associated with a high diagnostic sensitivity and specificity of ICA and autoantibodies to specific autoantigens which primarily include GAD65, insulin and ICA512 (IA-2). The nature of the initiating or triggering antigen(s) still remains to be determined. A large variety of autoantibodies have been reported (Tables II and III). Some of these antibodies have been confirmed by many investigators (Table II), others have not (Table III). It is reasonable therefore to discuss, in immunology of diabetes in 1998, whether the autoantigen has been identified and prepared in large quantities ("recombinant autoantigens") or not ("candidate autoantigens") . A different type of autoantigens are those detected by T-cells (T-cell autoantigens) (Table III). These autoantigens or T-cell epitopes differ whether the peptides are presented by HLA class I or class II molecules. The T-cell responses are different as well, since the former are recognized by cytotoxic T-lymphocytes (CTL), and the latter by T-helper cells.

The presence of an islet cell autoantibody signifys that an autoimmune reaction has taken place. We view the islet cell autoantibodies as markers of this anti-islet autoimmune reaction. Once a triggering antigen has been identified, it will be critical to elucidate the mechanisms by which that antigen is able to induce β-cell killing and subsequent development of autoreactive antibodies and T-cells. In the following we will discuss the different autoantigens identified so far and future directions for immunology of diabetes in 1998.

Table II. Recombinant β-cell autoantigens used in standardized radioimmunoassays for conformation-dependent autoantibodies

Antigen	Autoantibody assay
Insulin	Standardized radioligand (^{125}I-insulin) assay detects insulin autoantibodies (IAA) in about 50% of new-onset Type 1 diabetic children. Less in adults
GAD	Standardized radioligand (^{35}S- or ^{3}H-GAD65) assay detects autoantibodies (GAD65Ab) in about 80% of new-onset Type 1 diabetic children and adults. GAD67Ab are found primarily in GAD65Ab-positive sera
ICA512/ IA-2	Standardized radioligand (^{35}S- or ^{3}H-IA-2) assay detects autoantibodies (ICA512Ab or IA-2Ab) in about 50% of new-onset Type 1 diabetic children and adults. ICA512Ab are unique to Type 1 diabetes, develop after GAD65Ab and are closer to the clinical onset
IA-2β Phogrin	IA-2β is 74% homologous to IA-2 and the two molecules share most of the autoantibody epitopes. Radioligand (^{35}S- or ^{3}H-IA-2) assay detects autoantibodies (IA-2βAb) in about 50% of new-onset Type 1 diabetic children and adults. Remains to be standardized

A. Recombinant Autoantigens

Autoantigens associated with the pathogenesis of Type 1 diabetes have been identified using methods described by one of us by using serum or plasma by immunoprecipitation.[14,101] The highest diagnostic sensitivity and specificity have been achieved when the autoantibody analyses are carried out as radioimmunoassay with recombinant autoantigens.[12,102]

Table III. Candidate recombinant islet cell autoantigens with low or controversial diagnostic sensitivity for Type 1 diabetes

Antigen	Nature, location and autoantibody assay
ICA69	Western blotting but not radioligand assay detected ICA69Ab in 30% of ICA-positive first-degree relatives later developing Type 1 diabetes. Assay not standardized
38 kDa jun-B	T-cell responses found in recent-onset Type 1 diabetes patients. Immunoblotting detected jun-BAb in 33% of new-onset patients. Assays not standardized
Carboxy-peptidase H	Immunoblotting detected CPHAb among ICA-positive first-degree relatives but a radioligand assay failed to detect an increased frequency of CPHAb in new-onset patients. Assays not standardized.
Heat shock protein	ELISA test with recombinant murine hsp60 indicates an increased frequency of antibodies in Japanese type diabetes and rheumatoid arthritis patients. Assay not standardized.
Aromatic l-amino acid decarboxyl-ase (AADC)	Radioligand (^{35}S-AADC) assay detects autoantibodies (AADCAb) in 51% of APS-1 patients without and with Type 1 diabetes but not necessarily in new-onset Type 1 diabetic patients. Assay not standardized
DNA topoisomer-ase II	Autoantibodies to both full-length and fragments of DNA topoisomerase type II have been detected in 48% of Type 1 diabetes patients. Assay not standardized
Glima 38	Amphiphilic 38K membrane glycoprotein expressed in islet and neuroendocrine cells. Glima 38Ab were reported among 19% of new-onset Type 1 diabetes patients.
Imogen 38	Imogen 38 was detected in a peptide display library screened with a T-cell clone from a Type 1 diabetes patient. T-cell and autoantibody determination remains to be determined

Three autoantigens, GAD65, insulin and ICA512 (IA-2 and IA-2β), are showing the most reproducible results (Table II).

i. Insulin

Insulin autoantibodies (IAA) were not convincingly demonstrated until ^{125}I-insulin was used in a radioligand binding assay.[11] In the first study, it was carefully documented that the patients with IAA had not been given insulin before the clinical diagnosis. The prevalence of IAA at clinical diagnosis was about 40%; however, later studies in an assay with prolonged incubation increased the IAA frequency at onset.[103,104] The overall prevalence is dependent on age since IAA are more common among young children than among adolescents or adults.[105,106] So far, it has not been possible to distinguish IAA from insulin antibodies formed in response to daily insulin injections.

