

Antigen-Based Prediction and Prevention of Type 1 Diabetes

Jacob Sten Petersen

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Hagedorn Research Institute, Gentofte, Zymo-Genetics, Seattle, and Novo Nordisk, Bagsværd.

Correspondence: Novo Nordisk AS, Novo Nordisk Par, 2760 Måløv, Denmark.

Official opponents: Allan Flyvbjerg and Bente Klarlund Pedersen.

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2. INTRODUCTION

Type 1 (insulin dependent) diabetes develops as the result of a cumulative autoimmune-mediated destruction of the pancreatic beta cells mainly in genetically predisposed individuals. The disease becomes clinically manifest when 50-90% of the beta cells are destroyed (1) following a long prodromal period (months to years) during which autoimmune phenomena are often present, including mononuclear cell infiltration in the islets of Langerhans and circulation of islet cell autoantibodies. This review will provide a brief introduction to type 1 diabetes followed by an in-depth discussion of the current knowledge of antigen-based prediction and prevention/intervention of autoimmune diabetes focusing on glutamic acid decarboxylase 65 kDa (GAD65), one of the main autoantigens.

3. EPIDEMIOLOGY, GENETICS, ENVIRONMENTAL/ETIOLOGICAL FACTORS AND PATHOGENESIS OF TYPE 1 DIABETES

3.1 Epidemiology

Worldwide the incidence of type 1 diabetes is increasing and today the prevalence is estimated to be 2 million individuals in the Western world. Overall it is estimated that the incidence will be 40% higher in 2010 compared to 1997 (2). The incidence is, however, highly variable among different populations. The incidence of type 1 diabetes is 0.1/100,000/year in certain regions of China and 40/100,000/year in Finland; a 400 fold variation (2, 3). The incidence seems to be increasing especially in countries with a low disease incidence, and furthermore there is a trend toward earlier onset of clinical disease (3).

A geographic North-South gradient was previously used to explain the variation in incidence with a high incidence in Scandinavia and low in Southern Europe. However, Sardinia has almost as high incidence of diabetes as Finland and Estonia has a 75% smaller incidence rate than the closely situated Finland (3, 4). Thus the huge variation in incidence seems to be following ethnic/racial distributions reflecting a combination of genes and environment, rather than geographical location.

3.2 Genetics

Many genes have been associated with the development of type 1 diabetes. The human leucocyte antigens (HLA) was the first genetic association reported almost 30 years ago, and HLA is still the genetic factor with the strongest association, accounting for about 45% of genetic susceptibility for the disease (5). The HLA genes can not only provide susceptibility towards development of diabetes, but

they can also provide protection. The strongest susceptibility is with HLA DR3, DQ2 (DQB1*0201) and HLA DR4 (DRB1*0401), DQ8 (DQB1*0302) haplotype in Caucasian populations. In contrast the DR2, DQ6 (DQB1*0602) haplotype is negatively associated with type 1 diabetes. Further, adding to the complexity, is that the HLA association varies among different populations, e.g. in Asian populations DRB1*0405 is the major susceptibility haplotype (6). Although the function of the HLA genes are well known in presentation of peptides to T-lymphocytes, their specific contribution to the pathogenesis of type 1 diabetes remains elusive.

Many other chromosomal loci have been associated with development of type 1 diabetes but only a few genes have been identified from non-HLA loci. The IDDM2 locus on chromosome 11P5.5 contributes approximately 10% toward genetic disease susceptibility (7). This locus is a polymorphic region that maps to a variable number of tandem microsatellite repeats (VNTR) with short class I VNTR alleles predisposing to disease, while the longer class III alleles are dominantly protective. The mechanism of VNTR alleles regulating the disease susceptibility is believed to occur by transcriptional effect on adjacent genes associated with development of type 1 diabetes. Supporting this hypothesis is two studies demonstrating that the expression of both insulin mRNA and insulin in human fetal and post-natal thymus are associated with the VNTR class III allele (8, 9). The thymus expression of insulin is only a fraction of what is seen in the beta cells. Therefore, variation in insulin expression could have a great impact on the thymic education of T-cells and consequently maintenance of tolerance toward the beta cells. The level of insulin mRNA in VNTR I/III heterozygotes was in these studies demonstrated to be approximately 2.5 fold higher than in VNTR class I homozygotes. It was speculated that such an increase in expression may be sufficient to induce negative selection in the thymus, resulting in deletion of autoreactive T-cells. Such findings support that insulin may be an important autoantigen in the beta cell destructive process associated with type 1 diabetes. Different therapeutic approaches to induce tolerance to insulin will be discussed in details in section (5, IX, X, 10).

Another locus associated with type 1 diabetes, although not analyzed in all populations, is IDDM12 encoding genes of CTLA-4 and CD28 (11, 12). When CTLA-4 expressed on activated T-cells binds to B7 on antigen presenting cells it triggers apoptosis of the activated T-cells (13). Supporting a role of CTLA-4 in the development of type 1 diabetes comes from studies of CTLA-4 knockout mice, which have islet infiltration of lymphocytes as well as 100 fold increase of IgG (14, 15). The genes for the IDDM9 and IDDM18 loci have been identified to be CD80/CD86 and IL12 p40, respectively, and are also important molecules in regulating the immune system (16, 17).

Besides the loci discussed above, some 16 other chromosomal regions have been linked to the development of type 1 diabetes (18). However, only 9 additional loci show statistically significant evidence for linkage to the disease (19). Some of these loci have been associated with genes that could play an important role in the development of type 1 diabetes, e.g. IDDM4 has been speculated to be associated with the Fas-associated death domain protein (FADD), which is important in regulating apoptosis not only in T-cells but also in the beta cells (19, 20). However, positional cloning is required to finally confirm this association of FADD with IDDM4.

3.3 Environmental/aetiological factors

Despite a significant amount of research aiming at identifying potential etiological factors little is known about the nature or the time of initiating etiologic events. Studies in monozygotic twin pairs demonstrates that the crude concordance rates of type 1 diabetes are no higher than approximately 50%, indicating that environmental factors are involved in the pathogenesis of the disease (21-24). Environmental factors that have been associated with development of diabetes include viral infections (e.g. Coxsackie virus and cytome-

galovirus (25, 26)), vaccinations (e.g. mumps vaccination (27, 28)), diet (e.g. breast feeding versus early introduction of cow's milk (29, 30)) and toxins (e.g. N-nitroso derivatives (31)). However, prospective studies in the USA (DAISY (32, 33)) and Germany (BABYDIAB (34)) have failed to demonstrate any association of early cow's milk introduction, breast feeding, enteroviral infection and exposure to vaccinations. The reason for this discrepancy is not known but it indicates that identifying common environmental factors causing or accelerating disease is complicated if at all possible.

Traditionally it has been postulated that environmental agent(s) could trigger the onset of type 1 diabetes in genetically susceptible individuals (35). However, the above observations support a more complex model for disease development, wherein a multiple interplay between genetic factors such as immune dysfunction, beta cell defects and multiple environmental factors are responsible for disease initiation and progression (Figure 1) (36). Thus, rather than exposure to a single environmental agent on a genetically susceptible background, environmental encounters could act to promote or attenuate disease during different stages of development with effect dependent upon both timing and quantity of exposures (36). In support of environmental factors ability of being able to attenuate/prevent disease is that multiple infections early in life have been demonstrated to be associated with a reduced risk of developing type 1 diabetes (35, 37, 38). This could also explain the increase in incidence of type 1 diabetes observed during the last decades as healthcare and standards of sanitation have improved significantly in the Western world.

3.4 Pathogenesis

The pathogenesis of type 1 diabetes is strongly associated with the development of autoantibodies to several autoantigens, the three main ones being insulin, glutamic acid decarboxylase 65 kDa (GAD65) and protein tyrosine phosphatase-2 (IA-2/ICA512).

Autoantibodies can develop from birth the highest prevalence being between 3 months and 3 years of age. The clinical disease onset peaks around puberty (39-44), indicating that the disease process can start early in life and takes several years to result in insulin dependence. The first autoantibodies to develop, especially in infants are often insulin autoantibodies followed by GAD65 autoantibodies and then later IA-2 autoantibodies. Thus IA-2 autoantibodies appear to be more of a marker of later stage beta cell destruction (43). In adults the appearance of autoantibodies is more random although IA-2 also develops later than insulin and GAD65 autoantibodies. Furthermore, the prevalence of insulin autoantibodies decreases with age. Most individuals progressing to clinical onset of disease express multiple persistent autoantibodies and only few individuals expressing multiple autoantibodies escape clinical onset of disease (VII, 45, 46).

Although autoantibodies are early markers of beta cell autoimmunity and their presence indicates a significant correlation with disease progression/risk, development of clinical type 1 diabetes is believed to be dependent on T-cells and antigen presenting cells (APC) (47). There are several lines of evidence from human studies supporting this. T-cell specific immunosuppressive drugs like cyclosporine or monoclonal antibodies against the T-cell receptor CD3 have both been shown to delay the disease progression (48-50). In the case of cyclosporine this delay in disease progression was not accompanied by inhibition of autoantibody levels (III, 51). Longitudinal studies on autoreactive T-cell in islet transplanted type 1 diabetic patients have demonstrated a strong association between graft function and T-cell autoimmunity to beta cell autoantigens (52). Antigen presenting cells (APC) are not only important for activating the T-cells but may also directly play an important role in the destruction of beta cells (53, 54). Several studies have demonstrated that various cytokines, in particular interleukin 1 (IL-1) secreted by APC's directly can kill the beta cell. Interferon-gamma (INF- γ) secreted by APC does not only act in synergy with IL-1 to mediate killing of the beta cell directly but also up-regulates MHC class I thereby making the beta cell a moving target for activated cytotoxic T-cells recognizing autoantigens displayed by MHC class I (54, 55). In terms of uncovering the mechanism of beta cell killing, several studies have suggested that the Fas-Fas ligand pathway may also play an important role. It has been demonstrated that IL-1, INF- γ and TNF- α all secreted by the APC's induces Fas expression on the beta cells (56-58). Even high levels of glucose associated with the development of diabetes have been shown to induce Fas expression on beta cells (59, 60). Since CD8, CD4 and APS can express Fas-ligand the beta cell expressing Fas becomes a easy target for Fas-Fas ligand mediated killing. A study, analyzing pancreas biopsy specimens from recent onset patients have also confirmed that islets containing infiltration of mononuclear cells express Fas on the beta cells and Fas-ligand predominantly on the CD8 positive T-cell as well as on CD4 positive T-cells and macrophages (61). The results suggest that an interaction between Fas on the beta cells and Fas ligand on the infiltrating cells might trigger apoptotic beta cell death eventually leading to clinical onset of type 1 diabetes.

