IDENTIFICATION, CHARACTERIZATION AND APPLICATION OF AUTOANTIGENS IN TYPE 1 DIABETES MELLITUS

Henk-Jan Aanstoot

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IDENTIFICATIE, KARAKTERISERING EN TOEPASSINGEN VAN AUTOANTIGENEN BIJ TYPE 1 DIABETES MELLITUS

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PROMOTIECOMMISSIE

PROMOTORES:	Prof. Dr. H.K.A. Visser
	Prof. Dr. H. Galjaard
CO-PROMOTOR:	Dr. G.J. Bruining
OVERIGE LEDEN:	Prof.Dr. S.W.J. Lamberts
	Prof Dr. R. Benner

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'Door de vreugde van de ontdekking verblind, zullen wij niet uit het oog verliezen, dat het insuline nog slechts een eerste schrede is op den weg eener oorzakelijke behandeling der suikerziekte'

A.A. Hijmans van den Berg, 1925, Utrecht , *Voordrachten over suikerziekte*, 278.

opgedragen aan de grote mensen van morgen

CONTENTS

Outline of this thesis

Type 1 diabetes mellitus or insulin dependent diabetes mellitus is a disease characterized by the selective destruction of insulin producing ß-cells in the islets of Langerhans. The exact cause of this destruction is unknown, but is mediated by cells of the immune system. The immune system has a remarkable ability to recognize self from non-self, thereby providing a constant surveillance-mechanism that protects us for foreign invaders. This mechanism has failed in type 1 diabetes and attacks and destroys ß-cells. The autoimmune basis of type 1 diabetes is described in chapter 1. The central problem in autoimmunity is why and how the immune system sometimes fails to distinguish between self and non-self. Re-instructing the immune system or blocking faulty immune reactions could result in primary- or secondary prevention of type 1 Diabetes Mellitus. This requires however, the molecular dissection of the process, including the identification of ß-cell proteins involved in the autoimmune reactions. This thesis aims to contribute to this by the identification and characterization of two humoral autoantigens in type 1 Diabetes Mellitus, a 64kD and a 38kD protein. These aims are specified as follows:

1. Identification of the 64kD autoantigen in type 1 Diabetes Mellitus.
2. Biochemical and Cell biological characterization of the 64kD protein.
3. Identification of the 38kD autoantigen in type 1 Diabetes Mellitus.
4. Biochemical and Cell biological characterization of the 38kD protein.
5. Analysis of the frequencies of autoantibodies to the 64kD and the 38kD autoantigen at clinical diagnosis and in the prediabetic period.
6. Assessment of the predictive value of these autoantibodies, in particular in relation to other markers of autoimmune ß-cell destruction.

In chapter 2 experimental work is presented that identified the 64kD autoantigen as the neurotransmitter γ-amino butyric acid (GABA) synthesizing enzyme Glutamic Acid Decarboxylase (GAD). This identification was the result of the combination of clinical and laboratory data. The 64kD/GAD autoantigen was found to be a target for humoral autoimmunity in both type 1 Diabetes Mellitus and in the neurological disease Stiff-man syndrome. Differences between the two diseases in antigenicity towards GAD were found. From biochemical and cell biological studies presented in chapter 2 it was concluded that the 2 forms of GAD, found in pancreatic islets of Langerhans were identical to the 2 forms found in brain. One form of GAD has a molecular weight of 67kD and is usually referred to as GAD_{67}. This molecule is water soluble. The second form of GAD was originally described as the 64kD autoantigen. The calculated molecular weight is 65kD and hence this

molecule is now referred to as GAD_{65}. GAD_{65} can be soluble and membrane bound. We found that membrane binding of GAD_{65} is a two-step process. The second step involves palmitoylation of the protein. The membrane binding allows the GAD_{65} molecule to combine with the membranes of synaptic vesicles in neurons or microvesicles of endocrine cells. Taken together, these findings have important implications for the understanding of why and how this antigen becomes a target for the immune system and provide a basis to study the function of GAD and GABA in these cells in more detail.

Autoantibodies are important markers for ongoing destruction of ß-cells. The identification of 64kD as GAD allowed to develop recombinant expression systems producing GAD that can be used in autoantibody assays. In chapter 3, the development and use of an improved immunoprecipitation method for the detection of autoantibodies to GAD is described. In addition, this method was compared with the original 64kD autoantibody assay where human or rat islets were used. The new recombinant GAD bases assay yielded a more sensitive test. More important is the predictive value of autoantibodies for the clinical onset of type 1 Diabetes: In a population of schoolchildren the predictive value of GAD autoantibodies was analyzed, and was found to be better than prediction with a traditional method. In chapter 4, studies on a 38kD autoantigen are presented. After establishing the optimal conditions for the partial purification of this protein, sera were analyzed from patients and prediabetic individuals (individuals from whom serum samples were available from the time preceding the clinical diagnosis). Although the frequency of autoantibodies to 38kD is low, the analysis contributed to the predictive value since individuals could be identified who were positive for 38kD autoantibodies, but negative for any other autoimmune marker used in prediction. Although the frequency of autoantibodies is low, this protein can still be considered a candidate primary autoantigen. Characteristics of the 38kD protein were studied and described, but the molecular identity has not been resolved yet.

In summary, the experimental work helped to identify and characterize two autoantigens in type 1 diabetes. Until now, experiments on pathophysiology and assays on prediction lacked such a ß-cell specific autoantigen. The findings described in this thesis can be applied in experiments where ß-cell specific antigens are used. The hypothesis can be tested if these autoantigens are involved in the primary events leading to type 1 diabetes. Finally, this work will allow the development of prediction tests, which use ß-cell specific autoantigens, are quantifyable and have an increased sensitivity and specificity.

Chapter 1

GENERAL INTRODUCTION

1.1 Diabetes Mellitus

1.1.1 The Diabetic Syndrome.

Diabetes mellitus is a syndrome of a disturbed energy homeostasis caused by a deficient production or action of insulin. Insulin is a hormone involved in glucose homeostasis and is produced by the ß-cells in the islets of Langerhans in the pancreas. Diabetes mellitus encompasses a group of disorders with an inability to properly metabolize glucose, leading to the characteristic feature of hyperglycemia. Type 1 or insulin dependent diabetes mellitus, the focus of this thesis, is the most common endocrine disorder in childhood and adolescence and is, in frequency, the third most common chronic disease in children after chronic obstructive pulmonary diseases and epilepsy (1). With an annual incidence rate among people under the age of 20 years of roughly 10-20 (1 new case per 5000-7000 children per year) it is 3-4-fold more common than chronic childhood diseases as cystic fibrosis or leukemia and it is nearly 10-fold more common than nephrotic syndrome, muscular dystrophy or lymphomas in this age group (1). Whether frequent or not, all chronic diseases have important consequences on physical and emotional development in children and adolescents affected. In addition, it has an impact on parents and sibs. Although symptomatic treatment is available, no cure exists. Long-term complications, which are frequent, put an additional burden on the patients. Taken together, type 1 diabetes mellitus is a disease with strong repercussions on the lives of individuals affected, and it is a major health care problem.

1.1.2 Classification of Diabetes Mellitus.

The endocrine pancreas has been implicated since a long time in the pathogenesis of diabetes mellitus. Increasing clinical and pathophysiological knowledge of this organ resulted in the distinction of three major categories of the diabetic syndrome, type 1 diabetes, type 2 diabetes and a third group of several different diabetic syndromes. In addition gestational diabetes is recognized as a separate entity. This classification is based on clinical (phenotypic) characteristics (2). Analysis of the etiologies at the molecular level might lead to a different classification.

Type 1 or insulin dependent diabetes mellitus (IDDM) is characterized by the inability of the body to produce insulin, due to the loss of insulin producing ß-cells.

This lack of insulin results in dependence on exogenous insulin, which can be partial, as is often the case shortly after diagnosis, but usually becomes complete within 2-7 years. The onset of type 1 diabetes is often insidious and can occur at any age. Without the administration of insulin, type 1 diabetes is fatal. It is predominantly this form that affects children and adolescents. Type 1 diabetes accounts for 5-10% of all cases of diabetes mellitus in industrialized countries (3).

Type 2 or non-insulin dependent diabetes (NIDDM) accounts for 90-95% of diabetes mellitus cases. Patients are generally, but not always, obese and may be non-symptomatic. This form has two components: 1) insulin resistance and 2) the failure of ß-cells to compensate for the resistance. This disease starts usually after the age of 30 years. Although some people with NIDDM use insulin, this is mainly to achieve a better glucose homeostasis and is not of vital importance. Usually classified as a form of type 2 diabetes is Maturity Onset Diabetes in Youth (MODY) (4). Recent data showed a linkage of the glucokinase locus on chromosome 7p to this disease (5) or, in other families with the Adenosine Deaminase gene on chromosome 20q (6). Nonsense mutations in the glucokinase gene have been reported in non-insulin dependent patients (7).

Some studies have suggested that the division between type 1 and type 2 diabetes may be less clear than suggested. In particular in older individuals, the development of type 1 diabetes may mimic that of type 2. A number of type 2 patients fail to respond to treatment with oral hypoglycemic agents and show features of type 1 diabetes, such as islet cell antibodies (ICA's, see chapter 1.5) (8). It has been estimated that 1-2% of apparent non-insulin dependent diabetes patients become insulin deficient (9). Although most NIDDM patients have a relative, rather than an absolute deficiency of insulin, some NIDDM patients develop an absolute insulin deficiency within a few years after instigation of oral therapy. This type of diabetes has been called type 1 1/2 and while the clinical features at first resembled much of those found in type 2 diabetes, these patients show several aspects of type 1 diabetes (10, 11) such as low C-peptide levels, low body weights, Islet cell autoantibodies, other organ specific autoantibodies and a high frequency of HLA DR haplotypes (12) (see chapter 1.5).

The third group of diabetes mellitus includes a mixture of diabetic syndromes including diabetes resulting from or associated with other specific syndromes or conditions (sometimes referred to as secondary diabetes), such as diabetes in patients with cystic fibrosis (13), hemochromatosis (14) and some syn-

dromes with known chromosomal defects with an increased frequency of diabetes. In Trisomy 21, (Down's syndrome) (15) this is primarily autoimmune (insulin dependent) type 1 diabetes. In other syndromes such as Turner's syndrome (16) the association is with non-insulin dependent diabetes. A very rare cause of insulin dependent diabetes is due to pancreatic agenesis (17). In the 1980 and 1985 World Health Organization (WHO) classification of diabetes mellitus included malnutrition-related diabetes mellitus as a new entity of diabetes (18) of which the etiology is unknown and both exocrine and endocrine tissue of the pancreas is involved (19). A syndrome of transient diabetes mellitus of the newborn exists that seems to be due to a delay in maturation of the ß-cell (20).

The heterogeneity of clinical presentation in type 1 diabetes suggests the possibility of multiple mechanisms as etiologies. This was recently confirmed by the finding that mitochondrial DNA deletions are associated with familiar forms of insulin dependent diabetes (21). The disease started in the twenties and thirties. This indicates again that insulin dependent diabetes mellitus might not in all individuals be caused by autoimmunity and warrants further studies on etiology.

No cure exists yet for type 1 diabetes mellitus, but the disease can be treated by frequent insulin injections. Although the daily applications of insulin might suggest a relatively stable life, it is in fact a constant challenge, especially for children, parents and sibs. Insulin therapy is only a simple imitation of the body's superb fine-tuned mechanism of hormone secretion and glucose homeostasis. Insulin injections will usually prevent the acute life threatening complications, but do not exclude the development of acute and long-term complications. Other therapies used in type 1 diabetes mellitus, such as immunosuppression and transplantation are not causal and have several disadvantages such as their costs and complications. It is conceivable that only prevention will change this substantially . However, primary prevention requires adequate prediction. Useful strategies for prediction and prevention will only be developed if the pathophysiological mechanisms of type 1 diabetes are dissected at the molecular level. The work described in this thesis aims to contribute to this.

1.2 Type 1 Diabetes Mellitus.

1.2.1 Epidemiological Characteristics.

Epidemiological surveys revealed that some features of type 1 diabetes have a striking similarity to infectious diseases, such as rheumatic fever / rheumatic heart disease, which occurs after streptococcal infections (22). This disease showed a dramatic decline in incidence when preventive approaches designed from epidemiological and microbiological data became available and applicable. Thus, proper analysis of the epidemiology of diabetes could help to identify risk factors involved in the etiology (23) and help to develop preventive strategies.

Most of the studies describing the incidence or prevalence of type 1 diabetes are conducted in children and collection of epidemiological data has been difficult since only a few countries have complete population based registries available for the analysis of type 1 diabetes. A WHO project (DIAMOND) is initiated to collect epidemiological data on type 1 diabetes from around the world (24). The next paragraphs outline some important epidemiological findings.

Type 1 diabetes shows a specific age distribution. Although slightly different per country, roughly 40-50% of the cases occur before the age of 15 years, 30-40% occurs between 15 and 34 years of age and the remainder of the patients will develop type 1 diabetes later in life (9). Several studies indicate a very low incidence before the age of 5 with a more or less linear increase until puberty, where the peak incidence is found (25). In a recent study from Finland however, the incidence was extremely low under the age of 1 year but reached already almost its maximum in the 1-4 year old group (26). The puberty peak, also reported in earlier work from Finland (27), was lacking. This might reflect a trend that the onset of the disease is shifting towards the younger age group, but more research is needed to substantiate this hypothesis (25). It is also conceivable that the onset of diabetes in the very young children has a different etiology and therefore follows a different pattern.