The radioimmunoassay for insulin reached the highest diagnostic sensitivity and specificity in several international standardization workshops.[12,107] After the ELISA tests or similar solid-phase analyses had failed,[108,109] there are two steps to the IAA radiobinding assay used. The first is to treat the serum or plasma with acid charcoal to displace antibody-bound insulin and to remove free insulin, which otherwise would compete with the radioactive insulin. The samples are next incubated with or without an excess of non-radioactive insulin to demonstrate specific binding. Free and antibody-bound insulin is finally separated by polyethylene glycol (PEG) in the presence of carrier immunoglobulin.[12] Prolonged incubation and the use of larger volumes of sera seem to increase the sensitivity of the assay.[104,106,110]

The role of insulin as an autoantigen in the pathogenesis of Type 1 diabetes is still not understood. It remains to be determined when and why (prepro)insulin is processed and (prepro)insulin peptides presented. Perhaps the most immunogenic molecule is proinsulin or preproinsulin.[111,112] An IAA epitope was tentatively located to the B chain using insulin analogues and mutated insulin in the IAA assays.[113] The association between IAA and HLA may help to define epitope restriction. Among first-degree relatives to IDDM patients,

IAA was reported to be associated with DR4.[114] A possible DR4 subtype or a linked DQA1 allele needs to be identified.[115] Among new-onset Type 1 diabetic children, IAA was associated with DQ8 rather than DQ2.[64] Further studies are required to identify IAA autoepitopes and their HLA restriction. Many more B-lymphocytes are committed to production of anti-insulin IgG among new-onset IDDM patients compared to controls.[116] It is not yet clear to what extent the immune response to injected human insulin differs from endogenously autopresented insulin or proinsulin.

The diagnostic sensitivity and specificity of IAA for IDDM have been assessed in numerous studies of selected patients.[104,110,117] population- and Diabetes Registry-based studies of consecutively diagnosed patients and matched controls,[64,105,118,119] as well as among first-degree relatives progressing[103,120,121] or not[122,123] to Type 1 diabetes (Table IV). While the diagnostic specificity is high (99%), there are major differences in sensitivity (40–80%) dependent on the type of assay used.[105,106,118,124] Although Type 1 diabetes primarily develops among children and young adults who do not have a family history of the disease, there are more studies on select first-degree relatives followed to diagnosis than on individuals in the general population. IAA has low predictive value (3%) in children and young adults in the general population.[64,105] Large follow-up studies of marker-positive individuals detected by screening the general population[125,126] will be required to define the predictive values of IAA for Type 1 diabetes alone or in combination with other autoantibodies (GAD65 and ICA512) and genetic markers.

Table IV. Diagnostic sensitivity, specificity and predictive value in autoantibody assays with recombinant β-cell autoantigens

Autoantigen	Sensitivity	Specificity	Predictive value
Insulin	40–80%	99%	30%
GAD65	70–80%	99%	60%
GAD67	10–20%	99%	Very low
IA-2	50–60%	98–99%	30%
IA-2β	50%	99%	30%

The predictive values are estimated from studies of ICA-positive first-degree relatives, not for individuals in the general population. GAD67-specific autoantibodies are rarely found in Type 1 diabetes. Their presence signifies high-titer GAD65 autoantibodies.

A recurrent problem in Type 1 diabetes research is the difficulty in identifying and cloning T-cells with specific T-cell receptors (TCR) to autoantigen peptides or epitopes. Experiments in the mouse are less problematic and T-cell clones have been produced in the spontaneously diabetic NOD mouse.[127,128] Insulin, perhaps proinsulin,[129,130] is an important autoantigen in Type 1 diabetes. The positive predictive value of IAA for Type 1 diabetes in the general population is still not fully established but current data indicate that IAA in combination with GAD65 or ICA512 may have predictive values for Type 1 diabetes among first-degree relatives as high as 50–70%.[27] The mechanisms of insulin autoantigen processing, HLA class II presentation and susceptibility in DR4-DQ8-positive individuals need to be established.

ii. Glutamic Acid Decarboxylase (GAD)