As discussed above, the autoimmune-mediated destruction of the beta cells appears to involve basically all facets of the immune system, and in combination with a large individual variation of different environmental/etiological factors makes it difficult to accurately predict and intervene in the disease process. However, during the past decade significant progress has been made in our ability to predict the disease and in our understanding of the disease process, thereby allowing better prevention and intervention therapies to be developed.

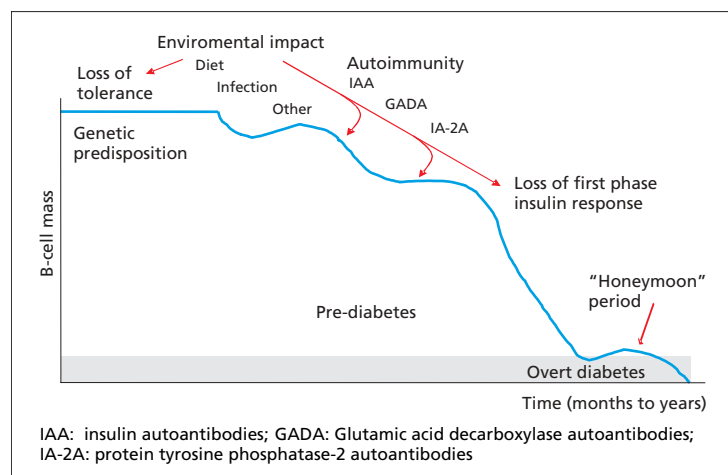


Figure 1. Pathogenesis and natural history of type 1 diabetes. The development of type 1 diabetes is believed to be a very complex interplay between multiple genetic and environmental factors that over months to years are leading to clinical onset of disease. The development of disease is associated with the presence of circulating autoantibodies, first single autoantibody positivity and then, as disease progresses, multiple autoantibodies will appear. Modified after Eisenbarth (36).

4. ASSAYS FOR PREDICTION OF TYPE 1 DIABETES

4.1 Autoantibody assays

Cytoplasmic islet cell autoantibodies (ICA's), measured by immunohistochemistry on sections of pancreas, were discovered in 1974 (62). In the nineties several autoantigens were identified at the molecular level facilitating the development of new assays (63, 64). These include assays detecting autoantibodies directed against insulin, GAD65 and IA-2/ICA512 (Table 1). Several studies comparing the original ICA assay with the new assays have suggested that these assays are more easier to standardize, more effective and less time consuming (VII, 65-68). Additional autoantigens have been identified, however, they are less well characterized, less specific, and/or less prevalent, e.g. heat shock protein (60) (HSP60), Glutamic acid decarboxylase (67) kDa (GAD67), Glima 38 and ICA69 (Table 1).

The platform for the majority of today's biochemical autoantibody assays used to predict progression to type 1 diabetes was initiated with two publications describing the use of recombinant GAD65 labeled with ³⁵S by cDNA coupled *in vitro* translation and transcription (II, 78). This was the first publication of a simple, reproducible and quantitative radioligand assay that could be used in larger scale screening as demonstrated in several autoantibody workshops (65, 66). Similar assays, based on the same concept of *in vitro* transcribed and translated antigen in the presence of a radioligand are now also used for detection of other autoantibodies, including IA-2 (68, 85), Phogrin IA-2β (92, 107). These assays have also been employed in Addison disease to detect autoantibodies to 21-hydroxylase (108).

There are several advantages using radio-labeled *in vitro* transcribed and translated autoantigens for detection of autoantibodies. Time-consuming and expensive purification steps, as well as labeling procedures can be avoided by generating the radio-labeled antigen from the corresponding cDNA. Autoantibody recognition of autoantigens associated with development of type 1 diabetes seems to selectively recognize conformational dependent epitopes. Therefore, purified recombinant autoantigens which are radio-labeled, biotinylated or used directly in ELISA have not been quite as successful since these procedures are impacting the conformational dependent epitopes, thus resulting in less sensitive assays (65, 66, 108) although progress is being made (81). The direct radio-labeling during biosynthesis using *in vitro* transcription and translation does not seem to harm critical conformational dependent epitopes which otherwise could be effected by iodination/biotinylation procedures or partly lost when purified recombinant GAD65 is absorbed to an ELISA plate.

Although the first standardization workshop held in the mid 1990 rapidly was followed by new workshops making a significant impact on our ability to predict and characterize the disease we have not yet, with a few local exceptions, implemented large scale screenings in the general population where more than 85% of new cases occur. Several factors complicate the development of autoantibody assays that are suitable for screening of the general population and there are several reasons why this is a difficult task (summarized in Table 2). The main obstacles have been standardization of assays. However, impressive progress has been made in this field, and there is hope that the standardization workshops will resolve some

Table 1. Type 1 diabetes associated autoantigens and assays for detection of the autoantibodies.

Antigen	Autoantibody assay	References
<i>Standardized validated assays</i>		
Insulin	Standardized radioligand assays based on ¹²⁵ I-insulin. Specificity 99%. Predictive value 30%. Sensitivity 40-80% dependent on age since the prevalence of IAA decreases with age	65, 69-71
Glutamic acid decarboxylase 65 kDa (GAD65)	Standardized radioligand assays based on ³⁵ S or ³ H GAD65. Sensitivity 70-80%. Specificity 99%. Predictive value 60%. Other assays, particular ELISA assays, are in general less predictive mainly due to lower sensitivity although new studies are showing some promise	II, 65, 72-81
ICA512/IA-2	Standardized radioligand assays based on ³⁵ S or ³ H IA-2. ICA512 develops after GAD65Ab closer to clinical onset of disease. Sensitivity 50-60%. Specificity 99%. Predictive value 30%	65, 82-86
<i>Autoantigens with low or controversial diagnostic value</i>		
Glutamic acid decarboxylase 67 kDa GAD67	Radioligand assays based on ³⁵ S GAD67. Autoantibodies approximately 10-20% of recent onset type 1 diabetics. Mainly GAD65Ab cross reacting due to 45% sequence homology	87-89
Phogrin IA-2β	Radioligand assays based on ³⁵ S or ³ H IA-2. Phogrin is 75% similar to ICA512 and share most of the autoantibody epitopes (see above)	90-92
Heat shock protein 60 (HSP60)	ELISA assays using murine heat shock protein 60 (HSP60). Autoantibodies in approximately 15% of type 1 diabetics and in 20% of patients with rheumatoid arthritis	93
Islet cell autoantibody 69 (ICA69)	Western blotting and immunoprecipitation of ³⁵ S ICA69. Autoantibodies in 5-30% of recent onset type 1 diabetics, and up to 20% and 6% of patients with rheumatoid arthritis and healthy controls, respectively	94-96
Glima 38	Immunoprecipitation using radiolabeled ³⁵ S methionine labeled islet as antigen source to detect a amphiphilic 38 kDa membrane glycoprotein. Glima 38 autoantibodies is found in approximately 20% of recent onset type 1 diabetics	97, 98
DNA topoisomerase II	Radioligand assays based on ³⁵ S or ³ H DNA topoisomerase II. Approximately 50% of type 1 diabetics have autoantibodies. Sequence homology with HSP65 and GAD (up to 64%), but unknown if the autoantibodies are cross reactive	99, 100
Bovine serum albumin (BSA)	Immunoassays and Western blot analysis to analyze anti-BSA antibodies in sera from diabetic patients have demonstrated elevated levels compared to healthy controls. Have been difficult to reproduce by other groups	101-106

Idea after 195.

Table 2. Challenges with current autoantibody assays in relation to population based screening.

Challenges	Potential solutions
Assay variation in sensitivity and specificity	Use validated and standardized biochemical autoantibody assays with a specificity higher than 99%
Assays are difficult to standardize	Only use assays that are amenable for standardization, e.g. not ICA
Autoantibodies do not appear at a specific age, or in any specific order across different age groups and populations	Use multiple testing over time
The prevalence of any specific autoantibody in the general population is significantly higher than the disease incidence	Screening for multiple autoantibodies will be necessary. This will increase the specificity but decrease the sensitivity
Some patients have no autoantibodies	Employ other assays capable of detecting individuals at risk, e.g. HLA typing and glucose tolerance test. However these assays are significantly more time consuming and expensive, thus can not be as easily applied for population based screenings

of these issues (65, 66, 109). More prohibitive in terms of applying autoantibody screening to the general population in a cost-effective manner are the following reasons unfortunately inherited in the natural history of the autoantibodies.

First, the prevalence of any specific autoantibody in the general population is significantly higher than the disease incidence (see references in Table 1) meaning that if autoantibody screening were to be the only basis for identifying individuals amenable for a potential intervention therapy, many more would be treated than would ever develop the disease, which can be difficult to justify unless the treatment is completely safe and inexpensive – and this is not likely to be the case. Since the relatively high prevalence of beta cell autoantibodies in the general population seems to reflect the presence of real autoantibodies and thus is not a technical problem with detecting false positive (references in Table 1) this issue will be difficult to solve. In theory these limitations in prediction can be overcome by measuring more than one marker, e.g. both an autoantibody marker and a genetic marker like HLA or two or more autoantibody markers. Fewer than 0.33% (1/300) individuals in the general population express more than a single beta cell autoantibody; this prevalence is similar to the risk for type 1 diabetes in the US thus supporting the approach of measuring more than one marker (VII, 110). However, it must be realized that the increase in specificity is on behalf on a decrease in the sensitivity.