The incidence of type 1 diabetes varies enormously between different areas of the world. The highest incidence is reported in Finland with 35.2 cases per 100,000 children under 14 years of age (26). Other Nordic countries have similar high rates: Sweden 22.6, Norway 17.6, Denmark 13.7 (28). In other parts of the world a low incidence rate has been found, such as Israel (4.3) and France (3.7)

and Japan that has the lowest reported incidence with 0.8 per 100,000 (28). The analysis of all the incidence data in several areas of the world is suggestive for a gradient in North-South direction. High incidences are found in countries closer to the earth's poles and lower incidences in countries close to the equator (29). The increasing number of diabetes registries, established in recent years show, however, that it is not just a simple gradient: 'Hot-spots' with high incidence rates can be found in areas with an overall low incidence of type 1 diabetes. An example of this is Sardinia, which has an extreme high incidence of 30.0/100,000 (31).. The incidence rate in the mainland of Italy ranges between 4-10 (30). Another spot with a very high incidence was found in Poland (32).

The incidence of type 1 diabetes shows an increase in several countries over the last decades (25, 33-37). A mostly linear increase in type 1 diabetes risk was found over the past 20 years in children under 15 years of age (25). This increase has, however, not been reported from all areas (38, 39). A recent cohort study in Dutch army conscripts found cumulative incidences of 1.85 per 1000 in 1960, slowly increasing to 2.12 per 1000 in 1970. The overall increase was calculated as 4.4% per year (40). These data were confirmed and extended by a recent national survey on the epidemiology of type 1 diabetes in the Netherlands (41). This increased incidence over time in the Netherlands is similar to that in other European countries.

In chapter 1.5 the strong association between genetic factors and type 1 diabetes will be discussed, originally based on epidemiological data showing that the disease tends to cluster in families. However, in a large prospective nationwide, Finnish study, it was shown that of all the new cases of type 1 diabetes, only 11.2% were multiple cases, affecting first degree family members (26). Similar results were suggested earlier (42). Thus, although a determinant for the disease, the predictive power of a family history is limited and most cases of type 1 diabetes occur sporadically in the population.

A seasonal pattern was observed in the incidence of type 1 diabetes in older children and adolescents. A significant lower incidence was found in spring and summer. This seasonal dependence has been found for populations both north and south of the equator (34, 43, 44). It is also suggestive for an environmental factor. This will be discussed in detail in chapter 1.5.

In conclusion, type 1 diabetes has specific epidemiological characteristics. They indicate that genetic and environmental factors contribute to the disease. Further and detailed analysis of these features may help to unravel the etiology of type 1 diabetes in a similar way as has been done for rheumatic fever and other infectious diseases.

1.2.2 Impact on Health Care.

The diagnosis of type 1 diabetes has, without doubt, a tremendous impact on somebody's life. Morbidity and mortality of acute and chronic complications are high and this puts an additional burden on both personal life as well as on the health care system. Type 1 diabetes is disproportional associated with premature mortality and with high morbidity. Death rates are 8 - 10 times that of the general population (45). More than 2% of affected individuals die each year (46). Already at the age of 25 years, individuals with type 1 diabetes have an increased frequency of hospitalizations compared to non-diabetic controls for ophthalmic (5 x more frequent), neurological (9 x more frequent), renal (14 x more frequent) and cardiovascular complications (3). Hospital stays of type 1 diabetic patients are longer (47). The total cost increases with the age of the patients and is almost 3 times higher for patients over the age of 65 years compared to patients under 20 years of age.

Type 1 diabetes has an additional impact on health care. The necessary control of food intake, sleep, injections, daily activities and other things, results in additional stress and psychosocial problems. Especially in adolescents, these problems often need extra medical attention. Moreover, living with diabetes has implications for employment, travel, and life insurance.

These facts implicate that type 1 diabetes absorbs a substantial part of health care budgets, especially since the incidence increases. In sum, the morbidity and mortality statistics urge for careful planning of both medical care and research intended to reduction of complications and in particular aimed at primary prevention of the disease.

1.2.3 Natural Course and Complications.

After diagnosis and treatment of the acute metabolic derangement, the life expectancy of diabetes is mainly determined by the occurrence of secondary (long-term) complications. These can be divided into consequences of macrovascular and microvascular disease. Macrovascular disease results in coronary heart disease (CHD), cerebrovascular accidents (CVA's) and gangrenous disease of the extremities. Microvascular disease includes retinopathy, nephropathy and neuropathy. Both groups of complications occur very frequent. Where most studies describe features of only one of these complications, only a few have examined the epidemiology, interrelationships and correlates of these complications. In the large follow-up study from Pittsburgh (48) 657 type 1 diabetic patients were studied with a mean duration of diabetes of 20 years. Table 1.1 summarizes this study, showing that complications are very frequent. The sex-differences suggest that hormonal changes may have some influence. The severity of these complications as well as the speed with which they develop varies widely (49, 50). It ranges from minor retinopathy without the need for intervention to kidney dialysis or amputations of extremities. The fact that the same clinical complications occur in both type 1 and type 2 diabetes strongly supports the idea that metabolic (glycemic) control is a major factor.

Several studies indicate that duration of diabetes and the cumulative effect of prolonged metabolic aberrations are the main determinants of this risk, especially in microvascular complications (51-53). Glycemic control may be important, but large prospective studies are needed to prove that tight control results in fewer complications and have not been performed. One large collaborative study, originally intended as a long term study (54). has recently been stopped prematurely. Although not yet published, the results indicate that good glycemic control was beneficial to reduce long-term complications (DCCT study; data presented at the 1993 American Diabetes Association meeting, Las Vegas). Two groups of patients were studied. Those with good glycemic control showed a lower incidence of complications over a 10 year study period. Although this study is the first that clearly shows that glycemic control is likely to have an effect on the occurrence of complications, this issue is still open. More data are needed from patients who developed the disease at young age: in children, the goals of glycemic control aimed for in this study are almost impossible to reach. Moreover, even with strict metabolic control, complications can occur as is illustrated in descriptions of patients with type 1 diabetes of less than 3 years duration, with good

TABLE 1.1
Study of Complications After Various Years of Type 1 Diabetes Mellitus (48).

MACROVASCULAR DISEASE

Cardiovascular disease	10%	of men after 30 years of type 1 diabetes
	30%	of women after 30 years of type 1 diabetes

MICROVASCULAR DISEASE

Background retinopathy	99%	after 20 years of type 1 diabetes
Proliferate retinopathy	70%	after 30 years of type 1 diabetes
Kidney disease (from microalbuminuria to dialysis / insufficiency)	84%	of men after 30 years of type 1 diabetes
	59%	of women after 30 years of type 1 diabetes
Polyneuropathy	72%	after 30 years of type 1 diabetes

to extremely good metabolic control, but already with complications such as neuropathy and retinopathy. This suggests that additional factors are involved, a fact that is supported by the differences in incidence and severity of complications observed in monozygotic diabetic twins (55).

The pathological hallmark of diabetic microvascular disease is thickening of the capillary basement membrane (56). This process is found in all affected organs, resulting however in a wide variety of clinical symptoms in the three target organs: retina, kidney and peripheral nerves. The mechanisms involved are not clear. Insulin does not seem to play an important role, since the tissues affected are not dependent on insulin for their glucose uptake, suggesting that hyperglycemia itself plays a major role. Several mechanisms for the effect of hyperglycemia on the development of diabetic complications have been suggested, but

no definitive consensus has been reached. Hyperglycemia is likely to interfere with several enzymatic systems in the cell, either by direct inhibition (57), by osmotic effects of intermediate accumulations like sorbitol (58), glycosylation of the protein (59), inhibition of oxygen free radical scavenger mechanisms (60) or by combinations of these factors. Autoimmunity, the mechanism by which type 1 diabetes develops, has been implicated in the etiology of peripheral neuropathy in type 1 patients (61). The identification of neuronal enzymes in this autoimmune mechanism of diabetes (62) urges for further evaluation.

In summary, diabetic complications are a frequent cause of morbidity and mortality in type 1 diabetes. They are likely to be caused by the interaction of multiple metabolic and other factors. Epidemiologic data support a common background for the different forms of microvascular complications. Hyperglycemia plays a major role and probably initiates changes in the capillary basement membrane that results in a cascade of effects, influenced by other factors. Diabetic patients, with a detected microvascular complication, are 5-10 times more likely to develop another complication as well, compared with patients free of complications (48, 63). The high mortality and morbidity associated with diabetic complications is another justification for the development of preventive measures.

1.3 The Islets of Langerhans

1.3.1 Anatomy of the Islets

In type 1 diabetes, the pathological hallmark is the infiltration by mononuclear cells of the islets of Langerhans in the pancreas. Before discussing this process, called Insulitis, and its implications for further studies on the pathogenesis, the normal islet anatomy and physiological implications will be outlined in this section.

The human adult pancreas contains 10^5 to 10^6 islets (64). They are scattered through the parenchyma (exocrine tissue) of the pancreas, vary in size and cell number with a mean of 140 μm and about 1000 cells per islet (65). In the adult, the endocrine cell mass occupies 1% of the total organ (66), but in the embryological phase this might be up to 10%. In fish, one endocrine pancreatic organ called the Brockmann body (67) can be found. The advantage of having the endocrine cells scattered in islets throughout the pancreas, as is seen in higher animals,

is not clear. It is suggested that the interaction between acinar (exocrine) cells and islet cells (66) and intra-islet regulation are more effective in the case of dispersed islets. Four major endocrine cell types can be found in the islets and table 1.2 summarizes some characteristics.

TABLE 1.2
Characteristics of the four endocrine cell types of the endocrine pancreas (islets of Langerhans)

Cell type	hormone secreted	% of islet cell mass	localization in islet
α-cell	glucagon	15-20	cortex
ß-cell	insulin	60-80	core
δ-cell	somatostatin	5-10	cortex
PP-cell	pancreatic polypeptide	15-25	cortex

The islets of Langerhans have a typical architecture where ß-cells are predominantly found in the core of the islet and α-, δ- and PP-cells occupy the cortical (periphery) areas. This is most outspoken in the rat, where the α-cells can be outlined as a ring around a core. In other animals and humans it is less outspoken.

1.3.2. The Micro Society in the Islets of Langerhans

Islets are innervated by different neuronal systems. Claude Bernard (1813-1878) already demonstrated that innervation is very important for glucose homeostasis: lesions in the medulla resulted in hyperglycemia (Piqure diabetique) (68). Both parasympathetic (cholinergic) as well as sympathetic (adrenergic) nerves enter the islets. An additional innervation by other systems such as GABA-ergic

(69) as well as peptidergic neurons (70) has been described. The role of these nerves in the regulation and their exact target cell are unknown.

Islets contain a dense capillary network. Afferent arterioles enter the islet in the periphery but do not have contacts with endocrine cells until they branch in the ß-cell core of the islet. This branching results in a dense capillary network, resembling that of the kidney glomerulus. This makes the endocrine pancreas a well supplied organ. With 1% of the organ volume it receives 10% of the pancreatic blood flow (66). The capillaries are lined with fenestrated endothelial cells, allowing a rapid exchange of metabolites and products. The typical anatomical pattern of the arterioles and capillaries results in a blood flow that is directed from ß-cell towards the periphery, with direct consequences for the regulation of hormone secretion (71). The ß-cells are in close contact with these capillaries and show a polarized morphology. In the rat, 8-10 ß-cells form a tube-like structure around a capillary. Most of the insulin granules are grouped in proximity of this capillary (apical) side of the cell. The other side of the ß-cell also faces a capillary (72). The close relationships between ß-cells and capillaries were concluded from studies in rodents where the three dimensional structure of islets was reconstructed as well as from physiological studies that measured hormone responses upon injections of the counter hormones in different areas of the islet (73). ß-cells therefore can influence α- and δ-cells by the release of substances into the capillaries. The situation in human islets is less clear and the capillary network seems to be even more complicated (64).

The islet cells are furthermore characterized by intensive cell-to-cell contacts that allows paracrine signaling. ß-cells can depolarize and have a synchronized electrical activity, providing an alternative way of cell-cell signaling (74). The neurotransmitter GABA is present in ß-cells (75) and $GABA_A$ receptors have been identified on α-cells (76). This provides a mechanism for paracrine signaling. Some recent findings and implications of the GABA-ergic mechanism in ß-cells are discussed in chapter 3. A link between the autoimmunity and the GABA-ergic system in the ß-cell has been made (62). In addition to these communication systems, the islet cells have gap junctions, specializations between homologous and heterologous cells that allow passage of molecules up to Mw 1200 Dalton (64). Finally, to form and maintain this specific construction, islet cells must express adhesion molecules. Non-ß-cells predominantly express the 135kD isoform of the neural cell adhesion molecule (NCAM)(77), while E-cadherin (Uvomorulin) is involved in the ß-cell core interactions (78). Expression of adhesion molecules in

islet cells is dependent on the developmental stage of the islet cells (79). The advances in adhesion molecule research including studies on cell-cell interactions that play a role in autoimmunity (80), urge for further studies. Recent work describes prevention of an autoimmune disease by blocking a specific integrin receptor (81) and the use of antibodies to two adhesion molecules (ICAM-1 and LFA) in blocking cardiac allograft rejection (82). The autoimmune disease pemphigus vulgaris is caused by an autoimmune reaction towards an adhesion molecule (83). Such data clearly exemplify the importance of adhesion molecules.

The anatomical organization of islets results in a micro society of interacting cells. There is evidence supporting the idea that the organization of this micro society is of physiological importance. This is illustrated by experiments where islets were dissociated, resulting in a loss of regulated function. Spontaneous reaggregation results both in the restoration of the typical anatomy (with ß-cells in the core and the other endocrine cells in the cortex of the islet) as well as a return of the normal physiological pattern of basal and glucose stimulated insulin secretion (84-86). Not only do differences exist between the islet cells, also within the population of ß-cells there is anatomical and functional heterogeneity (69) .

In summary, the islet is a micro society of cells with abundant possibilities for interaction. The central role of the ß-cell is striking. Heterogeneity within the ß-cells is important when considering the pathology of type 1 diabetes, where some islets and ß-cells seem much more vulnerable than others in the same pancreas.