Cloning of GAD in human islets showed that the previously described 64K protein[14] represented a novel isoform of GAD, GAD65,[16] also present in human brain.[131] The two isoforms of GAD—GAD65 and GAD67—are both expressed in neurons and are both catalyzing the formation of the main neuroinhibitor γ-aminobutyric acid (GABA) from l-glutamate.[132,133] The role of GAD65 and GABA in the islets of Langerhans is still unclear.[134–136] It remains to be determined if β-cell GABA is important to signaling, for fuel, for growth or all three. Following the demonstration of GAD65 as the major autoantigen in Type 1 diabetes,[16,18,19,47,137] special interest has been focused on this autoantigen. The use of GAD65[138,139] or GAD67[140] to prevent or delay the onset of diabetes in the spontaneously diabetic NOD mice did not decrease the interest. The observation that GAD65 plays a primary role in the initial stages of NOD mouse Type 1 diabetes pathogenesis is still being pursued by several investigators. The BB rat is also investigated; however, in this more robust model of Type 1 diabetes, the disease process was not halted by early intavenous or intra-

thymic GAD65 injections (Bieg and Lernmark, unpublished observations).

The GAD65 and GAD67 isoforms associate into dimers of approximately 120K, but differ in their interaction with the GAD co-factor, pyridoxal l-phosphate (PLP). GAD67 mRNA levels are relatively stable while GAD65 mRNA seems more regulated.[135,141–144] GAD65 consists of two subunits—an α and a β subunit,[145,146] whereas GAD67 is recognized as a single entity following immunoprecipitation or Western blotting.[137,147] Further studies are needed to clarify which transcription factors regulate the expression of the two GAD isoforms, including elements that may be sensitive to glucose and pro-inflammatory cytokines. The mechanisms of intracellular sorting[148] as well as the possibility that GAD65 can be discharged from the β-cells[149,150] also needs to be clarified.

Although high activity of GAD, GAD65 mRNA and high concentrations of GABA have been detected in islets at levels comparable to those in the central nervous system (CNS), the importance of GAD and GABA for the function of the pancreatic β-cells remains unclear.[151,152] GABA can be used for both regulatory and trophic reactions, and both paracrine and metabolic effects of islet GABA have been reported.[152,153] Co-secretion with insulin does not seem to occur[151] and insulin secretagogues do not affect GABA secretion.[154] It was proposed that GABA inhibition of arginine-stimulated glucagon secretion would be explained by the binding of GABA to $GABA_A$ receptors present on α-cells.[155] However, inhibiting the binding of GABA to its receptor with bicuculline, which specifically interferes with the postsynaptic $GABA_A$ receptors, did not influence glucose-induced inhibition of glucagon release.[154] It has been speculated that GABA is important to β-cell energy metabolism by generating NADH and ATP in the GABA shunt.[152,156]

The autoantibody response to GAD in Type 1 diabetes was complicated since immunoprecipitation assays demonstrated the combined precipitation of GAD65 and GAD67 in metabolically labeled rat but not human islets of Langerhans.[137,157,158] Comparing GAD65 and GAD67 expressed after transfection to a fibroblast cell line[137] or expressed by *in vitro* transcription and translation[18,159] revealed that 70–80% of Type 1 diabetes sera recognized GAD65 whereas only 10–20% recognize GAD67.[159–161] Shortly after the report that the 64K protein had GAD activity,[15] several assays to detect GAD autoantibodies were developed including ELISA,[162] enzymatic activity in immunoprecipitates[163,164] or radioimmunoassays[165] using widely different antigen sources including brain homogenates and recombinant GAD. The availability of recombinant human GAD65 cDNA made it possible to label GAD65 with either ^{35}S, ^{3}H or ^{14}C by coupled *in vitro* transcription and translation.[17–19] This approach allowed the development of precise and reproducible radioligand binding assays to detect GAD65Ab as demonstrated in two international standardization workshops[9,10] This assay system is now widely employed to detect a variety of autoantibodies including ICA512.[27,28,166] ICA69[167] or 21-hydroxylase in Addison's disease.[168] The advantage of the *in vitro* generated labeled autoantigen seems to be the direct labeling during biosynthesis which may not harm critical conformational epitopes otherwise affected by iodination or biotinylation and perhaps lost when recombinant GAD is absorbed to ELISA plates.