Secondly, the autoantibodies do not appear at any specific age (VII, 40, 41, 111) making multiple round of screening in the same population necessary over time adding significant cost to identifying individuals at risk of developing type 1 diabetes.

Thirdly, approximately 7-15% of recent onset or pre-diabetic individuals do not have any autoantibodies at all (VII, 112). A potential solution for this problem is to use other assays capable of detecting individuals at risk, e.g. HLA typing and glucose tolerance test. However, these assays are more time-consuming and expensive than screening for autoantibodies. Therefore, at present these tests can not be justified for screenings of the general population.

4.2 T-cell assays

Autoantibodies are not believed to play a significant role in the auto-immune destruction of the beta cells (113), although they have made significant contributions to improve our capabilities to predict the disease and boosted the identification of potential T-cell autoantigens. Actually they have been the main source with very few exceptions. However, this focus on autoantigens recognized by autoantibodies may have de-routed in the task aiming at identifying potential important T-cell autoantigens that can provide information on the pathogenesis and aiding in designing future antigen-specific prevention/intervention therapies. Having demonstrated that several beta-cell autoantigens selectively seem to be recognized by T-cells and not by autoantibodies supports this view (114-116). Further-

more several reports have described an inverse correlation between B- and T-cell responses to beta cell autoantigens, including insulin and GAD65 (117, 118).

In contrast to the successful standardization of many of the autoantibody assays, attempts to standardize T-cell assays for identification of individuals at risk of developing type 1 diabetes have been hampered by several factors (119). These factors include lack of reproducible assays, lack of appropriate autoantigens and low precursor frequencies of circulating autoreactive T-cells combined with lack of access to the inflammatory lesion in the pancreas. However, with the rapid development of new technologies, such as detection of cytokines secreted from T-cell by using enzyme-linked immunosorbant spot assays (ELISPOT) (120) and HLA tetramers (121) that allow a quicker detection of peptide-specific T-cells compared to the traditional proliferation assays, the stage is being set to improve the qualitative and quantitative detection of autoreactive T-cell associated with the development of type 1 diabetes. Therefore, future T-cell assays are likely to play an important role in 1) identification of new autoantigens, 2) monitoring the potential effect of immunotherapy and 3) and potentially aiding in identifying individuals at risk of developing type 1 diabetes.

4.3 Predicting type 1 diabetes in different populations

The development of new biochemical assays based on recombinant beta cell autoantigens, e.g. GAD65, insulin and IA-2, has resulted in a tremendous step forward in our ability to identify individuals at risk and understand the natural history of type 1 diabetes. The following section will review some of the main findings in different populations.

4.4 Prediction of type 1 diabetes in the general population

Due to the problems discussed above regarding screening in the general population, we and others have conducted limited screenings for GAD65, IA-2 and/or insulin autoantibodies (122, 123). In a study consisting of more than 1000 unselected school children from the Netherlands, we tested for the presence of GAD65 and IA-2 autoantibodies (122). Development of diabetes was recorded during a 7 year follow-up period. No children were positive for both autoantibodies without developing diabetes, strongly supporting, as suggested above, that two or more autoantibodies could be employed in screening in order to increase the specificity. Although the number of children that developed diabetes during the follow-up is too low to make any firm conclusions, the positive predictive value for GAD65 autoantibodies alone was 40%. For IA-2 autoantibodies alone or the combination of GAD65 and IA-2 antibodies, the positive predictive value was 100%. However, the latter prediction is at the cost of low screening sensitivity since 50% of the children progressing to diabetes would not have been identified by using positivity for both GAD65 and IA-2 autoantibodies as prediction criteria.

4.5 Prediction in relatives to type 1 diabetic patients

In contrast to general population screenings, involving screening of thousands to millions of individuals and many years of follow-up, it is much easier to investigate the sensitivity, specificity and predictive power of autoantibody screening strategies in first degree relatives of diabetic subjects.

In a study of an unselected Finnish population of 755 siblings to type 1 diabetic children, ICA, insulin, GAD65 and IA-2 autoantibodies were characterized (VII). Thirty-two siblings progressed to diabetes within the 7.7 year follow-up period. The positive predictive value of ICA, IA-2, GAD65 and IAA were 43%, 55%, 42% and 29%, respectively, and their sensitivities 81%, 69%, 69%, and 25%, respectively. Thus these data demonstrate that by using a single autoantibody assay, many individuals that would never develop type 1 diabetes will be identified to be at risk for developing the disease. However, the disease risk is strongly correlated to the number of autoantibody markers present in a given individual, probably because it reflects a spreading of the immune response as the disease progresses. Therefore, prediction has also shown to be greatly improved by considering combinations of autoantibodies as demonstrated in several studies (VII, 85, 112, 124, 125-129). In all these studies two or more autoantibodies provided a higher predictive value than a single autoantibody alone. In the Finnish study the positive predictive value in siblings with one or multiple autoantibodies were 10% and 61%, respectively and when combining, e.g. GAD65Ab and IA-2Ab, the sensitivity was 81% and the positive predictive value was 41%, which is comparable to ICA (VII). Thus this document that the "golden" standard ICA could be replaced by GAD65 Ab and IA-2Ab screening in a large population study of unselected subjects with an adequate length of follow-up.

The predictive value may be increased even further if only individuals with high titre autoantibodies are considered as described for ICA (130). Similarly, the positive predictive values of IA-2Ab and IAA increased when higher cutoff limits are used, but on the expense of a lower sensitivity VII. Unlike the other autoantibodies, the levels of GAD65Ab do not differ significantly between progressors and non-progressors. Consequently, raising the threshold for GAD65Ab only results in a lower sensitivity (VII).

The Finnish study did also address the necessity of multiple autoantibody screenings over time (VII). Six point three percent of the progressors had all four antibodies, 65% had three antibodies, 9.4% had two antibodies, 3.1% had one antibody and 15.6% did not have any of these antibodies in their initial blood sample suggesting that more than 10% would never have been identified if only one screening was applied, numbers that are similar to what has been reported in other studies (Figure 2). However, multiple autoantibody testing over time increases the predictive value. Accordingly, in the Finnish study, 97% progressors had ICA, 87.5% had IA-2, 87.5% had GAD65Ab and 81% had IAA on one or more occasions during the follow-up before the diagnosis of type 1 diabetes (VII). Taken together all progressors had one or more of the autoantibody markers antibodies during their pre-diabetic follow-up. An analysis like this will, however, be difficult to conduct in the general population because of the time and cost it takes to identify a pre-diabetic individual.

4.6 Prediction of latent autoimmune diabetes of adults

Historically type 1 diabetes has been considered to be a disease with clinical onset predominantly in children, therefore the name "Juvenile Diabetes". However, recent studies support a different view suggesting that the disease can occur at any age and that the incidence may be significantly higher in adults. Depending on the population studied, 5-30% of patients initially diagnosed with type 2 diabetes may actually have type 1 diabetes (131-133). These patients have first been identified by their expression of autoantibodies to GAD65 at onset of type 2 diabetes (131). Clinically these patients are initially treated with traditional oral anti diabetic

drugs stimulating insulin secretion. However, they will relatively fast become completely insulin-dependent in order to maintain glucose control, i.e. type 1 diabetic patients (131, 133, 134). The slow onset has led to the disease designation Latent Autoimmune Diabetes of Adults (LADA) (132). In a study from Finland screening recent onset type 2 diabetics (n = 1122) as well as individuals without type 2 diabetes but with impaired glucose tolerance (IGT), (potential pre-diabetics, n = 383), 9.3% and 4.4% were positive for GAD65Ab, respectively (134). In contrast, IA-2 or ICA is less frequent and their presence is mainly associated with GAD65Ab positivity. The GAD65Ab positive individuals from this study had significantly lower fasting C-peptide and lower insulin response to oral glucose and elevated frequency of HLA susceptibility alleles associated with the development of type 1 diabetes compared to the GAD65 negative individuals. An even larger study (UKPDS) (133) investigating the time to insulin dependence after being diagnosed as type 2 diabetic, clearly supported our original finding that GAD65Ab positivity will result in a rapid progression to insulin dependence compared to GAD65Ab negative type 2 patients (131). In the UKPDS study, the proportion of the 3672 individuals having ICA was 6% and GAD65Ab was 10%, with 12% of patients having either ICA or GAD and 4% having both autoantibodies. Ninety-four percent of patients with ICA and 84% of patients with GAD65Ab required insulin therapy by 6 years after clinical onset of type 2 diabetes, compared to only 14% of those without these autoantibodies, strongly supporting that these patients may in fact be type 1 diabetics with a slow progressing disease. Since the incidence of type 2 diabetes is 10 fold higher than type 1 diabetes the LADA patients could comprise significantly more than half of all patients with type 1 diabetes. The LADA patients may represent an excellent alternative to the hard to identify pre-diabetic individuals since they, like the pre-diabetic, have several years to insulin dependence. Therefore, the LADA patients could in the future dramatically change the way we view autoimmune diabetes and also have implication for future prevention/intervention therapies.