1.3.3 Islet Development and the Relations Between Neurons and ß-cells

Since the structure of the islets has implications for its function, it is conceivable that the embryo-fetal development of the human islets is of paramount importance for its structure, function and pathology. Some considerations on this point are given in this section. First, the islet is formed from single endocrine cells, that are able to differentiate and divide and thereby eventually form a cluster of cells called the islet of Langerhans. Second, embryological development of cells and tissues takes place during a certain time-frame. Although learning of the immune system continues throughout life, the embryo-fetal period is crucial. Expression of antigen during that period teaches the immune system to recognize them as self antigens or non-self 'invaders'. It should be noted that the term

'antigen' implies an immunological reaction. In fact antigens are normal cellular components, that only by virtue of an immunological reaction become 'antigen' in a strict sense. Third, knowledge of the development of ß-cells will result in the unraveling of the process of proliferation and differentiation of these cells. Such knowledge is pertinent for the development of strategies that will facilitate regeneration ß-cells after an attack, a situation that does occur naturally after partial pancreatectomy. In addition, the selective destruction of ß-cells seems to be a process that spreads out over a long period of time. The rapid onset of diabetes in some children under the age of 1 year and the finding of persistent neonatal diabetes not due to agenesis of islets (Bruining, Aanstoot, unpublished observations), urge for studies that analyze the role of developmental factors in ß-cell heterogeneity and this destruction. Finally, endocrine cells have several features in common with neurons. Most autoimmune diseases involve neuroendocrine tissue illustrated by the enzyme Glutamic Acid Decarboxylase, which is a target for studies in this thesis and which is present in neurons and ß-cells. Some of the shared features between these cells are especially outspoken during the embryo-fetal development of the islets.

The human pancreas develops from two diverticulae of the embryological gut during the 4th week of gestation in the human embryo. A ventral and dorsal primordium grow out and fuse at 7-8 weeks. At this stage all four major hormones can already been found. Glucagon is the earliest hormone detected by immunohistochemistry at 6 weeks of gestation (87). Islets are not present at that stage and the endocrine cells are scattered through the exocrine tissue, as single cells, especially in the area of ducts, or as small groups of cells (islet-like clusters) (88). Although endocrine cells have several features in common with neurons, they are believed to origin from endodermal precursors. However, several polypeptide hormone producing cells are suggested to develop from a neural crest precursor cell. Their similar characteristics lead to the concept of APUD cells (Amine Precursor Uptake and Decarboxylation (89)). While cell-tracing methods demonstrated the neuroectodermal origin of several APUD cells, a similar origin for endocrine cells of the pancreas could never be proven (90). Moreover, other studies supported an endodermal origin of the endocrine pancreas (91-93). Recently, using sensitive PCR combined with microsurgery techniques, Gittes and Rutter were able to identify mRNA for the islet hormones in the mouse embryo at very early stages (at somite stage 10, day 8) (94). They showed that at 20 somite stage (day 8.5-9) hormonal mRNA's were present in foregut cells that would later form the pancreas anlage. Areas that are located outside this zone were negative

for these mRNA's. The same study showed that cell-specific gene expression in the endocrine pancreas anlage began at a premorphogenetic phase i.e. before the organ itself started to form. It is conceivable that some of the foregut cells are pluripotent precursors of the endocrine lineage. Evidence for a common endocrine pancreatic precursor cell comes from studies in transgenic mice harboring hybrid genes containing the rat insulin II promoter gene linked to the coding sequence of SV40 large T antigen (Tag). Tag is primarily a nuclear, nonsecreted antigen. Pancreatic ß-cells in transgenic animals coexpressed the endogenous insulin gene and the transgene suggesting a common precursor for these cells. Subsequent work in the rat-insulin promoter-Tag (RIP-Tag) transgenes (95) showed that a period of coexpression of neuroectodermal markers and hormones takes place. Early endocrine pancreatic precursor cell express glucagon. Some cells will continue to express only glucagon during their entire life span and will give rise to α-cells. Other glucagon expressing cells will start coexpression of insulin. These cells will become committed to become insulin producing beta cells after loosing their glucagon expression. A fraction of these insulin/glucagon cells stop expressing glucagon and start to coexpress PP or somatostatin next to insulin. They are committed to become PP- or δ-cells. Interestingly, those early stages of differentiation show coexpression of neuronal markers such as tyrosine hydroxylase (TH) and phenylethanolamine N-methyltransferase (PNMT, the final enzyme in the catecholamine synthesis) (96). In the final stages of differentiation TH expression was lost (97). In the developing human endocrine pancreas, cells expressing simultaneously two or three hormones have also been identified (88). This coexpression in the human endocrine pancreas was confined to the second trimester of gestation, although data from the first trimester are incomplete (88). Coexpression of endocrine hormonal and neuronal markers can also be found in developing sympathetic neurons and chromaffin cells (98). Although expression of a hormone might indicate an end-stage of differentiation, it has been shown in experimental animals that some differentiated cells (defined by insulin expression) have proliferative capacity (as determined by ^{3}H-thymidine incorporation) in situations such as partial pancreatectomy (99). Such cells were furthermore characterized by (re-)expression of Tyrosine hydroxylase. Earlier studies suggested that (both fetal and adult) endocrine hormone containing cells can proliferate in vitro, but only under stimulating conditions (100, 101).

During the formation of islets, it is conceivable that, parallel to neuronal development, more cells are formed than eventually needed. This is more or less illustrated by the relative large amount of islets in the neonatal pancreas. The most

extreme example of this are newborn calves. They show such an extreme amount of islets that they become visible on the outside of the pancreas as small red dots ('blutinseln') (66). In pregnant animals and humans, an absolute and relative increase in ß-cells can be observed (66). Not all cells formed are needed to function as an organ and a special gene-controlled mechanism of cell death is used to adapt the number of cells to the situation. Organ formation and organ sculpturing is fully dependent on this process of programmed cell death (PCD). This process of cell death is different from necrosis. Special gene products are needed to induce apoptosis, which takes place without induction of scarring (102-104). This process is most extreme in lower animals such as the nematode C. elegans. Some cells are formed in this organism that only live a completely predictable period of time. They solely function as a 'message' or 'organizer' for other cells. In higher animals and humans similar processes take place. Genes and gene products are identified which are required in this process. The gene product Bcl-2, which encoding gene is localized within the human MHC, is a protein that stops programmed cell death and prolongs the life of cells. Except for these gene products, other molecules such as Tumor Necrosis Factor-α (TNF-α) and Müller Inhibiting Substance (MIS) are known to induce apoptosis (105). The role of apoptosis both in autoreactive T-cells as well as in islet formation is not clear. The formation of islets, also involves cells that are present in higher numbers during formation than after birth (Krijger, RR de, Bruining, GJ, Aanstoot, HJ, unpublished observations). This is in particular true for cells expressing somatostatin. These observations suggest that becoming a single hormone secreting pancreatic islet cell depends on the temporal expression of several genes during a certain time frame in the islet development. The importance of timed expression for the development of immunological tolerance, which may play a role in the pathophysiology of diabetes, will be discussed in Chapter 1.5 .

Despite the endodermal origin endocrine cells and neurons do share several features. In addition to the already mentioned TH and PNMT, other neuroectodermal antigens are expressed in ß-cells, for example PGP 9.5 (106), Neuron Specific Enolase (NSE) (107), Synaptophysin (108), Tyrosine Hydroxylase (TH) (109), and Glutamic Acid Decarboxylase (GAD) (110, 111). Moreover, ß-cells also show functional characteristics of neurons. They are excitable cells, responding to chemical signals with depolarization (74) An other example of identical systems is a similar dual pathway of regulated secretion. All cells have a constitutive pathway of secretion. This includes the formation and transportation of small vesicles from the trans-golgi region to the plasmamembrane, responsible for the

delivery of membrane components to the cell surface (112). In addition to this system some cells specialize in regulated secretion. Endocrine cells and neurons therefore have a regulated secretion mechanism consisting of two additional pathways. The first system consists of small vesicles: neurons possess small synaptic vesicles (SSV's) that contain neurotransmitters, which are released at the synaptic cleft upon depolarization (113). SSV's are 20-40 nm in diameter. Endocrine cells have a similar vesicle population, which is slightly larger (30-40 nm) and are usually referred to as synaptic vesicle like microvesicles (SLMV's). They are believed to contain non-peptide messengers that are involved in paracrine regulation (for review see (114)). Next to this SSV/SLMV system, neurons harbor larger vesicles that contain peptide messengers such as enkephalines. The peptide contents results in a dense image of these vesicles in electron microscopy, hence their name Large Dense Core Vesicles (LDCV's) (114). Although it was thought initially that neurons were committed to the secretion of one type of neurotransmitter (i.e. GABA or acetylcholine or adrenaline), it has been shown that one neuron can release more than one transmitter simultaneously (113). Peptidergic transmitters, such as enkephalines, are stored and released by LDCV's. The counterpart of LDCV's in the endocrine cells is the secretory granule (SG). They contain peptide hormones. Thus, both cell types have two pathways for regulated secretion, the SSV/SLMV pathway and the LDCV/SG pathway. SSV's are characterized by specific marker proteins such as synaptophysin, synaptobrevin, synapsins and p65 (113). SLMV's contain (at least in some cell types) marker proteins identical to those in SSV's. For synaptophysin however, there seems to be an overlap in localization. This marker is reported to be found in both SLMV's and the secretory granules of α- and ß-cells (115). A comparable situation was found for the neuronal SSV marker protein Rab 3A, which was believed to be a selective marker for SSV's (116), but was also found in the secretory granules (chromaffin granules) of bovine adrenal cells (117). The GABA-synthesizing hormone Glutamic Acid Decarboxylase (GAD) is another example of a protein expressed in both neuronal and endocrine lineages. The role, function and localization of GAD is further discussed in chapter 2.

In conclusion, endocrine pancreatic cells originate from endodermal (foregut) cells, albeit that they possess several neuronal markers. The development and formation of, eventually, 4 endocrine cell types producing one hormone each, is still largely unknown, but they are likely to develop from one precursor cell. Islet formation is dependent on specific and probably timed expression of proteins such as neuroendocrine markers and adhesion molecules. The result of islet formation is a micro society of cells with several anatomical and functional interactions.

These features might have direct implications for the pathophysiological mechanisms that lead to type 1 diabetes as is illustrated by the role of a neuronal enzyme in the autoimmune process as described in the experimental work of this thesis.

1.4 The Pathology of the Pancreas in Type 1 Diabetes Mellitus

1.4.1 Studies in Post-mortem Pancreas Tissues of Diabetic Patients

The pathology of type 1 diabetes is characterized by a selective depletion of the ß-cells in the islets of Langerhans. However, the process of destruction can be very heterogeneous. This depletion is almost complete after more than 1 year of diabetes in most patients, but even after 20 years of type 1 diabetes, ß-cells can be found (118). In a pancreas of a patient with type 1 diabetes, Glucagon-, Somatostatin- and PP-cells are present in normal numbers and are distributed normally in the remainder of the islets (119). Earlier studies suggested a hypertrophy and hyperplasia of somatostatin producing δ-cells (120) and PP-cells (121), but quantitative immunohistochemical studies showed that this was related to atrophy of the exocrine pancreas (119, 122). The exocrine tissue shows atrophy which is reflected in a lower, but clinically not significant, level of serum amylase in diabetic patients (118).

Pathological analysis during the first period after clinical onset has been limited to patients who died in the acute phase or in the first year after onset (123-126). One report from Japan described histologic features of pancreas biopsies performed in the first 2-4 months after diagnosis (127). Although the pathological findings only provide us with a static view of the process, the studies indicate the existence of three types of islets in recent onset diabetes:

1) Most islets (approximately 70%) are ß-cell deficient (as defined by the lack of insulin immunostaining) and are identical to those seen in long-standing diabetes;
2) Some islets still contain ß-cells and appear normal;
3) A portion of those islets with remaining ß-cells show the characteristics of 'insulitis', an infiltration with inflammatory cells;

This indicates that the infiltration is a variable process in place and time. Insulitis can be found in about 80% of the studied post mortem pancreases. (128). One study did not find any sign of insulitis in recent onset diabetic pancreases (129). Although the pathogenetic importance of insulitis and type 1 diabetes was first

stressed by Gepts (123), other reports described insulitis years earlier (130, 131). The cells in the infiltrate are mainly lymphocytes and macrophages. The factors that trigger the invasion of these cells are unknown. The histology of insulitis can be very different between adjacent lobules of the pancreas (128). Although insulitis is the pathological hallmark of type 1 diabetes mellitus, a striking heterogeneity of the islet pathology is seen within one affected pancreas.

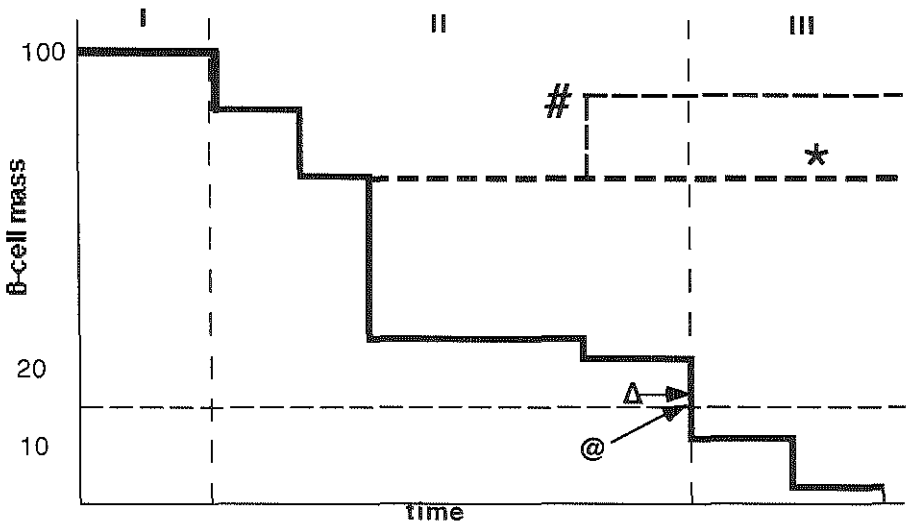
1.4.2 The Concept of Autoimmunity in Type 1 Diabetes Mellitus.