The highest diagnostic sensitivity of GAD65Ab has been found at onset of IDDM[159,160,163,167] and typically about 80% of the patients have GAD65Ab (Table IV). The sensitivity is lower in young boys[159,163] but does not seem to decrease with increasing age as is the case for IAA and ICA512Ab.[160,169] The frequency of GAD65Ab is increased among first-degree relatives and predicts IDDM.[27,47,167,170] The positive predictive value for IDDM of GAD65Ab alone or in combination with other islet autoantibodies is most likely as high as 50–60%; however, prospective studies in which all first-degree relatives and not only those identified because of ICA positivity and loss of β-cell function are needed. Investigations of antibody-positive individuals following screening in the general population are also needed. The diagnostic specificity is about 99% in the general population.[18,160] The frequency (about 1%) is the same as that of all newborns who will develop diabetes over a lifetime.[156] Although newborn children may have GAD65Ab,[171,172] it is unclear to what extent they predict Type 1 diabetes and if they are evanescent.[173] The presence of GAD65 autoantibodies at diagnosis has been suggested to be

associated with a more rapid loss of β-cell function.[19,174] Within 2 years after diagnosis, levels of GAD65Ab decrease somewhat, but the prevalence of GAD65Ab in long-term patients is still surprisingly high.[47,175]

The availability of simple, precise and reproducible assays has made it possible to analyze GAD65Ab and GAD67Ab in a large variety of disorders to better understand their diagnostic sensitivity. The presence of GAD65Ab is very high among patients with the autoimmune polyendocrine syndrome 1 (APS-1) whether the patients develop diabetes or not.[176,177] Both GAD65- and GAD67-specific autoantibodies were detected in Swedish non-diabetic patients with Grave's disease[178] and also in Japanese, who more often have both diseases.[174,179] GAD65Ab were not increased in frequency among patients with idiopathic Addison's disease without diabetes.[168,180]

The availability of GAD65 and GAD67 cDNA has made it possible to mutate GAD65 to study the autoantibody epitopes.[181–184] It is important in this respect that Type 1 diabetes-associated GAD65Ab do not recognize linear GAD65 epitopes, for example, when the antigen is blotted onto nitrocellulose since conformational epitopes are lost.[181,185] Deletion mutants which, for example, can be used with sera from stiff man syndrome[53,185,186] did not yield interpretable results with diabetes sera. Using 10 different human monoclonal ICA (MICA 1–10)[52,187,188] several GAD65 epitopes have been identified: N-terminal region (starting at position 39 for MICA 8/9); middle region (amino acid position 245–449 for MICA 4/6 and 10) and C-terminal region (amino acid position 450–570 for MICA 1/3 and 7). The results of these MICA isolated from three adult Type 1 diabetic patients were supported in a study with GAD65/67 chimeric molecules using sera from consecutively diagnosed Type 1 diabetic children,[182] demonstrating that the middle (E1) and C-terminal (E2) region antibodies dominate. In addition, it was observed that an increase in titer levels to the C-terminal (E2) region was associated with conversion to diabetes.[182] In these studies, the GAD65Ab epitopes are detected by IgG anti-GAD65; however, the epitope specificity as well as specificity for IgM, IgA and IgE as well as for IgG 1–4 subtypes need to be determined. It cannot be excluded that isotype- and subtype-specific anti-GAD65 immunoglobulin may better predict disease and reflect distinct pathogenetic processes. Since somatic mutations of the GAD65 antibody molecules most likely are antigen driven by CD4-positive T-cell-dependent mechanisms[188,189] it is also critical to determine GAD65 T-cell receptor epitopes.

Peripheral blood mononuclear cell (PBMC) reactivity to GAD65 at onset of Type 1 diabetes and in high-risk relatives has been reported.[190–192] Significant proliferation expressed as a stimulation index was reported in 47–67% of newly diagnosed IDDM patients, and was higher among ICA-positive (63–68%) compared to ICA-negative relatives (11%) without an effect of ICA titer, HLA type or gender.[190] GAD67-reactive PBMC have also been demonstrated in both pre-diabetic (40%) and diabetic patients (38%).[193,194] The relevance of T-cells proliferating in response to GAD67 is unclear and GAD67 immunodominant epitopes have not been identified.[195] Overlapping GAD65 peptides were used to study binding to HLA-DQ[196,197] and DR.[50] In the DQ molecules peptide-binding experiments, it was found that GAD65 peptides with strong homology to the PC-2 Coxsackie virus antigen were binders to DQ8 but not to DQ7 or DQ9.[69,197] No DQ-restricted T-cell proliferative responses have been detected so far to this epitope.[48–50] DRB1*0401-restricted GAD epitopes were, however, defined by T-cell hybridomas.[50,198] One of these T-cell epitopes was also detected by T-cells isolated from an adult Type 1 diabetic patient but none of the T-cell lines were proliferating in response to the Coxsackie B4 PCV-2 antigen peptide.[49] It is possible that the binding is not strong enough to induce tolerance by positive selection in the thymus. The mechanisms by which GAD65 autoantigen is processed and presented on HLA DR or DQ molecules remain to be elucidated. The possible role of molecular mimicry is still unclear.[47] Experiments in the spontaneously diabetic NOD mice suggest that immunizations with peptides from either insulin or GAD65 prevent Type 1 diabetes.[138,139,199] Peptides have been eluted and sequenced from both DQ8 and DQ2 molecules to define peptide-binding motifs of these IDDM-associated HLA class II molecules.[200] Such motifs may be useful to identify GAD peptides which may be involved in IDDM pathogenesis or perhaps of etiological importance. Similar approaches will be needed to determine GAD65 peptide binding to HLA class I molecules to define GAD65-dependent CTL.[201]