4.7 Prediction of type 1 diabetes in gestational diabetes

Gestational diabetes (GDM) complicates 1-3% of all pregnancies. The occurrence of GDM primarily predisposes to the development of type 2 diabetes (135) but is also associated with a 40 fold in-

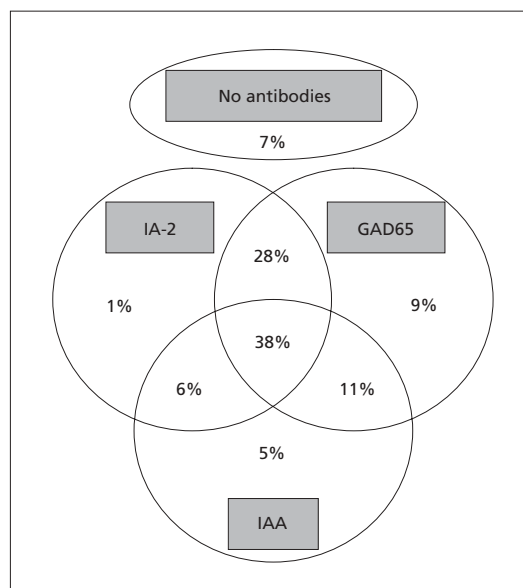


Figure 2. Prevalence of autoantibodies in prediabetics. The prevalence of antibodies in prediabetics (n = 105) who developed diabetes during follow-up. Identified from 3578 relatives data pooled from 4 different studies (85, 126, 127). Modified after (112).

creased risk of later developing type 1 diabetes (136). Few studies have demonstrated that women with GDM have an elevated frequency of GAD65Ab ranging from 2.2% in a Danish study to 10% in a German study (V, 137, 138). In the Danish study (V) 6 (4.3%) out of 139 GDM patients developed type 1 diabetes during a median follow-up of 6.3 years. Of the 6 individuals that progressed to type 1 diabetes 3 (2.2%) were positive for GAD65Ab at diagnosis of GDM compared to 0% of healthy pregnant women, i.e. resulting in a positive predictive value of 50% and a specificity of 100%. Similar numbers were obtained for ICA. Combining ICA and GAD65 Ab identified one additional patient developing type 1 diabetes (V). None of the GDM patients were positive for IAA, including the 6 individuals who later developed type 1 diabetes. The later development of the disease could be due to IAA being predominantly associated with early age of onset. Furthermore, by comparing the prevalence of GAD65 autoantibodies in insulin-treated GDM patients with non-insulin-treated GDM patients it has been demonstrated that the prevalence of GAD65Ab is 15% and 1%, respectively (139).

The lack of autoantibodies in the majority of women with GDM supports the notion that GDM is not caused by an autoimmune process. However, women with GDM who also have beta cell autoantibodies, e.g. GAD65Ab, have a higher risk of needing insulin treatment during pregnancy and developing type 1 diabetes later in life. Thus screening GDM patients for GAD65Ab may not only guide treatment but also identify women who could be eligible for preventive therapy.

4.8 Predicting the disease progression after onset of type 1 diabetes

Many studies have investigated different autoantibodies before and at onset of type 1 diabetes and characterized their ability to predict progression to onset of disease. Only a few studies have characterized autoantibodies in relation to progression of disease after onset, e.g. beta cell function and insulin requirements (III, V, 140, 141). As the onset criteria is not a specific point in time in relation to either the immune system or the beta cell mass, it is generally believed that there is no difference before and after onset in the ability of autoantibodies to predict progression. However, patients are being treated with insulin to compensate for their own inability to produce sufficient amounts of insulin to maintain normoglycemia at onset of disease and thereafter. Therefore, it can be argued that the period after onset of disease is very different both in terms of the metabolic demand on the remaining beta cell and in terms of the immune system, e.g. insulin will cause beta cell rest and compounds inducing beta cells rest, such as potassium channel openers (PCO), have demonstrated that they can delay progression of type 1 diabetes in humans after onset of disease (142, 143). Furthermore, several studies in animal models of type 1 diabetes have demonstrated a pronounced effect of insulin administrations on the immune system in terms of inducing tolerance to beta cells and thereby preventing/delaying the disease progression. Consequently, studying the progression in disease after onset in relation to the presence of autoantibodies may not only help guide future intervention studies but also provide a better understanding of the disease pathogenesis.

A study in recent onset type 1 diabetic patients treated with immunosuppression (Cyclosporin (CyA)) or placebo, demonstrated that both the frequency and titer of ICA and insulin antibodies (IA) (IA denominates the presence of IA after initiation of insulin treatment and IAA before insulin treatment is initiated) were significantly reduced in the cyclosporine treated patients (III, 48, 51). However, positivity for ICA or IAA before insulin treatment did not predict beta cell function in either the CyA or placebo-treated patients. In a subsequent study, we therefore, investigated if the presence or level of GAD65Ab could predict the outcome of immunosuppressive treatment as well as the beta cell function after onset of disease in the placebo treated patients (III). In contrast to ICA and IA the presence or level of GAD65Ab did not change during 12

months CyA treatment or 6 months after the CyA treatment was terminated. Furthermore, GAD65Ab positivity was not able to predict non-insulin requiring remission in the CyA-treated patients. The resistance of GAD65 autoantibodies to CyA therapy could be due to an increased antigen presentation of GAD65 compared to the ICA antigens, since high amount of GAD65 are present in the central nervous system (I, 63), which could release GAD65 continuously. Support for this speculation comes from a study demonstrating that 57% of type 1 diabetic patients with a mean duration of disease for >25 years are still GAD65 autoantibody positive compared with only 21% being ICA positive (144).

Even though no correlation between GAD65Ab and remission in the CyA treated patients could be demonstrated, the beta cell function was more than 30% lower in the GAD65Ab positive placebo-treated patients at 9 and 12 months after onset of disease compared to the GAD65Ab negative patients. Unlike GAD65Ab, the presence of ICA did not appear to be associated with decline in beta cell function after onset of disease like. The discrepancy between the ability of using GAD65Ab to predict beta cell function in the natural history of the disease after onset and not being able to do so in the CyA-treated patients is in accordance with similar studies of the predictive value of ICA for remission in the natural history of disease (145) and in CyA-treated patients (51). The explanation for this apparent discrepancy is most likely the different nature of the spontaneous remission in type 1 diabetes and that induced by immunosuppression. In the former, GAD65Ab are markers for the natural history of beta cell destruction whereas in the latter, the antibodies may not be related to the mechanism of action of immunosuppression that causes the remission.

In contrast, positivity for I-A2Ab was associated with a smaller improvement in beta cell function (C-peptide) during CyA treatment (146). This could potentially be explained by the fact that I-A2Ab appears later in the disease pathogenesis and consequently is a marker for more advanced disease progression/beta cell destruction and therefore more difficult to effect with CyA treatment.

The ability of GAD65 autoantibodies to predict the natural history of beta cell destruction/function is supported in a subsequent study (V) analyzing type 1 diabetes diagnosed during pregnancy. Such patients provide a unique opportunity to study correlation between autoantibodies and the final stages of beta cell destruction as they often have a long remission period after delivery, due to the high insulin demand during pregnancy (147). In this study (V) only analyzing GAD65Ab, we found that there was a difference in the non-insulin-requiring period after delivery in patients who were GAD65Ab positive (median 0.5 years) compared to patients who were GAD65Ab negative (median 2.6 years). In line with several of the studies reported in this review we were not able to demonstrate a correlation between the level of GAD65Ab and the length of the non-insulin-requiring period.

However, a study investigating patients with long standing type 1 diabetes (median 21 years) (141) was not able to demonstrate any correlation with persistent GAD65Ab and residual beta cell function. The explanation for this apparent discrepancy to our studies discussed above could be that the patients were investigated 21 years after onset of disease compared to only few years in the studies above. Furthermore, it may complicate the interpretation further that the autoantibodies were not measured at onset of disease but 21 years after onset at which point in time the prevalence has changed significantly, i.e. 82% compared to 32%, respectively.

Never the less the presence of GAD65Ab in type 1 diabetics, at least for the first few years, seems to be correlated with a more rapid decline in beta cell function after onset of type 1 diabetes which has also been extensively documented for GAD65Ab positive patients with type 2 diabetes (51, 131, 133). The implication of these data for future intervention studies could be that patients should be stratified for GAD65Ab positivity.

5. ANTIGEN-SPECIFIC PREVENTION AND INTERVENTION IN TYPE 1 DIABETES

The antigen-specific prevention and intervention discussed in this section will be focused on two of the main autoantigens, GAD65 and insulin as the involvement of other autoantigens in the disease pathogenesis have not been as well documented or their involvement in the pathogenesis of type 1 diabetes is controversial (Table 2). In order to set the scene for the later discussions of antigen-specific prevention/intervention in animal models and in human clinical trials, this section will first provide a brief introduction to the field of antigen-specific tolerance followed by a discussion of some of the animal models used in type 1 diabetes.

5.1 Antigen-specific tolerance induction

Antigen-specific tolerance defined as the absence of pathogenic autoimmunity (151, 162). The antigen-specific tolerance can be established and maintained by many different effector mechanisms as illustrated in Figure 3.

Administration of soluble antigens has been shown in several type 1 diabetes animal models to prevent beta cell destruction (IV, 163, 164) and has also been pursued in clinical trials, unfortunately without any effect. However, it remains unclear whether soluble antigens is an effective way of inducing regulatory T-cells (T-reg) (165), as only few studies have been able to demonstrate the presence of T-reg after exposure to soluble antigens (166). In diabetes, the presence of T-reg after parental insulin therapy has not been convincingly demonstrated. Besides the use of soluble antigens, adjuvants and cytokines given alone or together with antigens have also been demonstrated to promote the induction of T-reg (167-175).

Regulatory T-cells are not believed to be a special subpopulation of T-cells defined by a unique gene expression, but rather appears to be a quantitative difference in gene expression that is defining regulatory T-cells, also referred to as Th2 (normally associated with the peripheral tolerance) and Th3 (normally associated with mucosal

tolerance in particular oral) (176-179). Two main properties are shared by the regulatory T-cells: 1) They have an impaired capacity to respond to proliferative signals which makes them difficult to identify and isolate; and 2) they have an ability to inhibit other immune-cell functions, e.g. pathogenic T-cells associated with autoimmunity, either directly, through cell-to-cell contact, or indirectly, through secretion of anti-inflammatory cytokines. Some evidence suggests that also anergic T-cells share some of these properties (180).