The observations of insulitis and the selective depletion of ß-cells made Gepts to suggest (123) that the process of ß-cell destruction is slow and that it may take years to eventually destroy enough ß-cells to result in clinical symptoms. This is in keeping with observations that ß-cell dysfunction can be demonstrated up to (at least) 3 years before the clinical presentation (132), and the fact that after the onset of the disease, a residual ß-cell function may persist for several years. Furthermore, it was found that type 1 diabetes occurred more frequently in patients with autoimmune diseases and that diabetic patients often had organ specific antibodies (133). The identification of islet cell autoantibodies (134), association of type 1 diabetes with certain haplotypes of the human leukocyte antigen (HLA) system and the lymphocyte and macrophage infiltration of the islets, made it likely that type 1 diabetes was an autoimmune disease. The suggestion of Gepts of a chronic rather than an acute process was strengthened by the finding that circulating autoantibodies in relatives of patients with type 1 diabetes could be present long before clinical onset of the disease (135).

These observations suggested type 1 diabetes to be an autoimmune disease caused by a multifactorial complex of genetic, environmental and immunological factors. Once initiated the process is insidious, and it is conceivable that a considerable time can elapse before the clinical manifestations. Onset in very young children may indicate that the process can however be fast. The model shown in figure 1.1 was proposed by Bruining (136) and shows ß-cell mass plotted against time. Two phases of preclinical diabetes precede a third clinical phase (I, II and III). In phase I there is no evidence of ß-cell destruction and only genetic predisposition is present. Due to, presumably, environmental or other unknown factors, a selective attack towards the ß-cells starts. The duration of this second phase (II) is unknown and may span several years. It is conceivable that in some individuals this process might come to a halt (*), or that even some form of (persistent or

FIGURE 1.1:

Schematic representation of ß-cell mass during an autoimmune attack with increasing ß-cell destruction. X-axis represents time, Y-axis represents ß-cell mass (Adapted from (136), with permission). For details see text.



temporal) regeneration of ß-cells is possible (#). In general, the destruction will continue and eventually surpass a threshold of ß-cell loss that leads to the symptoms of diabetes (@). From animal studies it was suggested that after a 70-80% loss of ß-cell mass, diabetes ensues (137). This final phase might be triggered by a second insult, that leads to a relative fast loss in a short period of time (Δ) . Very often the onset of type 1 diabetes is preceded by a period of physical or psychosocial stress.

Insulin therapy is started, which results in 75% of children with newly onset diabetes in a short period of high endogenous insulin secretion called the *honeymoon* period. Exogenous insulin needs drop sharply, and in some children exogenous insulin administration can be stopped temporarily. However, this is of limited duration and eventually all ß-cells will be killed although this can take considerable time as shown by Rahier who identified ß-cells in a person after 20 years

of insulin dependency (118). The honeymoon period seems to be a feature especially found in children and young adolescents (138).

1.5 Studies on Etiology and Pathogenesis in Type 1 Diabetes Mellitus.

The studies on the pathology of newly onset type 1 diabetes strongly suggest that the selective depletion of ß-cells is due to an attack by mononuclear cells of the immune system. This observation was the basis for the concept of autoimmunity as the cause of type 1 diabetes mellitus (139, 140). This concept was based on the observations of infiltration in the islets and the occurrence of autoantibodies directed against islet cell antigens in the sera of patients with type 1 diabetes. Additionally, genes of the Major Histocompatibility Complex (MHC) showed to confer susceptibility for the disease, first shown by Singal and Blajchman (141). This suggested an autoimmune basis, since MHC products play a major role in the immune response. Moreover, type 1 diabetes is associated with other diseases that have an autoimmune basis such as thyroid disease (Hashimoto's and Grave's thyroiditis) and adrenal disease (Addison's disease). Other findings contributed to the concept, such as the response of newly diagnosed diabetes on treatment with immunosuppressives (142). This chapter will discuss the pathophysiology of autoimmunity. Since several studies use animal models of diabetes, some characteristics of these models will be introduced.

1.5.1 Animal Models of type 1 Diabetes Mellitus

Animal models of type 1 diabetes have been an important tool to study the pathogenetic processes since they facilitate to address questions that can not be answered in humans. The most widely used models are the NOD-mouse (Non Obese Diabetic mouse) (143) and the BB-rat (BioBreeding), (144), both having cellular and humoral autoimmune and genetic features of ß-cell destruction. Inbreeding resulted in a genetic background that coincides with a high incidence of autoimmune diabetes. NOD-mice have a unique MHC class II molecule, not present in other strains (145). Whatever drawbacks animal models may have for the study of a human disease, they clearly show that the process of autoimmunity is dependent on both genetic and environmental factors. Although genetically identical, not all BB-rats or NOD-mice develop diabetes. Moreover, in some animals in-

sulitis does develop, but it does not result in diabetes. BB-rats develop autoimmune diabetes in 40-90% depending on the colony (146). The diabetic prone rats have a lymphopenia and diabetes usually starts before they are 120 days old. The diabetes of BB- rats has been associated with an infection with Kilham's Rat virus, a parvovirus (147). In addition, diabetes in the NOD-mouse has been related to the presence of retroviral genes in the mouse genome (148) . Such findings clearly illustrates that environmental factors play a role in the animal models as well. In male NOD-mice, only a minority develops diabetes, while between 40 and 90% of the females develop the disease (149). The disease develops after 12-30 weeks. Several factors are known to influence the frequencies of insulitis and diabetes such as diet (150). The gender differences observed seem partly due to effects of sex-hormones (151) These two animal models, together with others (152), have had great influence on the research of type 1 diabetes and are mentioned in the next sections. Genetic, environmental and immunological factors have helped to provide evidence for the autoimmune nature of the disease.

1.5.2 Genetic Factors in Type 1 Diabetes Mellitus

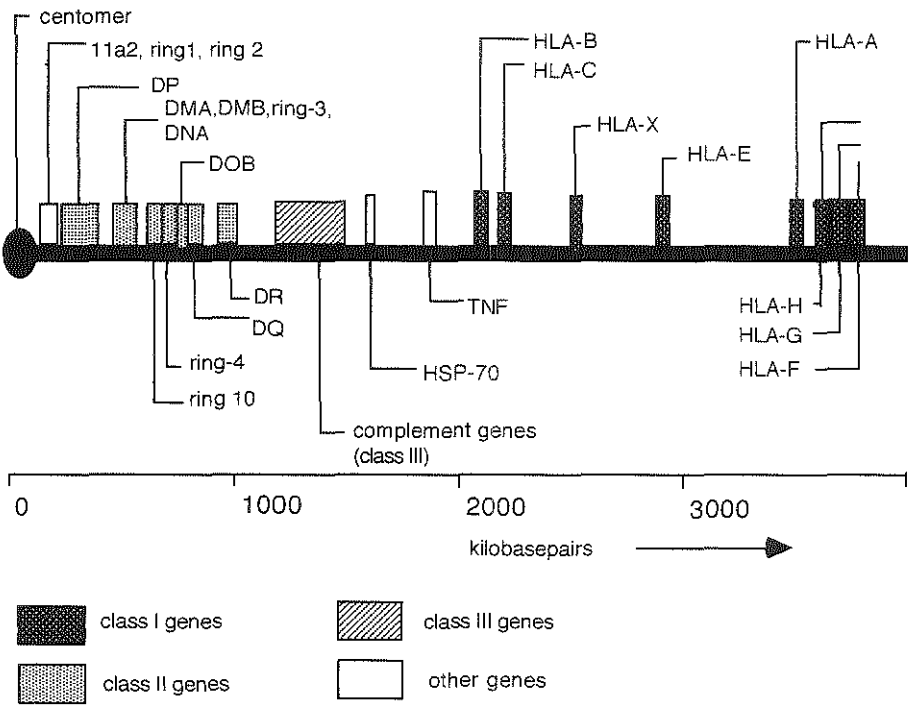
1.5.2.1 The Major Histocompatibility Complex (MHC).

Two functions of class I and II Major Histocompatibility Complex (MHC), in humans called HLA (Human Leukocyte Antigen) complex, have been mentioned in earlier sections. First, HLA molecules are involved in the shaping of the immunological make-up of self and non-self. Second, HLA-molecules are involved in subcellular peptide transport. As discussed in the epidemiology chapter, they confer susceptibility to type 1 diabetes mellitus. The genes encoding the human MHC are located on the short arm of chromosome 6. A simplified map of the MHC is given in figure 1.2. A detailed description can be found in (153)

Three major areas exist within the MHC, coding for Class I, Class II and Class III gene products. Class III products are complement factors and are not discussed here. HLA genes are highly polymorphic. Three groups of Class I genes were described: A, B, C, later followed by three other groups: E, F, G, the latter do not show polymorphism. Class I gene products are glycoproteins consisting of an α-chain with 3 extracellular domains, a transmembrane region and an intracellular cytoplasmic tail and are associated with β_2-microglobulin. HLA-G gene products are only expressed in extra embryonic tissues (154). HLA-E is ex-

FIGURE 1.2

Map of the short arm of chromosome 6 (human). Not all localized genes are shown.



pressed in several tissues and plays an additional role in embryological tissues. The function and structure of these molecules remain putative (155, 156). Class II molecules are heterodimers composed of an noncovalently bound α- and ß-glycoprotein chain. The chains have two extracellular domains of which the proximal (α2,ß2) form an immunoglobulin-like structure. The class II genes are clustered in 3 regions (HLA DR, -DQ and DP) intermingled with other genes. During biosynthesis of class II molecules a third chain, γ- or invariant chain associates temporarily with the class II α/β dimer. The γ-chain is responsible for the targetting the αβ dimers into the endocytic pathway and for preventing premature peptide binding The structure of Class II molecules resembles that of class I. The polymorphic areas of the HLA molecules line the protein binding groove and are ex-

tremely important for the HLA-peptide interactions. Class II molecules however, bind peptides of a slightly larger size than Class I (≈ 14 aminoacids) (157). Class I molecules contain peptides of 8-10 aminoacids in a real cleft , while in class II molecules, larger peptides bulge out on both sides ((158, 159) for review).

The HLA DR region has 6 loci, the non polymorphic DRA1 locus (encoding the α-chain) and five DRB loci of which DRB1 is the most polymorphic. The HLA-DQ subregion consists of two α- and two ß-chain loci: DQA1, DQA2, DQB1 and DQB2. DQA1 and DQB1 are polymorphic with 8 variants of DQA1 and 14 for DQB1. These genes correspond to nine serological recognizable specificity's of DQαβ heterodimers which are called DQw1 - DQw9.

The HLA loci are in close linkage. This results in inheritance 'en bloc', which is called a haplotype. The consequence of this is a strong linkage disequilibrium between certain alleles, which means that some alleles occur together more often than expected on basis of their gene frequencies. Certain haplotypes are therefore often maintained in the population. An example is the haplotype HLA-A1-B8-DR3-DQw2. The importance of HLA molecules in prediction and pathogenesis of type 1 diabetes is discussed in the next paragraph.

1.5.2.2 Genetics of Type 1 Diabetes Mellitus.

It was noticed in epidemiological and genetic studies that a pattern of inheritance existed for type 1 diabetes. Diabetes 'runs' in families, but the complexity of the inheritance resulted in the nick name the 'geneticists nightmare'. Also popular belief, such as 'it skips every other generation', indicates this problem. Studies in twins proved helpful in complex genetics and showed two important findings. Comparing the phenotypic similarity (concordance) in monozygotic and dizygotic twins it became clear that in type 1 diabetes the susceptibility and not the disease itself is inherited: monozygotic twins are concordant in only 30-55% of the pairs, when one child of a dizygotic twins has type 1 diabetes, the chances for the other are still high (55, 160, 161) The low concordance rate indicates that additional factors must come into play. This is further supported by the finding that the longer the time from diagnosis in the first (monozygotic) twin, the smaller the possibility for the other to get diabetes. Recurrence risks have been established and are given in table 1.3 (162). Several genetic markers have been evaluated as 'diabetes gene', but only the HLA system showed linkage to type 1 diabetes. After initial linkage

was described for the class I HLA loci HLA ß-15 (141) and HLA B8 (163) (with the strongest association with heterozygosity of B8/B15), a stronger

TABLE 1.3
Estimated recurrence risk (%) for relatives of type 1 DM probands

Relative:	Percentage:
Monozygotic Twins	30 - 55
Dizygotic Twins	15
Siblings	3 - 6.2
Children	3.4 - 5.4
Nephews and Nieces	1.5
General Population	0.15 - 0.4

association was found with MHC class II gene products of HLA DR3 and DR4 (164). About 90-95% of Caucasian patients with type 1 diabetes are either DR3 and/or DR4 positive compared to 40-60% of controls. The strongest association was found for HLA DR3/4 heterozygosity and in Sweden, with a high incidence of type 1 diabetes, 30-40% of the patients have this haplotype, compared to 3-8% of controls (165, 166). The haplotype HLA DR2 showed is associated with a very low risk of type 1 diabetes. Since most of the work was performed in Caucasian people it was an interesting observation to find that in other races different haplotypes are linked to type 1 diabetes. In the Chinese population the highest risk was in HLA DR3/9 individuals, while in the Japanese the highest risk was confined to individuals with HLA DR4/9. Numerous studies indicated that HLA DR4, DR3, DR1 and DR8 are associated with type 1 diabetes in a descending order.