CD8+ T-cells have been observed in insulitis but their peptide dependence and HLA class I restriction have yet to be elucidated.

iii. IA-2/ICA512

Antibodies to islet proteins were identified in human diabetic sera by trypsin treatment of the 64K immunoprecipitation product[202] also using radiolabeled rat insulinoma cells which lack expression of both GAD65 and GAD67.[20,203] The limited proteolytic cleavage of the immunoprecipitate revealed three different antibody specificities binding fragments of molecular mass (M_r) 37K, 40K and 50K. The 50K component was removed by GAD65 antibodies whereas the 37K and 40K fragments appeared to be derived from a different protein since neither recombinant GAD65 nor GAD67 competed for autoantibody binding to their 64K precursor.[204] Islet cell antigen 512 (ICA512) was independently identified from an islet cDNA expression library by screening with sera from Type 1 diabetic patients.[21,205] ICA512 was also isolated and expressed in a human insulinoma subtraction library, and designated islet antigen 2 (IA-2).[23] The 3.6 kb cDNA of IA-2/ICA512 showed a 979-amino acid protein homologous to receptor-type protein tyrosine phosphatases (PTP).[23,24] The IA-2 gene is on chromosome 2q35.[206] IA-2 has also been found in normal human brain, pituitary, pancreas and different cell lines.[207] The function of IA-2 in the islet β-cells remains unknown, but transmembrane PTP may regulate cell growth and proliferation, cell cycle and cytoskeletal integrity in response to external stimuli.[208] Since IA-2 is an integral membrane protein in the insulin secretion vesicle it is possible that it is of importance to signal internalization of vesicular membrane components.[207]

Further analysis of the association between IA-2 and the 37K and 40K tryptic fragments of immunoprecipitated 64K revealed that the 40K moiety is a product of the intracellular domain of IA-2.[22,24] The 37K fragment was identified as derived from a different, although related protein, IA-2β (see below). IA-2 and IA-2β share common epitopes.[209,210]

Initially autoantibodies against islet tryptic fragments of 37K/40K protein were detected in 50% of new-onset IDDM patients and 67% of pre-diabetic twins.[20,211] Recent data with IA-2 demonstrate a diagnostic sensitivity of about 50–60% and specificity of 98–99%[22,212,213] (Table IV). Several investigators believe that the combined GAD65Ab, IAA and IA-2Ab tests may replace ICA testing.[27,28,213] Although the cytoplasmic PTP-like domain is homologous to other tyrosine phosphatases, phosphatase activity has not been detected.[21,22] The autoantibody reactivity is directed to the cytoplasmic domain. In new-onset IDDM sera there are antibodies which react to at least four cytoplasmic domains.[214] Two were mapped to the juxtamembrane domain (amino acid positions 605–620 and 605–682) and an additional two in the PTP-like domain (amino acid positions 777–937 and 687–979). The majority of IDDM sera (83%) reacted at the PTP-like domain followed by the juxtamembrane (56%) and reactivity with both (39%).[209,214] This heterogeneity is not understood but may be explained by HLA restriction since IA-2 (ICA512) in IDDM is associated with DQ8.[213,215] Before a discussion of diagnostic sensitivity, specificity and predictive value of ICA512/IA-2 autoantibodies for IDDM, a novel isoform of IA-2, IA-2β, will next be discussed.

iv. IA-2β

Another transmembrane PTP, IA-2β, was identified from cDNA libraries of a mouse neonatal brain,[24] human colon carcinoma cells[25] or of human islets.[26,216] The intracellular domain of the two β-cell IA-2 isoforms shows 74% amino acid identity compared to only 26% in the extracellular domain. Similar to IA-2, IA-2β is primarily expressed in pancreatic islets and brain. IA-2β, also referred to as IAR or phogrin, cDNA was used in *in vitro* transcription and translation to prepare a radiolabeled autoantigen to demonstrate that about 50% of new-onset patients have IA-2β autoantibodies.[22,25,26,216] Most IA-2 autoantibody-positive sera react with both isoforms; however, some IDDM sera have been found which distinguish between unique epitopes on either IA-2 or IA-2β.[26]

The successful cloning of IA-2β also seems to solve the problem of the origin of the 37K fragment[22] since limited proteolysis suggests

that the IA-2β is the precursor of the 37K fragment.[24,25] IA-2β autoantibodies have a diagnostic sensitivity of about 30–50% and a specificity of 99% (Table IV). It needs to be carefully determined if IA-2βAb should be included in the panel of autoantigens to increase the predictive value for Type 1 diabetes. It will, for example, be of interest to test to what extent an IA-2/IA-2β hybrid molecule can be constructed and used as an autoantigen that would maximize IA-2βAb sensitivity and specificity. Future studies also need to define T-cell epitopes and the apparent effect of age at onset on diagnostic sensitivity.