Another attractive way of promoting tolerance induction is to present the antigen via "privileged" sites where the induction of tolerance is the rule rather than the exception. These sites are not surprisingly part of the reproductive organs (181, 182) and the interphases between self and non-self, in particular mucosal surfaces but also the interior chamber of the eye (183-187). Due to the ease of administration, induction of tolerance via the oral mucosal route has been explored extensively in many autoimmune diseases including diabetes (IX, X, 186, 188-190).

Oral tolerance is mediated by the Gut-Associated Lymphoid Tissue (GALT) that primarily function to protect the host from ingested pathogens but it also has the inherent property of preventing the host from reacting to ingested food antigens. Unlike the systemic immune system that function in a sterile milieu and responds vigorously to foreign antigens, the GALT guards organs which are replete with foreign antigens. It follows that upon encounter with this enormous antigen stimulus, the GALT must efficiently select the appropriate effector mechanism and regulate its intensity to avoid bystander tissue damage and immunological exhaustion. Thus, mucosal (oral) administration of antigens may result in non-responsiveness of the GALT but also in the development of regulatory T-cells capable of maintaining peripheral immunological tolerance, in case intact food antigens should escape from the gut to the peripheral. This process is referred to as oral tolerance (151, 179, 191, 192). This profound difference between GALT and systemic associated immune responses is most likely associated with the way

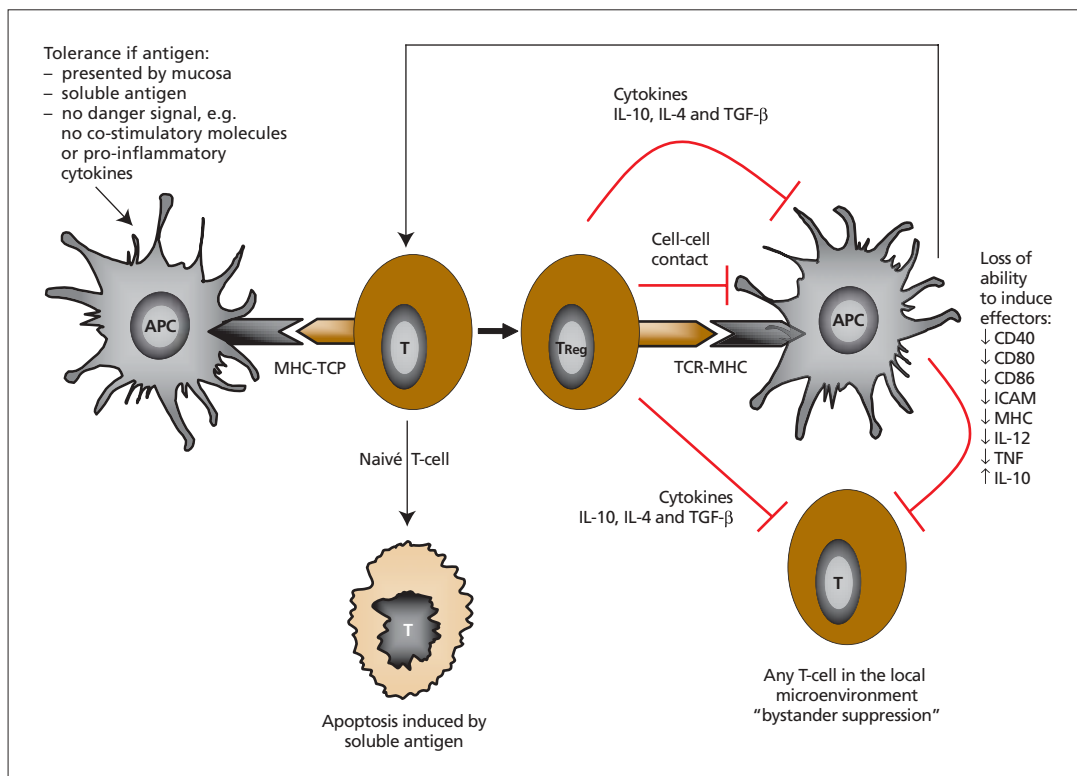


Figure 3. Mechanisms of tolerance induction. Lymphocytes can be deleted by apoptosis after exposure to soluble antigen. Lymphocytes that encounter antigen in the absence or with insufficient co-stimulation signals from APC's, e.g. non-professional APC's such as intestinal epithelial cell in the gut, antigens formulated in incomplete Freund's adjuvant (IFA) and/or anti-inflammatory cytokines such as IL-4, IL-10 or TGF- β can become anergic, i.e. inactivated or induced into regulatory T-cells (T-reg).

APC's present antigens to T-cells, i.e. in contrast to the systemic APC's, mucosal APC's seems to predominantly induce T-reg (Figure 3). There are several explanations for this. First, intestinal epithelial cells have been demonstrated to present antigen via MHC class II. These non-professional APC may lack proper co-stimulatory molecules thereby promoting T-reg. Secondly, the cytokine milieu in the gut mucosa is strongly biased towards anti-inflammatory cytokines like IL-4, IL-10 and TGF- β , which all are associated with promotion of T-reg (151, 179).

5.2 Animal models for type 1 diabetes

There are several animal models for human type 1 diabetes, each with their individual strengths and weaknesses. The following section will introduce some of the most frequently used animal models (see overview in Table 3).

The non-obese diabetic (NOD) mouse develops autoimmunity and beta cell destruction resembling type 1 diabetes in humans (193) (Table 3). One of the most striking similarities between this animal model and the human disease is the close resemblance between the murine and human MHC susceptibility molecules I-A^{g7} and HLA-DQ8, respectively. However, in addition to autoimmunity against the beta cells, the NOD mouse seems to have a general immune abnormality resulting in a mild degree of lymphopenia, aberrant cytokine signaling and other autoimmune diseases such as sialitis, thyroiditis and gastritis which are apparently unrelated to the development of autoimmune diabetes, differing somewhat from type 1 diabetes in humans.

Even with these differences the NOD mouse animal model is the most extensively used model of type 1 diabetes. It has, however, become increasingly clear that the NOD mouse alone cannot be used to predict the outcome of prevention and intervention strategies in humans, mainly because the disease is so easily prevented, i.e. more than 125 different approaches have been successful in preventing the progression of beta cell destruction (193). This has led to misconceptions and erroneous extrapolations resulting in false expectations with regard to the promise of immunotherapy preventing/curing type 1 diabetes.

There are many reasons why it has not been possible to extrapolate finding from the NOD animal model, the main reason is assumed to be that type 1 diabetes is both a complex and multifactorial disease in this model in terms of the genetic and environmental contributions (193, 200). The NOD mouse model, and any other of the animal models described in Table 3, only reflects one genetic variation of the disease and only a few environmental components since most colonies today are maintained under stringent specific pathogen-free conditions. Another reason is that there have been only few attempts to standardize work with this animal model (201, 220), which is as important as the autoantibody standardizations workshops.

With the appreciation of the complexity in using the NOD mouse model as a surrogate for the human disease comes the realization that it is essential to extend mechanistic studies in the NOD mouse to other animal models of type 1 diabetes. The main rat model is the BB/Wor diabetes-prone rat that develops diabetes spontaneously like the NOD mouse model (Table 3). There are several reasons for this model not being used more. Fewer immune reagents compared to what is available for the mouse and the fact that maintenance cost of rats are considerably higher compared to mice are important factors limiting the use of this model. In addition, development of diabetes is associated with severe T-cell lymphopenia, thus making immunointervention studies difficult to interpretate (216). In concordance with this it has been difficult to conclusively demonstrate humoral and T-cell responses to autoantigens in this animal model (VI, 205). However, the Komeda diabetes-prone rat is a new promising rat model spontaneously developing autoimmune diabetes without lymphopenia this model may overcome some of the issues associated with lymphopenia (221).

There are also several environmental-induced models of type 1 diabetes, e.g. streptozotocin (STZ) (222), Alloxan induced diabetes (223), BB/WOR diabetes-resistant induced with immunomodulation and virus (224) and lastly, a relatively new and promising mouse model, the LCMV model (209, 212).

The LCMV model is based on transgenic H-2d positive mice expressing the nuclear protein (NP) of the lymphocytic choriomeningitis

Table 3. Comparison between autoimmune diabetes in animal models and humans

	Human	NOD-mouse	BB-rat	LCMV
Genetic predisposition	Multigenetic	Multigenetic	Few genes	No (only viral transgene)
MHC association	Yes	Yes (I-A ^{g7})	Yes	No
Spontaneous disease	Yes	Yes	Yes	Disease induced with LCMV
Environmental influence	Yes	Yes	Yes	No
Onset of disease	Anytime but peaks in puberty	10-35 weeks of age	8-15 weeks of age	4-8 weeks after LCMV infection
Gender bias	No	Yes	No	No
Insulinitis	Yes, but not peri-insulinitis	Yes	Yes	Yes
Other autoimmunity	No, rarely	Yes	Yes	No
Autoantigens	GAD65, IA-2 and insulin + others	GAD65, IA-2 and insulin + others	Difficult to demonstrate	GAD65 and insulin
Lymphopenia	No	Mild	Severe (T-cell)	No
Effective prevention	No (s.c./oral insulin and nicotinamide)	Multiple, e.g. insulin and GAD65	Insulin	Insulin and GAD65
Effective intervention	Yes with Cyclosporin A and CD3	Yes with Cyclosporin A CD3 and insulin	Potassium channel openers (PCO)	Not done
Incidence	0.01-0.4%	60-90% females 30-60% males	70-100%	90-100%
References	54, 194-199	164, 193, 200-203	164, 164, 164, 164, 204-219	164, 208-212

gitis virus (LCMV) under the rat insulin promoter (RIP-NP) (187, 208, 209). When infected with LCMV, these mice clear the virus infection and in the process they develop a strong immune response against the NP protein. Thus within 3-8 weeks after virus infection the mouse develops diabetes due to a strong CD4 and CD8 response directed to the NP expressed in the beta cells. Insulinitis begins only when the systemic antiviral response reaches its peak and continues well after the LCMV infection has been cleared (187, 225). Therefore, the localized, beta cell-specific autoimmune process can be viewed as a true autoimmune process that follows kinetics completely different from the systemic anti-viral immunity although initiated by a response to the viral (self) NP transgene. Indeed antigenic spreading to insulin and GAD65 is observed during the pre-diabetic phase (226), pointing to the importance of these two auto-antigens. The LCMV model is, for the above reasons, a good model for autoimmune diabetes and because it comprises many features found in human diabetes without being immunocompromised like the models that spontaneously develops the disease (Table 3). Another distinct advantage of the LCMV model is that the time-point for induction of the autoaggressive, LCMV-NP specific response can be chosen experimentally (LCMV infection), and the NP-specific destructive CD4 and CD8 lymphocytes can be traced reliably (187, 227).