The importance of genes in the DQ region was first described by Owerbach and colleagues, using restriction fragment length polymorphism (167). A BamHI 3.7 kb fragment was found more frequent in DR4 patients than in controls. Cloning of this fragment revealed, that it flanks the coding region of the first and second

domain of an HLA DQβ-chain. The stronger association with diabetes indicates that the DQ locus is closer linked to a presumable diabetes susceptibility locus(28, 168). Further work showed that the DQβ-chains of haplotypes associated with type 1 diabetes (such as DR4-DQw8, DR3-DQw2, DR1-DQw5) were different at aminoacid position 57 when compared with haplotypes negatively associated with type 1 diabetes (such as DR4-DQw7, DR2-DQw1.2, DR2-DQw1.12) (169). This position is located in the groove of the MHC molecule, thus interfering with peptide binding. Alleles encoding aspartic acid (asp) at position 57 of the DQβ chain were suggested to confer resistance to type 1 diabetes, while alanine, serine or valine in this position coded for susceptibility (169-171). Subsequent work showed that arginine (arg) at position 52 of the DQα-chain was associated with susceptibility, whereas an other (non-arg) residue at this position conferred resistance to type 1 diabetes (172). It is however, unlikely that only these two residues confer the risk for diabetes, since not all persons with haplotypes coding for both susceptible aminoacids develop the disease. Furthermore, Japanese patients do not show the association with the asp at position 57 of the DQβ chain (173, 174). The two DQ loci do however, provide a way to assess such HLA data in prediction. Simple assessment of ARG and ASP in the two DQ chains can be done with relative fast and cheap methods and provide an important risk marker for type 1 diabetes. In a study where different ethnic groups were compared, Giphart and colleagues found that the risk for diabetes increases from homozygote non-susceptible haplotypes via heterozygote susceptible to a group with the highest risk, the homozygotes with a susceptible haplotype for both the ASP and the ARG position (175). Although assessment of these aminoacid residues helps to determine the risk for the disease, it should be stated that, although may be important marker for type 1 diabetes susceptibility, they are not the only determinants (9).

The linkage of MHC class II markers with type 1 diabetes risk is not absolute, and suggests that further analysis of genes in the region may result in the identification of 'the' susceptibility gene and that all other described associations are simply linkages to this gene. A number of genes coding for proteins involved in peptide processing and transport can be considered as a candidate. Antigen processing involves first the generation of peptides from cellular proteins, a process that takes place by the LMP complex, a distinct subset of the cellular pool of proteasomes. Subsequently, such peptides are transported into the endoplasmic reticulum and combine with HLA class I molecules. The TAP-1 and TAP-2 molecules, formerly in humans referred to as RING 4 and RING 11 respectively (176-179) are involved in the transport into the endoplasmic reticulum (ER). Inside

the ER, binding to the class I molecules includes conformational changes that may facilitate their export to the cell surface. The TAP genes are characterized by a polymorphism comparable to other MHC genes. The recent observations that a defect in antigen binding and presentation by class I molecules might be involved in type 1 diabetes (180) could indicate that these gene products might play a role. In addition, the gene product Bcl-2, a protein that prevents programmed cell death (apoptosis) of lymphocytes (during the selection process) and other cells (ß-cells?) is also located within the MHC (153). Such a gene could play an additional role in autoimmunity, but no definite evidence has yet been found.

Other genes outside the MHC region have been found to additionally influence the susceptibility of type 1 diabetes. Using congenic strains of mice (NOD's back crossed with other strains with particular haplotypes) Todd and coworkers identified 11 additional loci on different mouse chromosomes that were associated with the inheritance of insulitis and/or diabetes. Two of these genes in NOD-mice, idd-3 and idd-4, located on chromosomes 3 and 11 respectively, are strongly associated although less than MHC genes (referred to as idd-1 by Todd and coworkers) (181). Idd-3 is associated with insulitis. It is located on mouse chromosome 3. An important factor associated with idd-3 is the gene that codes for the high affinity IgG2 receptor (FcGR1). A pathophysiologic role for this gene product in autoimmune diabetes is unclear. Idd-4 is associated with the disease in the youngest age group of NOD-mice and not in older mice. The observations argue for a complex polygenic regulation, where different stages of the disease are under control of different genes. Homologues of these genes in humans are only partly identified and their role in (human) type 1 diabetes is still unknown. Studies in humans have only involved multiplex families (more than one sib or parent has the disease), which contribute to only about 5% of the cases (26). It is conceivable that the disease in these multiplex families represents a different subtype of type 1 diabetes, than what is found in the 95% remaining cases without a family history.

Until now, the analysis of the contribution of other genes is limited in humans. The polymorphic TCR genes were studied, but no correlation with the disease has been established, although this is still subject to controversy both in type 1 diabetes (182-184) as well as in autoimmunity in general (185, 186).

In summary, a strong association between genetic factors and type 1 diabetes exists for HLA genes, but they can only explain (a part of) the susceptibility to develop the disease. The HLA molecules have two important functions that could contribute to the disease. First, they are involved in the selection process of T-cells.

Second, their role is to present peptides to the immune system. No 'diabetogenic gene' has yet been described. The disease is polygenic and the inheritance is extremely complex. Although not conclusive, the data from all these observations do help in determining the risk for diabetes. The presence of predisposing or protective haplotypes can contribute in establishing the risk. Regarding other non-HLA genes within the MHC and non-MHC genes, more data are needed before conclusions can be drawn. Genetic analysis is extremely important in the prediction of type 1 diabetes and the study of its pathophysiology..

1.5.3 Environmental Factors in Type 1 Diabetes Mellitus.

The previous chapters suggest that type 1 diabetes is an autoimmune disease. Three phases could be recognized in the development of the disease (figure 1.1). Genetic factors determine susceptibility, indicated by phase I. Phase II is believed to start after the initiation by an environmental trigger. The nature of this trigger (or triggers) is unknown. Strong evidence that environmental factors play a role comes from studies in monozygotic twins, where the identical genetic background does not result in a 100% concordance. Moreover, the longer the time after the start of diabetes in one of the twin members, the lower the chances for the other one to develop the disease. In addition, only about 6-12% of the cases of type 1 diabetes occur in families with an affected parent or sib with the disease. Together with the epidemiological observations of increased incidence associated with epidemic-like outbreaks, seasonal variation and a general pattern that resembles infectious diseases, an environmental influences are very likely. Migration from an area with a low- to an area with a high incidence coincides with increased frequency of the disease, even without ethnic mixing. The identification of environmental factors is difficult. The long lag period between initiation of ß-cell destruction and clinical onset of the disease makes it difficult to pinpoint on factors that might have played a role in the initial process. A number of observations, however, support a causative role of the environment, including viral infections, chemicals, toxins and dietary constituents.

1.5.3.1 Viral Infections and Type 1 Diabetes Mellitus.

The first publication of a link between type 1 diabetes and viral infections dates from 1899, when Harris described a case of diabetes that started after a mumps infection (187). Evidence for a role of viruses include:

1) The presence of viral antigens in islets of newly diagnosed patients;
2) The presence of antibodies against viruses in the serum of newly diagnosed patients;
3) Observed ß-cell damage in children who died of overwhelming viral infections, and
4) The isolation of virus from the pancreas of children who died in the initial phases of diabetes with demonstration that this virus could induce diabetes in animals (thereby, fulfilling Koch's postulates).

Viruses linked to the pathophysiology of type 1 diabetes in man include mumps, coxsackie, rubella and cytomegalo virus (CMV) (188). The association with mumps comes from the prevaccination period, when mumps infection were found to result in type 1 diabetes in some cases (189-191). Islet cell autoantibodies (ICA's, see 1.5.7.1) were found after a mumps epidemic, but were not related to later onset of type 1 diabetes (192). Mumps virus can infect cultured human ß-cells and can up-regulate MHC class I expression in cultured human fetal islet cells (193) Coxsackie B4 virus was cultured from a newly diagnosed child who died from the complications of a diabetic ketoacidotic coma. This virus was propagated in ß-cells and could induce diabetes in mice. Coxsackie is normally tropic for exocrine tissue, but some strains seem to prefer endocrine cells of the pancreas (194). A large analysis of autopsy specimen from persons who died in initial periods of diabetes revealed no evidence for coxsackie B capsid protein (195). The responses of T-cells from diabetic individuals towards (in vitro) mumps and coxsackie infections seem to be HLA DR4 restricted (188). Congenital rubella infections are in 10-20% of the cases complicated by type 1 diabetes mellitus (196). A majority of the patients are HLA-DR3 (197).Since postnatal rubella is not associated with type 1 diabetes, the embryo-fetal period is of importance in triggering the disease. Cytomegalo virus infection often complicates treatments that result in a diminished function of the immune system (chemotherapy, transplantations). One case of CMV particles in ß-cells was described, and a subsequent analysis of 45 children who died of disseminated CMV infections revealed 20 with similar inclusion bodies (198, 199). These children did not have any evidence of ß-cell dysfunction. A monoclonal mouse antibody raised against CMV, reacted with a Mr.

38,000 islet antigen in humans and BB-rats (200). Recent, CMV was found to block the intracellular transport of peptide loaded HLA molecules, thereby interfering with immune surveillance (201). In NOD-mice a role for retroviral proteins was suggested (148). In BB-rats a relation with the Kilham virus (parvovirus) was made (147). The interpretation of such studies is, however difficult.

The mechanisms by which viruses can destroy ß-cells are:

a) direct ß-cell destruction due to infection followed by ß-cell lysis;
b) molecular mimicry, where a viral component produced in the ß-cell or elsewhere looks very similar, but not identical, to a ß-cell antigen and the immune reaction directed against the viral intruder also attacks the self antigen;
c) disturbance of the ß-cell metabolism and function, resulting in an increased or aberrant expression of a normally non (= sequestered) or almost non exposed ß-cell antigen;
d) A virus can interfere with immune cells as exemplified by CMV;

It should be realized that the infection and destruction process might take place shortly after the infection but also many years later. In a large population study 339 recently diagnosed children were evaluated for the effect of vaccinations on onset of type 1 diabetes. None of the vaccines (tuberculosis, smallpox, tetanus, whooping cough, rubella and mumps) appeared to be protective (202).

1.5.3.2 Chemicals, Food Components and Type 1 Diabetes Mellitus.

A striking example of environmental agents involved in type 1 diabetes came from accidents with the rodenticide Vacor® (N-3-pyridylmethyl-N'-p-nitrophenylurea). This substance is a nitrosurea compound. Accidental ingestion in humans lead to a syndrome of diabetes and severe neurological dysfunction (203). In vitro, the drug is toxic to human islets (204). Except for its presumed direct ß-cell cytotoxic effects, patients showed a secondary autoimmune reaction after ingestion of Vacor (205). Similar observations of cytotoxicity and autoimmune phenomena were found for the nitrosamine compound Streptozotocin. Moderate and high doses of this drug have a direct cytotoxic effect on ß-cells. Repeated low dose intoxications result in an MHC dependent attack on ß-cells. This autoimmune-like destruction was transferable by injecting the lymphocytes from animals treated like

this into healthy controls. In addition, anti-lymphocyte serum and total body irradiation prevented the occurrence of diabetes (206), also suggesting its autoimmune nature. Nitrosamine compounds are often produced in the human body from nitrites that are often used in food preservatives. One report suggested that ingestion of nitrites in food (Icelandic cured mutton) increased the incidence of type 1 diabetes (207). Epidemiological data are needed to support this hypothesis, which is largely based on animal experiments. Alloxan and pentamidine are other components causing ß-cell toxicity. Pentamidine is a antiprotozoal drug reducing the response of ß-cells on glucose and arginine. Direct ß-cell destruction was also seen after longer exposure to this drug (208).

Food components linked to type 1 diabetes include coffee and cow's milk. Epidemiological data show a parallel between coffee consumption per capita and type 1 diabetes (209). Breast-feeding during infancy, or late introduction of cow's milk is suggested to be protective (210, 211). Studies from Finland and Canada showed that newly diagnosed children had a high frequency of antibodies to a fragment of bovine serum albumin (BSA). The epitope responsible for specific diabetes related antibodies is called the ABBOS (Albumin Bovine Serum) fragment (212). The antibodies do not precipitate a protein in immunoprecipitations, but the authors were able to, indirectly, identify a protein of 69kD on western blot. Mimicry between the BSA and the 69kD protein is the mechanism suggested of autoimmunity in type 1 diabetes. Currently, other labs try to set up assays for ABBOS directed autoantibodies, but until now without success (Atkinson, personal communication). Since the epidemiological studies are retrospective, a causal role for the disease nor a protective effect can easily be drawn. The ABBOS data triggered a nationwide blind study in Finland, where babies will be fed BSA free- or BSA-containing formula. Apart from the ethical aspects of such studies, the presence of BSA in several foods and the possible transfer of BSA from the mother into the breast milk will make this study difficult to perform and analyze. In Sweden, where a national registry exists for type 1 diabetes, no evidence of a relation between cow milk and diabetes was found. However, a relation was found between the occurrence of type 1 diabetes and birth weight and weight gain in the first 6 months of life (J. Ludvigsson, personal communication).

Effects of chemicals and food components can have an effect by cumulative damage. Both a repeated dose or chronic exposure to a diabetogenic agent can result in enough triggers to start a sequence of events, eventually resulting in autoimmune diabetes.

Although a genetic susceptibility is a requirement for the development of type 1 diabetes, an additional environmental factor seems necessary. Since such factors could operate only over a brief period or could be repetitive, they are difficult to trace. Moreover, exposure does not necessarily result in diabetes, probably not even in those with a susceptible HLA-haplotype. The additional make-up of the immune system (see next section) is important. Viruses and toxins may function as environmental triggers, but it remains unclear which of these environmental factors contributes to (most of the cases of) type 1 diabetes.