B. Other Autoantigens

The following cloned proteins have been implicated as candidate autoantigens (Table III). The importance of this group of putative autoantigens remains to be determined since many are only based on a single publication which has not yet been confirmed. Since these autoantigens have been cloned it will be possible to resolve these uncertainties.

i. ICA69

The islet cell antigen of 69K (ICA69) was first detected in rat insulinoma cells by cross-reactivity with antibodies to bovine serum albumin.[217] ICA69 was cloned from a human islet cDNA expression library; the structural gene was designated ICA1, and it was mapped to human chromosome 7p22.[218] The open reading frame of ICA69 predicts a 482-amino acid protein with some sequence homology to bovine serum albumin that was proposed to trigger Type 1 diabetes.[217,219] There was no specificity in the expression since ICA69 mRNA was reported in human pancreatic islets and brain, and in rodent islet cell lines, testis, islets and brain.[218,220] Western blot analysis of human and mouse tissue also revealed high levels of ICA69 in brain, testis, pancreas and islet cell lines.[221] The function of ICA69 remains to be clarified.

Autoantibodies against ICA69 detected by immunoblotting with sera from pre-diabetic individuals and in recent-onset patients amounts to 20–30% and was independent of other islet autoantibodies such as ICA or IAA.[218] However, another study showed that less than 5% of newly diagnosed IDDM patients immunoprecipitated *in vitro* translated ICA69.[167] ICA69 antibodies were also reported in patients with rheumatoid arthritis.[222] The utility of ICA69 as a humoral marker for Type 1 diabetes is therefore controversial.

T-cell proliferation to ICA69 was most pronounced in recent-onset Type 1 diabetes patients compared to patients with the disease or to non-diabetic first-degree relatives.[223] An inverse correlation between T-cell and autoantibody responsiveness to ICA69 was also observed. Although available as a recombinant protein, the ICA69 autoantibody reactivity has not been easily reproduced and the role of ICA69 in the pathogenesis of Type 1 diabetes needs further studies.

ii. Glima 38

Glima 38 is a 38K amphiphilic membrane glycoprotein expressed in islet cells and neuronal cell lines and is immunoprecipitated in about 20% of new-onset IDDM children and 14% of pre-diabetic first-degree relatives[224] (Table III). A 38K protein had already been described in the initial study of the 64K autoantigen.[14] Glima 38 shares the neuroendocrine expression pattern characteristic of GAD65 and IA-2. Deglycosylation with *N*-glycanase reduced the molecular mass to 22K. Glima 38 has yet to be cloned and the immunoprecipitation assay in Triton X-114 is preliminary to establish diagnostic sensitivity and specificity of glima 38 autoantibodies for IDDM. While the prevalence of glima 38 autoantibodies in new-onset IDDM patients was 19%, it is possible that the combination of GAD65Ab, IA-2Ab and glima 38 will yield a diagnostic sensitivity well above 90%. The specificity needs to be established since in the initial study too few healthy controls were tested.[224] Sera which were positive by immunoprecipitation were negative in a Western blot analysis, indicating that the Type 1 diabetes autoantibody epitopes are conformational.[224] The presence of autoantibodies to the glima 38 autoantigen has not been confirmed by others.

iii. 38K-jun-B

T-cell reactivity to the early-response nuclear transcription protein jun-B (38K) was reported in Type 1 diabetes[225] (Table III). Using a 180 N-terminal amino acid recombinant jun-B preparation, peripheral blood T-cell reactivity was demonstrated in 71% of recent-onset IDDM patients, 50% ICA-positive first-degree relatives, 25% other autoimmune disease subjects but not in healthy controls. Autoantibodies against jun-B were reported to co-precipitate in 33% of GAD65Ab-positive sera from IDDM patients. The jun-B antigen is most likely different from glima 38 since the former is a non-glycosylated nuclear protein and the latter a glycosylated membrane protein. Antibodies against an islet protein of 38K were found in Type 1 diabetes patients with cytomegalovirus antibodies. Jun-B shares sequences with human cytomegalovirus and also with related herpes virus antigens.[225] Again, jun-B autoreactivity in Type 1 diabetes is only reported once and confirmatory studies are needed.

iv. Carboxypeptidase H (CPH)

CPH, also known as enkephalin convertase, is a 52K enzyme expressed in islet and neuroendocrine cells (Table III). CPH exists both as a membrane-bound and a soluble form which is co-secreted with insulin.[226] The serum used for screening an islet cell tumor library was from a single ICA-positive first-degree relative. Immunoprecipitation of the *in vitro* transcribed and translated CPH did not reveal a difference in antibody frequency between new-onset Type 1 diabetic patients and controls.[167] This autoantigen has not been subjected to autoantibody standardization.