In summary, it is clear that single experimental animal models of type 1 diabetes should not be used to determine the efficacy of prevention and intervention strategies in humans but minimum two different animal models such as the NOD and LCMV models should be used. By using this approach a better understanding of which therapeutic protocols that is reasonable to extrapolate to humans and which are not can be obtained. This will be exemplified in some of the studies discussed later.

5.3 Insulin specific prevention and intervention in animal models of type 1 diabetes

Gotfredsen and colleagues first demonstrated that hypoglycemic doses of insulin could inhibit beta cell destruction in BB-rats (164). This observation was then extended to show that only hypoglycemic doses of insulin could protect the BB-rat while lower doses were ineffective. Based on this and other studies it was concluded that insulin treatment appears to work primarily by beta cell rest in the BB-rat (228). Atkinson and colleagues similarly demonstrated that insulin administered subcutaneously could protect NOD mice from diabetes including non-hypoglycemic doses (163). Although a different approach than parental insulin injections, it has also been demonstrated that insulin or insulin B chain immunizations in incomplete Freund's adjuvant (IFA) can protect the NOD mice from development of diabetes (229). Wegmann and colleagues have demonstrated that a large proportion of the T-cells infiltrating the islets in NOD mice are recognizing the insulin B chain epitope B9-23, and that these T-cells can transfer disease into irradiated recipients indicating the importance of this epitope. They have also used the B9-23 peptide in antigen-specific therapy and demonstrated that immunizations in IFA as well as mucosal administrations (intranasal route) could protect NOD mice from development of diabetes (230). Many other approaches have been tried using pro-insulin, insulin or insulin fragments in antigen-specific therapy (Summarized in Table 4). Among these is the most prominent oral tolerance using insulin the reason being that protection has been clearly demonstrated to be mediated via regulatory T-cells that act as bystander-suppressors in the pancreatic draining lymph node, where they dampen auto-aggressive responses utilizing the IL-4/STAT6 signaling pathway (186, 187, 210). Since the mechanism is via bystander suppression this circumvent the need for identification of the initiating autoantigen(s). However, human trials, with this promising approach have failed, in basically all of the major autoimmune diseases, including diabetes.

5.4 Human trials using insulin immunotherapy

A small pilot study in humans by Eisenbart's group demonstrated that first degree relatives at high risk of developing diabetes were protected from progression in disease compared to historical controls when given parental insulin (243). In combination with some of the animal experiments discussed above (Table 4) this formed the basis for the large and well conducted Diabetes Prevention Trial-1 (DPT-1) which was initiated in order to determine whether parental insulin could prevent or delay the onset of overt diabetes in relatives of patients with diabetes. This tremendous effort involving screening of more than 84,228 first and second degree relatives for auto-antibodies followed by genetic, immunologic and metabolic staging to quantify their risks ending up with 339 randomized individuals to treatment with parental insulin. Unfortunately this huge and commendable effort recently concluded that it was not possible to prevent or delay the disease using parental insulin therapy (0.25U/kg/day, non-hypoglycemic dose) (244). This result was a great disappointment for the entire diabetic community since expectations had been very high. Can we explain why a therapeutic approach that has been demonstrated to work in several different animal models and in a small human pilot trial and subsequent supported by several publications in particular using the NOD mouse model did not work?

In order to explore this discrepancy we investigated the influence of insulin dose, treatment frequency and the contribution of beta cell rest on diabetes development in the NOD mouse animal model.

Treating NOD mice daily with a low dose of insulin (0.30U/kg/day) similar to the one used in the DPT-1 study showed no effect on diabetes development. Significantly higher doses were needed to see an effect, but even when using higher doses the treatment effect was strongly dependent on frequency of the insulin administrations, i.e. 2 times a week did not work whereas 5 and 7 times worked (10).

These studies demonstrate the critical importance of dose and treatment frequency and may explain why the DPT-1 study did not work, i.e. a too low dose of insulin was used. Unfortunately it will be difficult to do a study in humans with higher doses of insulin because of the risk of hypoglycemia.

In the second arm of the DPT study daily oral insulin administrations were studied. Like in the parental insulin arm this arm of the study (DPT-2) was unfortunately also reported to be without any effect in delaying or preventing the progression of type 1 diabetes in medium risk individuals (communicated in a session at the American Diabetes Association meeting 2003 in New Orleans, USA by Dr Jay Skyler). The result was, however, anticipated based on a previously clinical study. In this study comparable doses to that used in the DPT-2 trial was without effect in preventing deterioration in beta cell function (198). We also analyzed the influence of dose and antigens used, i.e. insulin species, since we did not believe that these issues have been properly addressed. Even though there are only few differences between porcine and human insulin the differences are profound in the doses required to induce oral tolerance and prevent the development of diabetes in both the LCMV and NOD animal models, i.e. porcine insulin has a maximal effect at 1 mg/dose and human insulin around 10 mg/dose (IX, 188). Unfortunately, it is not known which doses of mouse insulin I or II that would be required to obtain a similar therapeutic effect, but this point to the fact that one should carefully consider which antigen to use in future clinical trials. The importance of even minute differences, like between human and porcine insulin, is further supported in a recent study (245). In this study subcutaneous injection in incomplete Freund's adjuvant of the B9-23 insulin II, but not the B9-23 insulin I peptide, significantly protected NOD mice from diabetes. These two peptides are both endogenous mouse peptides and only differ in position B9. The protection afforded by the insulin 2 peptide but not the insulin 1 peptide in the NOD mouse was reflected by its predominant Th2 humoral response.

However, as humans only have one insulin gene, the most likely

explanation for the failure of the human oral insulin trials is the low doses of insulin used (Table 4). In our hands 10 mg/dose of human insulin is needed to prevent diabetes in the LCMV mouse model (IX) which is the same or higher than was used in the human trials (Table 4) and based on the relative difference between mouse and human gut sizes and body weights (several thousand fold), the effective insulin dose should have been significantly higher. Supporting the hypothesis are the results of a human exploratory trial using oral administrations of keyhole limpet haemocyanin (KLH) to suppress subsequent T cell responses following KLH immunizations, where doses of 50-100 mg/dose were effective (246). When we used KLH to induce similar oral tolerance to KLH in mice, we found that 50-100-fold less antigen is needed to obtain comparable reduction of the KLH specific response (1.5 mg KLH required in mice versus 50-100 mg in humans (IX)). However, since insulin is a different antigen than KLH it is difficult to extrapolate these findings to insulin and calculate an appropriate dose. Nevertheless a comparable dose of insulin, taking the KLH data in humans and mice into account, would have been between 330-660 mg/dose, clearly exemplifying that the dose used in humans in all likelihood has been too small.

Thus future clinical trials should either increase the dose of oral insulin significantly, which could be cost prohibitive, or alternatively develop adjuvants potentiating the effect of oral insulin.

5.5 Future directions for insulin specific immunotherapy in type 1 diabetes

The failure of the DPT-1 trial could be explained by the low dose of insulin used. It has been demonstrated that an inactive insulin analogue (X38) mutated at a single amino acid position thereby preventing binding to the insulin receptor, is as effective in preventing the development of diabetes in the NOD mouse as metabolically active insulin (232). Furthermore it has also been demonstrated that the non-metabolic active insulin B chain or insulin B9-23, can prevent disease as effectively as metabolically intact insulin (234). These data suggest that the preventive effect mediated by parental insulin administrations in the NOD mouse is selective via an immunological mechanism, i.e. because insulin is an antigen and not a hormone. In addition, using inactive insulin analogues will eliminate the risk of hypoglycemia and allow the use of significantly higher doses to explore the validity of this therapeutic approach in man.

The failure of the oral insulin trials could also be explained by the low doses used, as discussed above. Using the "right", i.e. high dose, will probably not be economically attractive. To overcome this problem we and others have investigated the effect of mucosal adjuvants. It has been demonstrated that a single dose of minute amounts (micrograms) of antigens conjugated to the receptor-binding non-toxic B subunit moiety of cholera toxin (CTB), can markedly suppress

Table 4. Insulin immunotherapies in animal models and humans.