1.5.4 The Immune System: Components and Mechanisms of Tolerance.

The major function of the immune system is to respond to foreign molecules (antigens), while avoiding reactions against molecules of the host itself (self-antigens). Autoimmunity implies a faulty reaction of the immune system. Every unknown molecule must be eliminated, which implies that a mechanism exists that is capable of distinguishing self from non-self. Tolerance to self is a prerequisite for survival. Earlier work (213, 214) suggested a clear and relatively simple mechanism for this: upon the entry of a foreign antigen, the body would react by producing antibody and by developing a cellular response, thus showing non-tolerance. In this scenario, autoimmunity was the result of a loss of tolerance to an antigen that was originally tolerated. Toleration was thought to occur entirely in fetal life. At that stage, cells that were reactive to self-antigens would be eliminated, but in some cases, forbidden clones of self-reactive cells could await the moment to attack. Although several aspects of this concept still hold, the mechanisms of tolerance are more complex.

The immune system recognizes antigens using three major recognition structures: immunoglobulin (Ig) receptors on B-lymphocytes, T-lymphocyte receptors (TCR's) on lymphocytes and cell surface molecules of the class I and II major histocompatibility complex (MHC class I , MHC class II). MHC Class I molecules can be found on most nucleated cells of the body, including adult ß-cells. They have a primary function in peptide transport. Fragments of proteins will bind to class I molecules, be transported and eventually presented at the cell surface. MHC class II molecules are only found on specialized cells of the immune system that take up, process and present antigen (antigen presenting cell, APC) to T-lym-

phocytes, B-lymphocytes and macrophages / dendritic cells. For both the Ig-receptors (215) and TCR's (216) a large repertoire is possible by programmed rearrangement and random assortment of the Ig- and TCR-genes. TCR's are phylogenetically linked to Ig-molecules, but have some different properties. T-lymphocytes recognize antigen fragments of 8-12 aminoacids, independent from their spatial configuration (primary epitopes), whereas B-lymphocytes recognize conformational epitopes and thus, require an intact three dimensional structure of the antigen. MHC molecules possess a binding cleft or groove for antigens (217) and T-lymphocyte activation is achieved upon presentation of an antigen at the surface of a cell in this cleft with subsequent recognition by the TCR. In order to result in activation of the T-lymphocyte, spatial interactions between MHC molecule, antigen and TCR must be optimal. Additional molecules are necessary for the function of the trimeric complex of MHC, antigen and TCR, such as adhesion molecules. Moreover, APC's do not only present antigen to T-cells, they also provide a so-called second signal, needed to obtain a proliferative response of the T-cells (218) (219).

Two classes of T-lymphocytes can be distinguished. One group consists of T-helper (T_h) cells. They express CD4 cell surface molecules ($CD4^+$) and can interact with MHC class II expressing APC's. The others are cytotoxic T-cells (T_c). They have the ability to express the surface molecule CD8 ($CD8^+$) and to kill other cells. CD8 positive cells can interact with MHC class I expressing cells. $CD4^+$ (T_h) cells are regulators and orchestrators of the immune system. Upon activation they can start to secrete factors (cytokines) that recruit and activate other cells: macrophages, T_c-cells, natural killer (NK) cells and B-cells. The activated cells are attracted to the site of the immune response which results in an infiltrate. This combined action results to the secretion of additional cytokines (lymphokines) that triggers the expansion of certain clones of B- and T-cells. Eventually, regulatory and suppressive mechanisms will limit and terminate the immune response. $CD4^+$ cells can be divided into two functionally distinct groups on the basis of their cytokine production. Th1 cells produce predominantly Interleukin 2 and interferon-γ, Th2 subsets produce Interleukin 4 and 6. There is still controversy on this subdivision. Phenotypic markers can also subdivide $CD4^+$ cells. The CD45 molecule exists in low (CD45RO) and high (CD45RA, B or C) molecular weight forms which are the result of alternative splicing. The CD45RA+ cells were found to be predominantly present in insulitis lesions in NOD-mice and seem to be the most important subgroup (220). It has been suggested that suppressor CD4+ T-cells do express CD45RA (220).

The result of all these components is a system that is diverse, able to encounter unknown antigens and to respond fast after a second encounter. However, tolerance to self antigens is required. Burnett suggested in 1959 that tolerance depended on clonal deletion of self-reactive cells, a process he proposed to take place in the thymus (214). We now know that selection processes indeed take place in the thymus for both B- and T-cells. Immature T-cell precursors derived from the bone marrow start expressing both CD4 and CD8 and arrive in the thymic cortex. They have the capacity to express TCR's for any antigenic determinant, but only those cells that actually do encounter self-MHC (presented by the thymic epidermal stromal cells) will continue the process of differentiation. This means that they become committed to express either CD4 or CD8. Cells that do not continue differentiation (and keep expressing both CD4 and CD8) will undergo programmed cell death (apoptosis) (221). Programmed cell death is gene regulated. One of the genes involved is the BCL-2 locus in the HLA region. Cells that continue the process of differentiation can encounter self-antigens in the thymic medulla, presented by dendritic cells. Dendritic cells are originally derived from the bone marrow. An initially, positively selected (on basis of its self-MHC recognition) cell that reacts with a very high-affinity to a self-peptide/MHC complex is eliminated. This process is referred to as negative selection. The theoretical result is that only T-cells that recognize foreign antigens in conjunction with self-MHC will survive. Autoreactive T-cells are however, present in many individuals as is illustrated by multiple sclerosis, where the frequency of autoreactive T-cells against the presumed trigger myelin basic protein is almost equal in controls and in patients.

Thymic mechanisms of selection can thus explain the achievement of tolerance to antigens that are present in the thymus. However, not every antigen will reach the thymus. Some antigens are only expressed in a certain time frame of development, others are situated (sequestered) in anatomical privileged sites. In addition, some antigens may be expressed by cells that have a low or absent MHC class I expression or express MHC that is incapable of presenting antigen in a proper way to the immune system. In view of this, mechanisms of peripheral tolerance were considered and supported by experimental evidence (222). Work in transgenic mice clearly showed the existence of clonal anergy in vivo as reviewed in (223, 224). This is defined as silencing of a lymphocyte without its actual destruction (225). Anergic lymphocytes are capable of binding the self-antigen-MHC structure with their TCR, but incapable of proliferating in response to this (223, 226). It is suggested that this anergy is due to the absence of a second signal,

needed to activate T-cells (218, 219). The mechanisms proposed for peripheral tolerance are however still open for debate and anergy may be more dependent on the thymus (227). Mice transgenic for an MHC molecule (K^b), targeted to the ß-cell by linking it to the insulin promotor, do express this MHC in their ß-cells, do not get infiltration of islets after immunization with K^b and become tolerant at the T-cell level. Crossing these mice with transgenes for the anti-K^b TCR, produces double transgenics. These double transgenics expressed, however, only small amounts of T-cells with the K^b TCR. This suggested that a few molecules of K^b must have been present in the thymus and were able to initiate deletion of T-cells with high K^b TCR's. Although conventional techniques such as immunohistochemical staining and northern blotting could not reveal K^b in the thymus, it was possible, by PCR, to show K^b in the thymus. Triple transgenes, in which the double transgenes were crossed with mice transgenic for interleukin-2 (IL-2) expressed in ß-cells, develop insulitis and no diabetes very fast. The autoimmunity did not seem to be related to K^b, but the expression of IL-2 in the context of antigen (K^b) and specific T-cells did stimulate K^b reactive T-cells in the double (K^b and anti-K^b TCR) transgenics. IL-2 may have overcome the anergy and activated the 'ignorant' T-cells. When single transgenic K^b (expressed in the ß-cells) mice were thymectomized (to prevent K^b expression in the thymus), irradiated, reconstituted with bone marrow from K^b TCR transgenes, and transplanted with a normal thymus, an immune response to K^b developed (228-231). This indicates that in this model, no anergy of K^b TCR cells develops actually, but that the system is more or less ignorant until a rather non-specific trigger appears. While, in strict sense, anergy can be regarded as the result of a lack of a secondary signal within the silent T-cell, ignorance could include the presence of other cells that suppress the T-cell. The mechanisms by which T-cell suppression works in achieving tolerance, are poorly understood. The existence of suppressor T-cells was largely based on indirect experimental evidence without a clear view of the characteristics of this cell type. Since no direct evidence for their existence could be found, but since regulatory effects upon more aggressive T-cells were found, they are also referred to as 'regulatory T-cells'. New data however, support the concept of suppressor T-cells (232, 233) and indicate that they can be partly identified on the basis of their cytokine production (234). Suppressor T-cells could therefore have important functions in limiting the reactions of autoreactive cells. In summary several mechanisms can induce or interfere with tolerance. At least four major groups can be outlined: 1) positive and 2) negative selection (both in the thymus) result in 'central' or thymic tolerance, 3) mechanisms such as anergy result in peripheral tolerance and 4) suppressor mechanisms can render T-cells ignorant and limit autoimmune reactions.

1.5.5 Tolerance and Autoimmunity.

Positive and negative selection is a first filter system to autoreactive T-cells. If cells escape these mechanisms, peripheral mechanisms such as anergy, ignorance and suppression reduce the dangers of autoreactive T-cells. Recent work emphasizes the fact that autoreactive T-cells can escape the self-censorship and play a role in the onset of autoimmune disease (235). Additional evidence that not all autoreactive T-cells are eliminated comes from experiments where T-cell responses are induced using pancreatic islets. All NOD-mice, irrespective of age, sex, and disease progression possess islet specific $CD4^+$ and MHC class II restricted T-cells. These T-cells were specific by virtue of the absence of reactions with other cell types. Such autoreactive T-cells were however, also found in other non-diabetic mouse strains indicating a normal occurrence of islet cell reactive T-cells (236). Similar findings were found in rats (237). Such data support that tolerance to some ß-cell antigens is not established. Failure of thymic tolerance is one mechanism of aberrant tolerance. Other mechanisms include anergy and suppression, but except for T-cell mechanisms, also antigen and APC related causes for tolerance ablation exist. Table 1.4 summarizes some mechanisms that could result in either autoreactive T-cells or the inability to induce tolerance (modified after (225)).

Antigen sequestration in place is the situation where an antigen is not shown to the immune system, due to its privileged anatomical localization. The antigen cannot travel to the thymus and the privileged site may include the inability of peripheral lymphocytes to reach it. Thus, sequestration can result in non-visibility and therefore intolerance. An example are the autoantigens against sperm found after vasectomy in men. The testis is an immunological privileged site. Following vasectomy, a striking accumulation of macrophages can be found around the obliterated vas deferens, not infrequently resulting in granulomas (238). Sperm components are absorbed into the body and such autoantibodies to sperm antigens can be found in 50-80% of men after vasectomy.

Sequestration in time means that, dependent on the timing of presentation of an antigen to the immune system, tolerance may or may not ensue. It is evident that the process of maintaining tolerance to peripheral self proteins has to be continued throughout life. However, the period during development seems to be of crucial importance for the induction of tolerance. Studies in twin calves showed already in 1945 that exposure of the immune system to foreign antigens in utero is

TABLE 1.4
Mechanisms involved in change of tolerance and autoimmunity (T-cells)

Lymphocyte related:

- failure of positive selection.
- failure of negative selection.
- failure of suppression mechanisms.
- failure of peripheral tolerance (anergy/ignorance).

Antigen related:

- sequestration in place or time
- molecular mimicry
- alterations of self antigens (different processing or different post-translational modification)
- combination of self antigen with a foreign determinant

APC related:

- aberrant MHC class II expression
- aberrant processing and presentation of antigen

sufficient to permit the development of long-term immunological tolerance (239). Moreover, studies in transgenic mice strengthened the importance of expression of an antigen during ontogeny to ensure the development of tolerance (240). By targeting expression of SV40 virus large T-antigen (Tag) to ß-cells in mice, and by using different promoter lengths of the insulin promoter, animal strains with both early embryonic as well as animals with late post-natal expression of Tag were obtained. Early embryonic expression resulted in life long tolerance, animals with late expression showed non-tolerance for Tag. Since Tag was selectively expressed in ß-cells, the animals developed autoimmunity towards the ß-cell. However, the animals did not die of diabetes. This was due to the oncogenic

properties of Tag, resulting in lethal insulinomas. Similar findings of the acquisition of self-tolerance to foreign genes came from other mouse lines transgenic for MHC II molecules (232, 241).

An other example of sequestration is found in paraneoplastic diseases. Tumors are able to express several genes that are normally not encountered in certain tissues. This may include sequestered antigens or antigens that are normally only present in low concentrations. In patients with paraneoplastic cerebellar degeneration, it was found that the tumor (ovarian, breast or endometrial in origin) was able to produce large amounts of a protein normally only present in Purkinje cells. The presumed sequestration of this antigen resulted in autoimmunity towards this antigen upon release of large quantities from the tumor, and subsequent cerebellar dysfunction (242, 243).

Molecular mimicry is a mechanism whereby viruses and other infectious agents can cause autoimmunity (244). They possess this function by sharing antigenic determinants with self antigens of the host. The foreign antigen should not be completely identical. In that case, tolerance should already have been established by the host, unless a second mechanism such as sequestration coincides. Depending on the antigenic differences or antigenic distance between the two components, the foreign antigen is able to 'fool' the immune system: the response to the foreign antigen coincides with a response to the self-antigen. A typical example of molecular mimicry is the epitope sharing between streptococcal antigens and endocardial structures in rheumatic fever (245).

Alterations of antigens can result in autoimmunity. A typical example is thyreoglobulin. After treatment with papain, that will break up the molecule, fragments appear that are able to induce autoimmunity. Injection of the (rabbit) hormone fragments without adjuvans in rabbits can result in both antibody production and thyroiditis in normally tolerant animals (246). Alterations can also include a different processing of a protein or a modified post-translational modification. Recently, it was shown that Cytomegalo virus (CMV) infections are able to modify the process of protein degeneration, which resulted in different peptides than normally encountered by the immune system (201). Such mechanism could be envisaged in diabetes and autoimmune diseases as well.