v. Heat Shock Proteins (HSP)

HSP are stress proteins ubiquitously produced by cells in response to, for example, an increase in temperature, cytokines or free radicals. HSP have been implicated in the pathogenesis of several autoimmune diseases. It is thought to be important to NOD mouse diabetes.[139,227] Sequence homology to GAD65 suggested molecular mimicry to an epitope of the 65 kDa HSP. HSP65 may be expressed on the β-cell surface and may be a target for islet cell autoantibodies.[228] Recombinant murine hsp60 used in ELISA (Table III) detected hsp60 antibodies in 16% of Type 1 diabetes and 20% of rheumatoid arthritis patients, 1% of healthy controls but not in slowly progressive Type 1 patients, Type 2 patients or in patients with autoimmune thyroid disease.[229] This report warrants further investigation also in other ethnic groups to establish the possible role of HSP as autoantigens in Type 1 diabetes.

vi. Aromatic l*-Amino Acid Decarboxylase (AADC)*

Patients with autoimmune polyendocrine syndrome type 1 (APS-1) are often positive for autoantibodies against GAD65 and an unrelated 51K β-cell protein.[230] The 51K protein was identified as AADC by screening a rat insulinoma expression library.[231] AADC catalyzes the decarboxylation of aromatic l-amino acids which are intermediates in the synthesis of catecholamines and indolamine neurotransmitters. AADC uses pyridoxal l-phosphate (PLP) as co-factor, and is a cytosolic enzyme. Apart from the active site, there is little sequence similarity between AADC and GAD65. AADC is also present in the peripheral and central nervous systems, liver, intestine and kidney. Using *in vitro* transcribed and translated AADC, AADC autoantibodies were detected in 51% of APS-1 patients but neither in 138 Type 1 diabetes patients nor in the controls[232] (Table III). This study seems to exclude AADC as an autoantigen in Type 1 diabetes. Since AADCAb are associated with APS-1[176] but not with Type 1 diabetes, this autoantigen may be useful to analyze differences in pathogenesis.

vii. DNA Topoisomerase II

An increased frequency of nucleoprotein antibodies in Type 1 diabetes and in first-

degree relatives was reported[233] (Table III). As a possible confirmation, it was recently found that 48% of Type 1 diabetic patients have autoantibodies to both full-length and fragments of DNA topoisomerase type II.[234] In contrast to ICA and IAA, the frequency of DNA topoisomerase type II antibodies was unaffected by gender, disease duration or age. Comparing the amino acid sequence of DNA topoisomerase type II with insulin, HSP65 and GAD revealed sequences which shared up to 64% homology. Whether the autoantibodies develop as a result of cross-reactive autoantibodies or show HLA-dependent specificity remains to be determined. It was speculated that the antigenicity of DNA topoisomerase type II is stronger due to its nuclear location and large molecular mass.[234] The recombinant protein needs be used in a standardized assay to determine the diagnostic sensitivity and specificity of DNA topoisomerase antibodies for Type 1 diabetes.

viii. Glycolipids: The Elusive ICA Antigen

It has long been debated whether the ICA indirect immunofluorescence reaction is explained by non-protein autoantibodies. Organic solvents were used to extract pancreatic tissue to produce glycolipid-containing fractions depleted of protein.[235,236] These fractions blocked the fluorescence reaction of ICA-positive sera to human pancreatic sections, suggesting that the target antigen of ICA was a sialoglycoconjugate. The autoantigenic epitopes were recovered by borohydride treatment, suggesting the presence of glycolipids rather than glycoproteins. Comparing the co-migration of both human whole pancreas and islet extracts with ganglioside markers suggested that islets differentially express monosialoganglioside.[236] A GM2–1 pancreatic islet ganglioside has been identified as a putative ICA autoantigen;[237] however, further studies are needed to identify this potentially interesting autoantigen.

ix. T-Cell Reactive Autoantigens

Islet mitochondrial antigen 38K (Imogen 38). A human diabetic T-cell clone was used to screen a recombinant antigen epitope library to identify Imogen 38[238] (Table III). The cDNA of Imogen 38 was isolated using a βTC3 mouse insulinoma cDNA library. Due to its broad tissue distribution, it was speculated that Imogen 38 is a target for bystander autoimmune attacks rather than a primary autoantigen.[238] However, mitochondrial antigens such as the pyruvate dehydrogenase complex are important to other HLA-DR3-DQ2-associated organ-specific autoimmune diseases such as primary biliary cirrhosis[239] and further studies are therefore required to disclose the role of Imogen 38 and similar T-cell-defined autoantigens in Type 1 diabetes.