Model	Antigen	Route	Protection from diabetes	Reference
BB rat	Insulin high doses	Subcutaneously	Yes	164, 228
BB-rat	Insulin	Mucosal (oral)	No	231
NOD mouse	Insulin	Subcutaneously	Yes	163
NOD mouse	Inactive insulin analog (X38)	Subcutaneously	Yes	232
NOD mouse	Insulin B-chain	Subcutaneously in IFA	Yes	229
NOD mouse	Insulin B9-23	Subcutaneously in IFA	Yes	229, 233
NOD mouse	Insulin B9-23	Subcutaneously no adjuvant	Yes	234
NOD mouse	Insulin B9-23 or B10-24	Mucosal (intranasally)	Yes	168, 233, 235, 236
NOD mouse	Porcine insulin	Mucosal (oral)	Yes	186, 237
NOD mouse	Pro-insulin	DNA, IM	Yes	238
NOD mouse	Pre-Pro-insulin	DNA, IM	No – acceleration of disease	239
LCMV model	Insulin B chain	DNA, IM	Yes	240
LCMV/NOD mouse	Human insulin	Mucosal (oral)	No	188
LCMV model	Porcine insulin	Mucosal (oral)	Yes	188
LCMV model	Human insulin high doses	Mucosal (oral)	Yes	X
LCMV/NOD model	Insulin conjugated to CTB very low doses	Mucosal (oral)	Yes	IX, 241
LCMV/NOD model	Insulin mixed with CTB low doses	Mucosal (oral)	Yes	X
PVG.RT1 rat	Insulin B1-18	Intratyptic	Yes	242
Human high-risk prediabetic Small study	Insulin low doses	Subcutaneously	Yes	243
Human recent onset (IMDIAB)	Insulin low doses	Mucosal (oral)	No	198
Human high-risk prediabetic DPT-1	Insulin low doses	Subcutaneously	No	244
Human medium risk prediabetics	Insulin low doses	Mucosal (oral)	No	*

*) Results communicated at the ADA meeting 2003 by Dr. Jay Skyler.

systemic T cell-mediated inflammatory/autoimmune reactions in naive as well as in immune animals (247, 248). Furthermore, in the NOD animal model it has been demonstrated that not only can the frequency of oral feedings be reduced, but the dose of insulin conjugated to CTB can be reduced up to 500 fold, compared to the maximal effective dose of insulin given alone (IX, 249, 250). In a series of adoptive transfer experiments, we have demonstrated that the mechanism by which CTB conjugated to insulin (CTB-insulin) protects against the development of diabetes is indeed via bystander suppression (IX, 241). Furthermore, the protection seems to be mediated via CD4⁺ T cell (IX, 251) positive for CD62L and α 4-integrin corresponding well with the mucosal origin of the regulatory T-cells 252. The mechanism of protection by the CTB-insulin regulatory CD4⁺ in the LCMV animal model seems to be via suppression of autoaggressive (LCMV specific) CD4 and CD8 responses in the pancreatic draining lymph nodes (IX) via inhibition of IFN- γ and probably mediated through IL-4 (251). We also demonstrated that the pronounced difference between the doses of porcine and human insulin needed to induce tolerance and protection from diabetes development was abrogated by conjugating porcine or human insulin to CTB (IX). Consequently, human trials using CTB conjugates would probably not depend on minor differences in antigenic sequences and thereby have a higher chance of success.

Previous studies by Weiner and others have suggested that the precise amount of fed autoantigen is crucial for obtaining suppression of autoimmune disease. In their experiments "intermediate" (0.5-1.0 mg/dose) but not high antigen dosages (>10mg) resulted in induction of regulatory lymphocytes (253). Too high amounts of oral antigen are thought to lead to deletion of antigen-specific T-cells including the regulatory T-cells, an outcome which is not desirable when the goal is to promote them (179). We have also demonstrate that higher doses of both insulin and the CTB-insulin conjugate are not able to mediate bystander suppression expanding Weiners findings to insulin and even to insulin conjugated to CTB (IX). This finding has implications for future clinical trials in type 1 diabetes, strongly suggesting that several doses should be tested in order to avoid ending with a dose that might be too high thereby only inducing deletion of insulin-specific T-cells and no bystander suppression and consequently little if any clinical effect.

Another issue hampering the therapeutic use of the CTB-insulin conjugate approach is the very difficult conjugation procedure between insulin and CTB. In small scale it is relatively easy to produce CTB-conjugated to insulin. However, large scale conjugation necessary for developing this approach for commercial use has unfortunately proven to be much more complicated. The main reason being that insulin is conjugated to the CTB pentamer through free amino groups of which CTB has more than 50 and with 3 amino groups on insulin this gives a very large amount of different conjugate species, i.e. $50^3 = 125,000$. Even if the conjugation can be controlled to some degree, it is very difficult to make a reproducible and reliable process required for testing in humans. (Karen DeJong and J.S. Petersen unpublished observations). To overcome this problem we have attempted to produce a genetic fusion protein between the insulin B chain and CTB. A preliminary analysis of this fusion protein suggests that this fusion protein may be a viable path overcoming some of the problems with the chemical conjugation (254).

Another potential solution for the chemical conjugation issue is to use the insulin and CTB as simple oral mixtures. The rationale behind this idea comes from the demonstration that CTB directly co-stimulates antigen-primed CD4 and CD8 T-cells (255) and also has a stimulatory effect on antigen-primed CD4 T cells (256). Thus it could be speculated that without conjugation CTB would work as a mucosal adjuvant potentiating the effect of oral tolerance. In accordance with this speculation we have demonstrated that CTB, even in its unconjugated form, function as a mucosal adjuvant increasing the specific tolerogenic effect of oral insulin by more than 10 fold on a dose-to-dose comparison, i.e. as little as 1 μ g CTB

mixed with 100 μ g insulin works significantly better than 1 mg of insulin (X). To obtain a comparable effect using human insulin, doses between 5 and 10 mg are needed (IX).

These findings suggest that CTB may be a suitable mucosal adjuvant for potentiating the effect of oral insulin tolerization but potentially also of other autoantigens, e.g. GAD65, thereby allowing this approach to be tested in human clinical trials using doses that are economically feasible.

DNA vaccination using the insulin gene represents another new approach. However, there are conflicting results with regard to the safety and efficacy of this approach (Table 4). In some reports using animal models of type I diabetes a good protection is seen (238) and in others an acceleration of disease is observed (239). The discrepancy between these results may be that different parts of the insulin molecule were used in the different studies. In addition, plasmid design and vaccination modalities may affect the clinical outcome of DNA vaccination (257, 258). In conclusion, before insulin DNA vaccination may be fully accepted as safe for use in clinical trials, additional animal studies should be conducted to further elucidate the mechanism behind the dual outcome of this approach in disease prevention and acceleration.

5.6 GAD65 specific prevention and intervention in type 1 diabetes

The following sections will discuss the use of GAD65 in prevention and intervention. However, since GAD expression is not as restricted as insulin, and the expression of an autoantigen can have significant implications for the interpretations of its involvement in the disease pathogenesis, a brief introduction to the GAD protein and its expression is given below.

In 1990 Baekkeskov and colleagues identified the 64 kDa autoantigen as GAD65, the biosynthetic enzyme of the inhibitory neurotransmitter gamma aminobutyric acid (GABA) (259, 260). To date, two isoforms of GAD, GAD65 and GAD67, have been identified and shown to be encoded by separate genes on different chromosomes (261). The GAD65 protein is amphiphilic and can be both membrane-bound and soluble, whereas GAD67, which shares 67 amino acid sequence homology to GAD65, is hydrophilic and soluble (262). Both isoforms are expressed in the GABAergic neurons of the brain as well as in pancreatic islets and to a less degree in thymus, testis, ovaries and stomach (263-265). More surprisingly there seems to be a significant variation of GAD65 and GAD67 expression in the islet of human and animals (I, IV, 265). Rat islets express both GAD65 and GAD67 restricted to the beta cells (I). In contrast, only GAD65 can be detected in human islet and is, in addition to beta cells, also localized to some alpha, delta and PP cells. The selective expression of GAD65 in human islets may explain why autoantibodies predominantly recognize this isoform of GAD. In mouse islets including the NOD mouse animal model of type 1 diabetes, the overall GAD expression is significantly lower than that observed in both human and rat islet (I, IV). Furthermore, the predominant isoform expressed in mouse islets is GAD67 expressed approximately at two fold lower levels than in rat islets (IV). GAD65 expression in mouse islets was difficult to detect and was found to be approximately 10 fold lower than seen in rat and human islet (I, IV). Even though the expression of GAD has been characterized, the role of GAD and GABA in the islets remains largely unknown, but the very high variability in expression pattern between different species could indicate that, in contrast to the brain, this is not an important enzyme for islet function. This speculation is further supported in a recent study, demonstrating that selectively suppressing the islet expression of GAD65 and GAD67 *in vivo* using antisense mRNA had no effect on the insulin production and glycemic control (266).

Injection of GAD65 intrathymically (267), intravenously (i.v.) (268) early in life or i.p. in neonatal NOD mice (IV) was not only demonstrated to tolerize the T-cell mediated immune response against GAD65 but also against other beta cell autoantigens, such as those against HSP65 and CPH, and consequently prevent/delay in-

sulitis and the progression of diabetes. In our study we followed NOD mice neonatally tolerized with GAD65 for almost the entire lifespan of the animals in order to investigate if neonatal tolerance induction to GAD65 is eventually broken. Unfortunately this turned out to be the case. At 72 week of age 60% of the GAD65 treated mice compared to 90% of the sham treated mice had developed diabetes (IV). However, the remaining diabetes-free NOD mice treated with GAD65 had a significant reduction in the intra-islet infiltration of mononuclear cells (insulinitis) at 72 weeks of age compared to non-diabetic controls treated with bovine serum albumin or sham. The controls all had more or less end stage islets, i.e. completely infiltrated by mononuclear cells. These data indicate that at least in some NOD mice, a life long tolerance to GAD65 can be successfully induced. However, the data also indicate that in order to achieve a more reproducible and sustainable tolerance induction, several treatments will probably be needed in order to maintain tolerance. In addition, oral administrations of GAD65-expressing transgenic potato plants, recombinant GAD65 and nasal administration of GAD65 peptides or intact GAD65 have also shown to prevent the development of diabetes in the NOD mice (150, 189, 190, 236). Furthermore, several approaches of DNA vaccination using the GAD65 gene has been shown to prevent development of diabetes in animal models but in one study GAD65 DNA vaccination accelerated the disease (Table 5). In order to address the potential safety problems with DNA vaccination in terms of disease acceleration, von Herrath and colleagues have demonstrated that co-immunization with an IL-4 expression plasmid reduces the risk of augmenting autoaggression against the beta cells and in this way increases the safety margin of DNA vaccination (257, 269), thus holding promise for this approach to move into clinical trial in the future.