Combinations of foreign determinants and antigens are exemplified by the drug-induced hemolytic anemia's such as after α-methyl-dopa (247). The complex

evokes an immune response. Due to its binding to erythrocytes, these cells will be lysed as innocent bystanders. Chemicals and drugs could have additional influences on posttranslational modification or processing of an antigen. They could decrease, increase or otherwise alter the amount of antigen presented to the immune system, thus interfering with tolerance.

Aberrant expression of MHC class II antigens has been extensively studied in type 1 diabetes after Bottazzo and colleagues suggested that aberrant expression of MHC class II (HLA-DR) by ß-cells was the basis for antigen expression by the ß-cell and subsequent autoimmunity (126, 248). Class II expression has been described in target cells of other autoimmune diseases . Expression of MHC class II by the ß-cell could directly present a ß-cell autoantigen and stimulate autoreactive CD4 T-cells. Foulis and coworkers reported findings in post-mortem tissue of newly diagnosed type 1 diabetic patients (249). The immunohistochemical staining for MHC class II was seen on ß-cells in islets that were not yet infiltrated by the insulitis, suggesting it was an early step in the destruction process and not due to secondary phenomena. The same type of islets showed hyperexpression of MHC class I molecules (96), but hyper expression of class I was also found on other endocrine cells in the islets. The concept of aberrant MHC expression by ß-cells was attractive. It would be a direct way to present a ß-cell autoantigen to CD4 positive T-cells, thus starting a cascade of reactions resulting in ß-cell destruction. It initiated studies using MHC transgenic mice. Lo and colleagues and Sarvetnick and colleagues targeted MHC class II expression towards ß-cells by using constructs of the MHC gene hooked to the insulin promoter (241, 250). Although the animals became diabetic, this was not due to an autoimmune disease, as was illustrated by the absence of insulitis. It was suggested that the aberrant expression of the MHC molecules interfered with the insulin secretion (251). The differences found between Bottazzo's model and the findings in the transgenes could be due to differences between these models and the natural situation. The hypothesis of Bottazzo describes newly induced class II expression. The transgenic models, in contrast, have constant (from early embryological life on) class II expression on ß-cells and this timing of class II expression might be critical. In addition, T-cells need a 'second signal'. As indicated before, recent work shows that both an appropriate presentation of the antigen as well as this second stimulating signal needs to come from a cell. In the absence of this second signal, unresponsiveness or anergy is likely to occur. It is unlikely that this second signal can be produced by the ß-cell. Large numbers of dendritic cells added to in vitro experiments can mimic such a second signal. However, dosages used for this, are far over

physiological levels. Other experiments also weakened the hypothesis. In NOD-mice, no expression of MHC class II on ß-cells was found. The cells in the islets that express class II were CD45 positive (bone marrow derived) immune cells. There was increased expression of class I molecules on ß-cells (252). In another study it was found that, at the electron microscopic level, some of the insulin positive, MHC class II expressing cells were in fact macrophages that had engulfed ß-cells and therefore stained for insulin. Experiments by Markmann and colleagues (253) showed that ß-cells were poor antigen presenting cells in vitro and, by lacking a second signal, induced anergy of T-cells instead of autoimmunity. Finally, it should be emphasized that class II - peptide associations usually involve exogenous peptides that are endocytosed and processed in a different way than endogenous peptides, of which the processing and presentation involves class I molecules. In order to function properly, it is important that the peptide meets the MHC molecule in the correct subcellular compartment. A number of newly identified gene products involved in this (176, 178, 179) is localized in proximity of the DQ locus of the MHC. Equally important is the fact that T-cell-APC interaction requires additional adhesion molecules. In conclusion, the expression of class II molecules by ß-cells is unlikely. If present, it does not necessarily result in correct presentation to T-cells, since the accessory system may be absent.

In sum, the unique features of the immune system to discriminate self from non-self implies a system of tolerance. It seems, however, tolerance is not always established and that tolerance may also be broken. Selection processes of lymphocytes as well as aberrations in antigen may be important. Factors of the antigen presenting cells play an additional role. The mechanisms involved in self- and non-self recognition work surprisingly well, considering the wealth of antigens in and around us and the relative low frequency of autoimmune disease. Probably the 'cost' of maintaining this system is autoimmunity. If any of the suggested mechanisms applies for type 1 diabetes is at present unknown. Genetic susceptibility, characteristics of the immune system and specific features of the ß-cell (such as sequestration in time and/or place of ß-cell antigens, mimicry of a ß-cell antigen with foreign molecules, post-translational processing and aberrations in this process) are cooperating factors in the pathogenesis of type 1 diabetes.

1.5.6 Cellular Autoimmunity in Type 1 Diabetes Mellitus: T-lymphocytes.

The current concept on the pathogenesis of type 1 diabetes is to regard this disease as a T-cell dependent and T-cell mediated disease. Evidence for a major role of T-cells in ß-cell destruction comes from several lines of research both in humans and in animal models ((254, 255) for review):

- The observations of insulitis were followed by analysis of the infiltrate which was found to contain T-cells and macrophages;
- Immunosuppressive drugs such as ciclosporine (= Cyclosporine A) and azathioprine, that are known to affect T-cells, delayed the onset of diabetes in NOD-mice and BB-rats and had a delaying effect on the last stages of ß-cell destruction in humans (256-258). In humans the benefits of such therapies are still controversial;
- The disease can be transferred in laboratory animals with purified T-cells (259).
- Diabetes can be prevented in vitro, using antibodies specific for T-cells or T-cell subsets (260, 261, 262).
- Diabetes can be prevented in susceptible animals by neonatal thymectomy (263).
- Islet cell specific T-cell clones can be isolated from NOD spleens (264-266) and islet cell specific T-cell clones can be isolated from inflamed pancreatic islets of Langerhans.
- $CD4^+$ and ß-cell reactive T-cells can be isolated from peripheral blood mononuclear cells (PBMC's) of newly diagnosed patients (267) and prediabetic individuals (268). Moreover, T-cell responses can be obtained from T-cells of newly diagnosed diabetic patients against specific antigens, such as GAD (269).
- T-cell clones reactive with an insulin secretory membrane protein of Mr 38kD can be isolated from newly diagnosed diabetic patients (270, 271).

Together with the specific T-cell clones for 38kD, the transfer experiments (and T-cell deletion or inhibition experiments) are important both as proof for the role of T-cells as well as for further analysis of the pathogenesis of the autoimmune process. Both $CD4^+$ and $CD8^+$ T-cell subsets are required for the initiation of the disease in laboratory animals (259, 272, 273). Several experiments however, show that the process is primarily $CD4^+$ T-cell dependent and not MHC (Class I) restricted. $CD4^+$ cells therefore orchestrate the autoimmune response (272, 274).

$CD8^+$ cells are cytotoxic T-cells. Such $CD8^+$ cytotoxic cells might be triggered to migrate to the islets after an initial effect ('help') by $CD4^+$ cells . However, in one study (274) it was shown that in syngenic islet transplants in NOD-mice, the destruction was completely $CD4^+$ dependent. In other models however, a clear differential effect was seen: $CD4^+$ cells were found earlier and around the islets, while $CD8^+$ cells actually infiltrated the islets (275) (276). Recently, some clarification of these controversial results on the role and necessity of $CD4^+$ and $CD8^+$ cells was provided. Adoptive transfer studies in neonatal NOD-mice indicated that homing of $CD8^+$ cells to islets is $CD4^+$ cell dependent (277). The models do however not allow tracking of adoptive transferred T-cells. Furthermore, none of the models document the ß-cell specificity of the islet destruction i.e. that somatostatin and glucagon cells are not destroyed. Christianson and colleagues used an NOD-mouse with SCID (Severe Combined Immune Deficiency) background (NOD-*scid/scid*) to analyze the role of different T-cell subsets (278). They used specific donor animals (NON-Thy-1^a) to isolate T-cells (from the Thy-1.1^+ thymic precursor) from spleen or islets that could be traced with monoclonal antibodies since the NOD-*scid/scid* recipients are Thy 1.2^+ in phenotype. However the scid mutation does not allow functional T and B cells. The NOD-*scid/scid* thymocytes are Thy 1.2^+ and all CD3 (TCR) and IL-2 receptor negative (279). $CD4^+$ cells from young prediabetic animals were not able to induce diabetes when transferred without $CD8^+$ cells (depleted using an anti-CD8 antibody). $CD4^+$ cells from diabetic (= older) animals could do this however, after the CD8 depletion. This suggests that older $CD4^+$ cells are likely to have been activated and amplified. $CD8^+$ cells alone did not transfer the disease. This study confirms the requirements of $CD8^+$ cells in early insulitis lesions. This is also illustrated in a study by Roep and colleagues, showing that ß-cell specific $CD4^+$ cells from patients at onset are capable of lysing macrophages that are pulsed with ß-cell antigen (280). In summary, the experiments do show that both cell types are needed for disease development.

One of the key questions in T-cell autoimmunity of type 1 diabetes however is, which antigen is responsible for T-cell activation. The first studies that addressed this question analyzed responses to insulin or insulin fragments. This was initiated by the ß-cell specificity of insulin and by the finding that autoantibodies to insulin are present in type 1 diabetes, thus suggesting that autoimmunity was most likely to be caused by breakdown of tolerance to insulin. No specific responses were found however, in newly diagnosed patients (281, 282). Subsequently, islet specific T-cells were generated from BB-rat spleens and pancreases after stimulation with rat insulinoma protein fractions (283). The target antigen of these $CD4^+$ T-

cells has not been identified. Studies in humans have been hampered by the lack of sufficient amount of ß-cell protein. The first identification of diabetes associated T-cell responses and their ß-cell target came from Roep and colleagues, who identified a T-cell clone reactive with a 38kD insulin secretory membrane protein (270). The 38kD specific T-cells were established in more diabetic patients (271). Current efforts are directed to identify the 38kD target molecule.

It was found that mycobacterial heat shock protein 65 (Hsp65) cross reacts with a ß-cell target antigen in NOD-mice (284). Specific T-cells were found, as well as autoantibodies. A specific peptide of Hsp65 was identified. T-cell clones recognizing this peptide mediate insulitis and hyperglycemia. Moreover, administration of the peptide itself to NOD-mice can down-regulate immunity to the 65kD heat shock protein and prevent the development of diabetes. Hsp65 (the human homologue is now referred to as Hsp60) is a ubiquitous protein and the mechanism of this effect is not clear. Hsp is expressed in virtually all cells and a limited immune response to one cell type is unlikely. Other mechanisms must play a role, one of them the similarity of Hsp with HLA DQ. Hsp 60 has similarities with several autoantigens in autoimmune diseases, but similarity scores rarely exceed 60, and similarity stretches are limited to 3 identical amino acids plus some flanking regions with similarities (285). No clear pathogenetic mechanism is known for a role of Hsp's in autoimmunity. The role of Hsp's as chaperones could result in a binding of potential antigens to these Hsp's, with aberrant processing and aberrant presentation to the immune system.

While antigens will be discussed in more detail in chapter 1.5.9, the third partner of the trimeric complex, the TCR, needs to be discussed. TCR's are highly polymorphic. Random selection and pairing of gene segments of the variable parts of the molecule, allows the development of a large number of T-cell clones, each with a unique TCR. Despite their extreme variability, these TCR's can be grouped into families that each use a single Vβ gene segment. Such Vβ's can be identified by monoclonal antibodies or oligonucleotides. In a model of multiple sclerosis, experimental allergic encephalomyelitis (EAE), T-cells that recognize the autoantigen of this disease (Myelin Basic Protein, MBP) are homogeneous in their Vβ usage. In this model, most MBP reactive T-cell clones have the TCR subtype Vß8.2. (182, 286). Specific monoclonal antibody therapy against this TCR prevents the disease (182). In diabetes of the NOD-mouse, both Vβ5 and Vβ8 TCR's have been suggested to be implicated in the disease (287, 288). However, McDuffie found in non-manipulated NOD-mice that lacked the Vβ5 and Vβ8 TCR's, that neither of

these Vß's is required for the onset of the disease (289). Other groups found that Vß usage of the first infiltrating T-cells was diverse (183, 290). Thus, no specific TCR is related to type 1 diabetes. If an antigen that triggers this disease is identified however, it is likely that specific TCR's will induce the strongest responses in the corresponding T-cells.

In summary, type 1 diabetes is both T-cell mediated and T-cell dependent. However, other components of the immune system have to be taken into account as well. The key issue in dissecting the mechanism of autoimmunity is to obtain candidate autoantigens that serve as primary triggers of the autoimmune reaction. Which mechanism changes tolerance or ignorance can only be solved when models become available that pinpoint to such antigens. The identification of proteins that are involved in the autoimmune T-cell reactions is therefore of paramount importance.

1.5.7 Other Cells of the Immune System.

Macrophages have been implicated in the development of type 1 diabetes. They are among the first immune cells found in the islets (291) and can function as an antigen presenting cell (APC) and a cytotoxic effector by releasing cytokines that attract other immune cells. In particular, dendritic cells of this macrophage/ monocyte lineage, present in the islets (292), can function as excellent antigen presenting cells. It is possible that such cells are indeed processing ß-cell antigens in such a way that autoreactive T-cells become activated. However, which factors are responsible for this are unknown. Even if these cells are the first present in the islets and are activating T-cells, unknown factors must initiate this. Among the possible mechanisms are increased cytokine levels and virus induced direct ß-cell lysis. A low-dose streptozotocin induced diabetes models showed similar characteristics of so called 'single cell insulitis' (293). Moreover, the function of macrophages can be impaired with silica particles, which resulted in prevention of diabetes (294, 295). Experiments from the group of Yoon suggested that macrophages were essential in the initiating phases of insulitis in NOD-mice and BB-rats (296, 297). Other evidence for a role of macrophages came from studies where blocking of adhesion molecules, involved in macrophage migration, also resulted in disease prevention (80). There is also evidence for a role of macrophages in ß-cell destruction. Macrophages can be found in the insulitis lesions, that engulfed ß-cells as seen by the presence of insulin crystals (298, 299).