5. THERAPY

A. Primary Intervention

Studies of immunology of diabetes in 1998 will continue with analyses of the healthy population to better understand possible ways by which islet autoimmunity can be prevented altogether. Since we do not fully understand if there is a single or multiple factors which trigger islet autoimmunity and the subsequent development of a progressive inflammatory lesion in genetically susceptible individuals, further studies on environmental factors are badly needed. Screening newborns for the islet cell autoantibody markers suggests that in some children, the autoimmunity might have been initiated already *in utero*.[171,172,240] Primary intervention will therefore depend on an increased understanding of a variety of risk factors which may initiate the disease and also on our ability to distinguish them from environmental factors which may accelerate islet autoimmunity.

B. Secondary Intervention

The development of reliable assays for humoral immune markers has been a most rewarding development in Type 1 diabetes research during the past few years. Further progress is expected if the standardized assays are disseminated to the medical community at large. Analysis of autoantibodies to GAD65, IA-

2 and insulin has shown that these markers may be present several years before the onset of the clinical disease. Early detection and treatment of relatives of Type 1 diabetic patients are now possible by GAD65, IA-2 and insulin antibody analyses. In 1998, it will be important to establish diagnostic sensitivity, specificity and predictive values in different countries and ethnic groups. Studies need to be expanded beyond first-degree relatives. In Swedish new-onset Type 1 diabetic children and young adults it was found that 87% had one or several autoantibodies at the time of clinical diagnosis.[169] Almost 20% had autoantibodies against all three autoantigens. Prospective studies are needed to establish the positive predictive value for Type 1 diabetes in the population. It will be an important task since the incidence rate varies with age[241,242] but the initiation of such studies is critical since more than 85–90% of new-onset Type 1 diabetes patients do not have a first-degree relative with the disease.[5,7,243]

Several autoantibody workshops have taken place in order to standardize the different antibody assays.[9,10] A first attempt has been made also to standardize the T-cell proliferation test, with ambiguous results. Before using these procedures for routine screening of, for example, school children or first-degree relatives for identifying subjects at high risk for the disease, it will be important to define assay quality and concordance by proficiency testing in particular, if immune intervention therapy is contemplated. Several prevention trials are already under way and these are reviewed elsewhere. In first-degree relatives with ICA positivity, a controlled, randomized clinical trial is in progress to determine if nicotinamide is able to reduce the conversion rate to Type 1 diabetes. This multinational study is important since it involves an attempt to screen for ICA and test β-cell function with standardized methods. The protocol used is a paradigm for future intervention trials with antigen-specific immuno- therapies, as has already been attempted in the NOD mouse and the BB rat with insulin or insulin fragments or with GAD.[199,244–246] These animal data and preliminary results in man[247] resulted in the Diabetes Prevention Trial (DPT-1) in which some 60,000 relatives to Type 1 diabetic patients are currently screened for ICA and randomized to both parenteral and oral insulin treatment. Several such studies, also including nasal insulin, are under way in other countries. Although the results are not going to be known in 1998, the protocols used serve as an important incitement to initiate similar clinical trials based on the new more reliable screening assays for autoantibody markers alone or in combination with genetic markers.

6. CONCLUSIONS

There are considerable gaps in our knowledge about Type 1 diabetes that we need to fill in 1998. Despite considerable progress in our understanding of genetic susceptibility factors in the HLA region we need to fully identify the other contributing genes and find out what they do. The environmental influences are unclear. Do they initiate or accelerate the disease process? It is speculated that multiple etiological factors may initiate a common pathogenic pathway which results in immune-mediated β-cell destruction. In 1998 we need to learn more about the possible importance of gestational infections, as well as isolation of viral DNA or RNA from the blood of new-onset patients or marker-positive individuals. Cloned autoantigens and their use in standardized autoantibody assays are reaching state-of-the-art but we need to maintain standardization workshops and proficiency testing. The diagnostic sensitivity and specificity of autoantibody markers for Type 1 diabetes are high, as are their predictive values. We need statistical methods to combine autoantibody information with genetic risk factors. Novel therapeutic interventions are now possible provided they are safe and ethical. Insulin is the therapeutic agent of choice since we have 75 years experience with this hormone albeit not in non-diabetic individuals. The 1998 research on the interaction between environmental factors and susceptibility genes to initiate β-cell-specific autoreactivity should allow the development of therapies for secondary prevention, perhaps also a cure for insulin-dependent (Type 1) diabetes.

Acknowledgements

The studies in the authors' laboratory were supported by grants from the National Institutes of Health (DK26190, DK42654, DK53004), the Juvenile Diabetes Foundation International as well as the University of Washington Diabetes Endocrinology Research Center (DK17047). DBS was supported by a fellowship from the Juvenile Diabetes Foundation International.

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