If GAD65 autoimmunity plays an important role in the disease pathogenesis, it should be possible to induce the disease by generat-

ing an immune response to GAD65. Several groups have attempted this and not been successful including our own (281). We injected several different mice strains with GAD65 in a strong Th1 adjuvant, i.e. complete Freund's adjuvant (282). We were not even able to see mononuclear cell infiltration in the islets despite a detectable T-cell response against the injected GAD65. The explanation for this failure in inducing disease is most likely that before T-cells reacting to GAD65 can home to the islets they need a homing signal, e.g. the presence of activated APC's presenting islet antigens including GAD65. A study demonstrating that diabetes can be provoked by immunizing NOD mice with GAD65 at 3 weeks of age when the islets only are infiltrated by APC's (283) supports this hypothesis. Furthermore, a CD4 T-cell clone specific for GAD65, isolated from a mouse in which diabetes was provoked by GAD65 immunizations, were capable of adoptively transferring insulinitis and diabetes into NOD-*scid* mice (283). However, these findings only show that GAD65 reactive T cells can be diabetogenic *in vivo* but do not indicate if the GAD65 autoantigen is the primary autoantigen initiating the disease process in NOD mice (283). More direct evidence for a primary role of GAD autoimmunity comes from a study of Yoon and colleagues demonstrating that complete suppression of both GAD65 and GAD67 expression by antisense-GAD expressed under the rat insulin promoter can prevent the development of diabetes and islet cell autoimmunity in general (266). This result is in discrepancy to a study selectively inhibiting the GAD65 expression in islets. In this study there was no effect on diabetes development (279). As mentioned above we have demonstrated that mouse beta cells predominantly express GAD67 and very low level of GAD65 (IV). Therefore it may be difficult to draw conclusions on the importance of GAD autoimmunity using only GAD65 knockout mice since GAD65 shares several epitopes with GAD67. Yoon and colleagues (266) have been met with considerable skepticism in the scien-

Table 5. GAD immunotherapies in animal models and humans.

Model	Antigen	Route	Protection from diabetes	Reference
BB rat	GAD65 from rat brains	i.p/neonatal	No	VI
NOD mouse	rGAD65	i.v.	Yes	268
NOD mouse	rGAD65	intrathymic	Yes	267
NOD mouse	GAD65 from rat brains	i.p/neonatal	Yes	IV
NOD mouse	rGAD67	i.p	Yes	270
NOD mouse	GAD65	Mucosal transgenic plants	Yes	190
NOD mouse	rGAD65	Subcutaneously in IFA	Yes	271
NOD mouse	rGAD65	Recombinant vaccinia virus	Yes	272
NOD mouse	GAD65 peptides	Mucosal (nasal)	Yes	150
NOD mouse	GAD65 +/- IL4	DNA, IM	Yes, but in some cases on with IL-4	239, 240, 273-276
NOD mouse	GAD65	DNA, IM	No acceleration of diabetes	277, 278
NOD mouse	GAD65/67 suppression of expression in beta cells	Antisense RNA	Yes	266
NOD mouse	GAD65 suppression of expression in beta cells	Antisense RNA	No	279
NOD mouse	GAD65 transgenic animals	Expression under invariant chain promoter	No	280
LCMV model	GAD65	DNA, IM	Yes, seems to work better together with IL-4	269
Human recent onset Type 2 diabetics GAD65Ab positive (LADA)	GAD65	Subcutaneous, in adjuvant (Alum)	Small effect in LADA patients	*

*) 2003/2004 ADA meeting, presented by Dr Åke Lernmark.

tific community since the absence of GAD expression in islet may render the beta cell resistant to apoptosis or induce an unknown metabolic effect protecting the islets, thus it can not be definitely concluded that GAD autoimmunity is of primary importance in the NOD mouse before this has been analyzed in more details.

A recent study has demonstrated that expression of a transgenic GAD65 construct, in NOD mice, with enhanced routing to the class II MHC loading pathway induced complete GAD65 specific tolerance to all epitopes without effecting the development of insulinitis and diabetes (284). The authors of this paper wrongly concluded, at least not supported by data, that GAD autoimmunity is of no importance for diabetes development in the NOD mouse. However, in view of the predominance of GAD67 expression in mouse beta cells (IV) one cannot exclude the fact that some diabetogenic T-cells selectively are recognizing GAD67 specific epitopes and thereby still being able to cause disease. This was unfortunately not investigated in this study. Therefore, it can "only" be concluded, based on this study, that autoimmunity to GAD65 is of little pathogenic importance in the NOD mouse.

How can these data be reconciled with the fact that various regimens of GAD65 administration result in prevention/delay of disease whereas tolerogenic expression of GAD65 has no effect on the disease development. It has been demonstrated that GAD65 administration generates regulatory T-cells that can be adoptively transferred to provide protection from development of disease, probably by local suppression of the immune response to beta cells in a bystander fashion as for oral tolerance (179). In contrast, transgenic expression of GAD65 was demonstrated to result in deletional tolerance, i.e. elimination of GAD65 specific T cells rather than generation of regulatory T-cells, thus the findings discussed above are not mutually excusable.

Most studies testing different GAD65 therapeutic approaches have mainly been done in the NOD mouse animal model. However, we have also analyzed the importance of GAD65 autoimmunity in the BB-rat (VI). In this study we were not able to detect any GAD65 T-cell reactivity in either naive or GAD65 immunized BB-rats. This indicates complete tolerance to the GAD65 antigen or that GAD65 autoreactive T cells are sequestered in the pancreas. In order to eliminate the possibility that GAD65 autoreactive T-cells are important in the pathogenesis, but not detectable in a conventional T-cell assay, BB-rats were also tolerized to GAD65, using a tolerance i.v. protocol demonstrated to work for another antigen (BSA) in the BB-rat and in the NOD mouse model (268). However, we were not able to demonstrate any delay or prevention of diabetes, even though the critical importance of islet cell antigens in the development of diabetes in the BB-rat has been demonstrated by Posselt and colleagues (215). In this study Intra-thymic injection of whole islets into 3 week old BB rats, i.e. similar age as the rats used in our study, completely prevented the development of diabetes, thus demonstrating that induction of tolerance to a specific beta cell autoantigen(s) can abrogate the autoimmune beta cell destruction in the BB-rat. Like in the study expressing GAD65 as a transgene in NOD mice (280), i.v. injection of soluble antigen is also believed to mainly induce deletion of antigen-specific T-cells (285). Thus these data can also be reconciled with the fact that GAD65 may be a good autoantigen to use for immunotherapy, even though they demonstrate that GAD65 autoimmunity is of little importance for diabetes development in both the NOD mouse and BB-rat. However, one cannot exclude that GAD67 may play an important role in the development of diabetes in the BB-rat as discussed for the NOD mouse, since the BB-rat also express relatively high levels of GAD67 in the beta cells and our study did not address tolerance to the isoform of GAD67 (VI).

Even though it remains to be elucidated what role GAD65, and in particular GAD67 have in the pathogenesis of type 1 diabetes especially in relation to animal models of diabetes which in contrast to human beta-cells, express GAD67, it is clear that GAD65 is a major autoantigen recognized by T-cells and/or autoantibodies from sev-

eral different animal models including humans with type 1 diabetes. It is also documented that immunotherapy with GAD65, especially therapeutic approaches aiming at inducing regulatory T-cells, are capable of delaying and/or preventing development of diabetes in animal models.

5.7 Human trials using GAD65 immunotherapy

Only one clinical trial in humans has tested if GAD65 immunotherapy can delay or prevent beta cell destruction in recent onset type 2 diabetics patients with GAD65 autoantibodies, i.e. LADA patients. Human recombinant GAD65 is formulated in the Th2 promoting adjuvant Alum and used to immunize LADA patients. Four different doses of 4, 20, 100 and 500 µg have been tested in groups of only 9 patients. Each patient received their first injection followed by at least one boost injection four weeks later. So far no safety issues have been observed at any dose level.

The only effect reported at the 2004 ADA meeting (Agardh et al. Diabetes 272-OR) was an improvement in C-peptide. In addition the dose response seems to be bell shaped, i.e. 4 and 100 µg/dose showed no response, while 20 µg/dose resulted in an improvement in C-peptide. Although these results give some promise, larger trials are needed in order to give a more firm indication of the success of this approach.

6. CONCLUDING REMARKS

If we look into the future, one may argue that predicting and preventing type 1 diabetes may be difficult and may not even be possible. However, 15 years ago we could hardly predict the disease and basically no autoantigens had been identified, at least not any that could be used in reliable, high capacity autoantibody assays. Furthermore, only few prevention/intervention therapies had been tested. Now 15 years later we can predict the disease although we are not quite ready to predict disease in the general population, but we have the tools to move forward as discussed in this thesis. Likewise, in terms of prevention and intervention, many new approaches have been tried successfully in animal models. Some of these have been tested in humans unfortunately without great success for reasons discussed herein. However, new approaches as proposed in this thesis such as inactive insulin analogs allowing higher, therapeutically more relevant doses to be tested and mucosal adjuvants may prove more promising. Furthermore, a few new approaches tested in humans look promising and have actually been able to arrest the disease progress, e.g. GAD65 immunizations and anti-CD3. If within the next decade we can accomplish the same progress as has been made in predicting and preventing the disease during the past decade, type 1 diabetic patients have every reason to be optimistic about the prospect for a cure.

THIS THESIS IS BASED ON THE FOLLOWING PUBLICATIONS:

- I. Jacob S. Petersen, Steven Russel, Michael O. Marshall, Hans Kofod, Karsten Bushard, Allan Karlsen, Bill Hagopian, Esper Boel, Åke Lernmark, Alister Moody, Thomas Dyrberg, Kim Hejnæs, Ole Madsen, and Birgitte Michelsen. Differential expression of Glutamic Acid Decarboxylase in rat and human islets. *Diabetes*. 42, 484-495. 1993.
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