Recently, it was reported from the NOD-*scid/scid* model, that chronic silica treatment in animals that received pathogenic T-cells, did not prevent diabetes (278). Chronic silica treatment did increase the number of macrophages in the islets and resulted even in granulomas without preventing insulitis. Thus, a primary role for macrophages is not yet established although their toxic effects may add to the destruction of ß-cells (see 1.5.8).

Several studies in BB-rats suggest a role for Natural Killer (NK)-cells may in ß-cell destruction. Treatment of BB-rats with antibodies specific for NK-cells, depletes these cells from the animal, and significantly reduced the incidence of diabetes (252). However, there is no direct evidence for the involvement of NK-cells in the ß-cell destruction in NOD-mice and in human IDDM patients (255, 300). The actual specificity of NK mediated ß-cell lysis and the mechanism by which NK cells could cause ß-cell destruction are unknown. It has been hypothesized that the metabolic state of the ß-cell may influence its susceptibility to NK mediated injury, presumably by inhibiting membrane repair mechanisms and/or effector-target cell binding (301). NK cells also produce lymphokines, which could add to their ß-cell cytotoxic action.

1.5.8 Cytokines.

Cytokines are produced by cells of the immune system. They are mediators of cell interactions and cytotoxicity. Interleukin 1 (IL-1) is one of the best studied cytokines in type 1 diabetes (302). Nerup and co-workers developed a model of type 1 diabetes based upon the action of cytokines (303-305). Il-1 receptors have been reported to be present on ß-cells (306). Macrophages can produce TNF-α, which can potentate the effects of IL-1. After initiation of cytotoxic effect and cytokines, free radicals are formed. The ß-cell has a reduced capacity for scavenging these compounds, thus further increasing their effects (307, 308). Moreover, IL-1 has an effect on insulin secretion (309). The model describing a role of cytokines requires the concomitant presence of T-cells, antigen presenting cells (macrophages) and triggers for cytokine release. In particular dendritic cells, with high numbers of cytokine receptors would be very active in presenting antigen to (autoreactive) T-cells. Release of ß-cell antigen(s), processed and presented by macrophages to T-cells initiates a self-perpetuating and self-limiting circuit of cytokine production, of which IL-1 is cytotoxic for ß-cells (310).

TNF-α and interferon-γ can up regulate expression of class I and class II MHC molecules on islets, but, as discussed, the role of aberrant class II is unknown. Induction of class I could help to increase expression of ß-cell antigens thereby surpassing minimal level to induce a T-cell response. In a transgenic model, where interferon was placed under the control of the insulin promotor in mice that were already transgenic for MHC class II expression (which results in non-autoimmune diabetes, see 1.5.4.1), and thus expressed in ß-cells, do develop an immune type of diabetes.

In summary, cytokine production, one of the results of T-cell activation does play a role in type 1 diabetes. If and which cytokines are involved in initial processes is unclear, but a modulating role in the disease process is very likely.

1.5.9 Humoral Autoimmunity and Type 1 Diabetes Mellitus.

Among the best studied abnormalities in type 1 diabetes is the presence of autoantibodies, both at clinical onset of the disease as well as in the preceding period of ß-cell destruction. The current model of autoimmune diseases suggests that tolerance to a self antigen is broken with the result of T-helper cell activation and a subsequent attack of a specific cell or tissue. In some autoimmune diseases the T-helper induction results solely in activation of cells of the B lymphocyte lineage followed by autoantibody production and such antibodies can be pathogenic as exemplified by pemphigus vulgaris. In this disease, a cell adhesion molecule, needed for a normal skin structure becomes a target of pathogenic autoantibodies (83). There is no cytotoxic T-cell infiltration. In other autoimmune diseases, such as type 1 diabetes, there is a predominant action of T-cells. As described, the disease is characterized by infiltration of lymphocytes and macrophages into the islets of Langerhans, followed by specific destruction of the ß-cell. Although autoantibodies are produced in type 1 diabetes, they do not play an evident role in the pathophysiology of the disease. Transfer of B lymphocytes into diabetes prone animals does not result in the disease (278, 311).

Additional evidence comes from pregnant diabetic mothers. Their babies do not develop diabetes although maternal IgG is present in their circulation. In addition, serum from newly diagnosed patients do not induce complement mediated lysis of ß-cells. However, one group reported an inhibitory effect of diabetic sera on insulin secretion, due to antibodies to the GLUT-2 glucose transporter (312). Although not pathogenic in nature, such antibodies could further deteriorate the

glucose homeostasis in patients. Others described antibodies that stimulate insulin secretion (313).

Analysis, identification and characterization of autoantibodies in type 1 diabetes has two major functions. First, autoantibodies were found to be predictive for the future clinical onset of type 1 diabetes. During the ongoing ß-cell destruction of phase II in figure 1.1, autoantibodies can be detected. Second, antibodies may be closely related to T-cell responses against an antigen. In a T-cell mediated disease, it is obvious that the T-cell triggering antigen is of paramount importance, but T-cells can induce ß-cell proliferation. Autoantibodies to such a trigger may reflect the antigen that initiated T-cell activation. Thus, identification, characterization and epitope mapping of candidate antigens of humoral responses may help to identify the primary T-cell antigens.

Like autoreactive T-cells can be found in normal individuals, a similar situation exists for autoreactive B-cells. Low levels of autoantibodies are described to be present in normal individuals (314). Such 'natural autoantibodies' are in general non-organ specific and of the IgM isotype. They are believed to be coded for by germ line genes (315). Origin and function may be identical to natural antibodies and may involve clearance of naturally occurring cell debris, participation in tissue regeneration, and regulation of immune-responses (316). However, it is clear, that natural autoantibodies are not associated with any pathological autoimmune processes, instead they may actually prevent the development of autoimmune disease (317).

In contrast to the IgM natural autoantibodies, those found in disease are generally of the IgG isotype and form a spectrum of organ specificity (318). One end of the spectrum consists of non-organ-specific autoimmune diseases such as systemic lupus erythematosus, and are associated with a wide range of non-organ-specific autoantibodies. The other end of the spectrum shows organ-specific autoimmune diseases such as myasthenia gravis, multiple sclerosis and type 1 diabetes mellitus. These diseases are characterized by antibodies that are organ restricted and highly specific for certain tissue antigens. There is definitely overlap in the spectrum.

Autoantibodies in type 1 diabetes were first described by Bottazzo and colleagues, who found that sera from patients with multiple endocrine abnormalities, including type 1 diabetes, could stain islets of Langerhans in an indirect

fluorescence assay, using donor pancreatic tissue as a substrate (134). After this, other antigens were identified, which will also be discussed in the next paragraphs.

1.5.9.1 Islet Cell Cytoplasmic Antibodies.

Bottazzo and colleagues described in 1974 that in sera from patients with poly-endocrine diseases (including diabetes) antibodies to islets could be detected using indirect immunofluorescence (134). The technique involves immuno-histochemistry on non-fixed human donor pancreas (Blood group O). Additional work showed that sera from a majority of type 1 diabetic patients has these islet cell cytoplasmic antibodies (319) (ICCA, usually referred to as ICA). Studies differ in percentage of positive patients at onset and range from 50-90% (320-322). The wide variation and bioassay character urged for standardization of the method. This was initiated by the Juvenile Diabetes Foundation International (JDF) by defining a standard serum. End-point titers are compared with the standard serum and values are expressed in JDF units, but even in these workshops the variation between participating labs is substantial (323, 324).

Although ICA's or autoantibodies in general, can help to discriminate between different types of diabetes, this is usually not needed. One of the most important application of autoantibodies is to use them to detect ongoing ß-cell destruction, thus to detect individuals on their way to clinical diabetes. This implies that normal populations must be screened for incidence of ICA's. Bruining and colleagues showed in a study of schoolchildren that a single determination of ICA only predicts half of the diabetes cases over a period of 10 years (325). Similar findings were reported from other groups (326-328). Although the Bruining study showed a very low incidence of ICA's in the schoolchildren population (probably due to their excellent pancreas substrate), the prevalence of ICA in the normal schoolchildren population is mostly reported between 2 and 4%(321). A tenfold higher incidence was found in first degree relatives of patients with type 1 diabetes (132, 322, 327, 329, 132). In these studies, the presence of ICA was associated with an increased risk of subsequent clinical IDDM. High titer ICA's (> 80 JDF units) confers a higher risk than low titer ICA (< 20 JDF) (324, 330). Furthermore, persistent ICA values are more predictive than intermittently positive (331). ICA's tend to disappear after onset of diabetes, reflecting the disappearance of the antigen from ß-cells that initiated their existence. Patients with poly-endocrine disease (332) have persistent ICA's long after onset of type 1 diabetes .

ICA's are not unique for type 1 diabetes (126) and as their name implies, ICA's recognize the whole islet of Langerhans and the target(s) for ICA's are not known. It is conceivable that several antigens which play a role in primary or secondary ß-cell immune responses will be visualized by this technique. It has been proposed that the target of ICA is a mono sialoganglioside (333). This was based on studies where several solvents and proteolytic enzymes were used on pancreatic sections. Such experiments suggested that ICA's bind to a sialic acid containing glycolipid moiety (334). and a ß-cell specific ganglioside GM2-1 has been proposed as the candidate. Others could not confirm this.

While normal ICA staining shows fluorescence of all islet cells (α–, β–, γ– and PP), a ß-cell restricted staining pattern was described recently. Sera from some ICA positive individuals only stain ß-cells (335-338) .This 'restricted' ICA staining was due to Glutamic Acid Decarboxylase(GAD) staining. Thus those sera strongly stained GAD in the indirect immunofluorescence technique. It was suggested that this restricted ICA staining pattern is associated with a protective haplotype (DQB *0602). Patients with this haplotype and restricted ICA pattern do not progress to overt diabetes, but do have strong GAD titers (339). Further studies are needed to analyze this interesting observation.

In summary, ICA's are present in a majority of diabetic patients at onset of the disease and they are present before the clinical diagnosis thereby providing a method to predict ongoing ß-cell destruction. They help to determine the risk for the development of type 1 diabetes in first degree relatives, but the predictive value of only ICA determinations in the population is not strong enough. High titers and repetitive positive titers are related to a short lag period before the clinical symptoms appear. ICA's generally show a decrease in titer after clinical onset. The lack of knowledge on their target(s) does not allow further characterization and analysis of the role of these targets in the immune process.

A special group of islet cell antibodies are the islet cell surface antibodies (ICSA's). They are characterized by their ability to bind to antigens on the surface of the ß-cell (340, 341). They are difficult to assay. However, the finding that the glucose transporter GLUT 2, located on the plasmamembrane, may be a target for autoimmunity in type 1 diabetes, urges for additional studies (312).

1.5.9.2 Insulin Autoantibodies.

Insulin antibodies were described in diabetes for the first time in 1963 (342). Insulin autoantibodies (IAA's) were described first in the insulin autoimmune syndrome (343). Insulin autoantibodies are defined as antibodies that bind insulin and occur in exogenous insulin naive subjects. The first association with diabetes in 1963 was largely ignored until 1983, when Palmer and colleagues found IAA's in 18% of newly diagnosed untreated patients (344). With standardization of the methods, IAA's can be found in up to 50% of newly diagnosed patients with type 1 diabetes (345). They are more frequently in young children. In the prediabetic individuals, IAA are often coinciding with ICA and may increase the risk for diabetes development (346). More recent data indicate that IAA's do not contribute strongly to the risk of diabetes in first degree relatives (347). In the Swedish Childhood Diabetes study data are available on the predictive values of IAA and ICA (348). ICA's are present in 84% of newly diagnosed subjects and 3% of controls. IAA's were present in respectively 43% and 1%. In the newly diagnosed patients, 40% were double-positive. No controls were double-positive and none has developed diabetes in the 3-5 years follow-up. With a sensitivity of 88% and a specificity of 96%, the predictive value for diabetes was only 3%. Moreover, in double positive patients the predictive value (at onset!) was 100% with 100% specificity and 40% sensitivity. Such data indicate that combination of ICA and IAA can add to the prediction, but that predictive values of each assay alone are relatively low in a population based setting.

Like with ICA's, IAA assays are standardized by JDF workshops. Until now the epitope responsible for the diabetic antibody recognition has not been found. This hampers the analysis in a standardized way, since other anti-insulin antibodies, not related to type 1 diabetes can be found. Only when using a radio immunoassay (RIA), the results are specific and sensitive enough (345). Some of the assays have incubation times of 7 days which indicates that the affinity of the antibodies for insulin is low, and might even reflect that they are actually not specific. A role of insulin in the pathogenic mechanisms of type 1 diabetes has not been elucidated. It is possible that these antibodies form as a result of insulin secretion in a damaged environment. Insulin is stored in granules in crystal forms and is therefore relatively immunogenic when release takes place outside the normal secretory pathway. This is somewhat supported by the observation that also antibodies to proinsulin are present in type 1 diabetes (349). The reason for the

high incidence of autoantibodies in children is unknown. IAA's at onset of the disease are not predictive for the formation of antibodies to exogenous insulin.

In summary, IAA's, when measured by RIA, are markers of autoimmunity in type 1 diabetes. Their analysis can help to improve the predictive power of antibody testing in type 1 diabetes. A primary role in the pathogenesis of insulin itself or of the IAA's is not likely.

1.5.9.3 Autoantibodies to a 64kD Autoantigen.

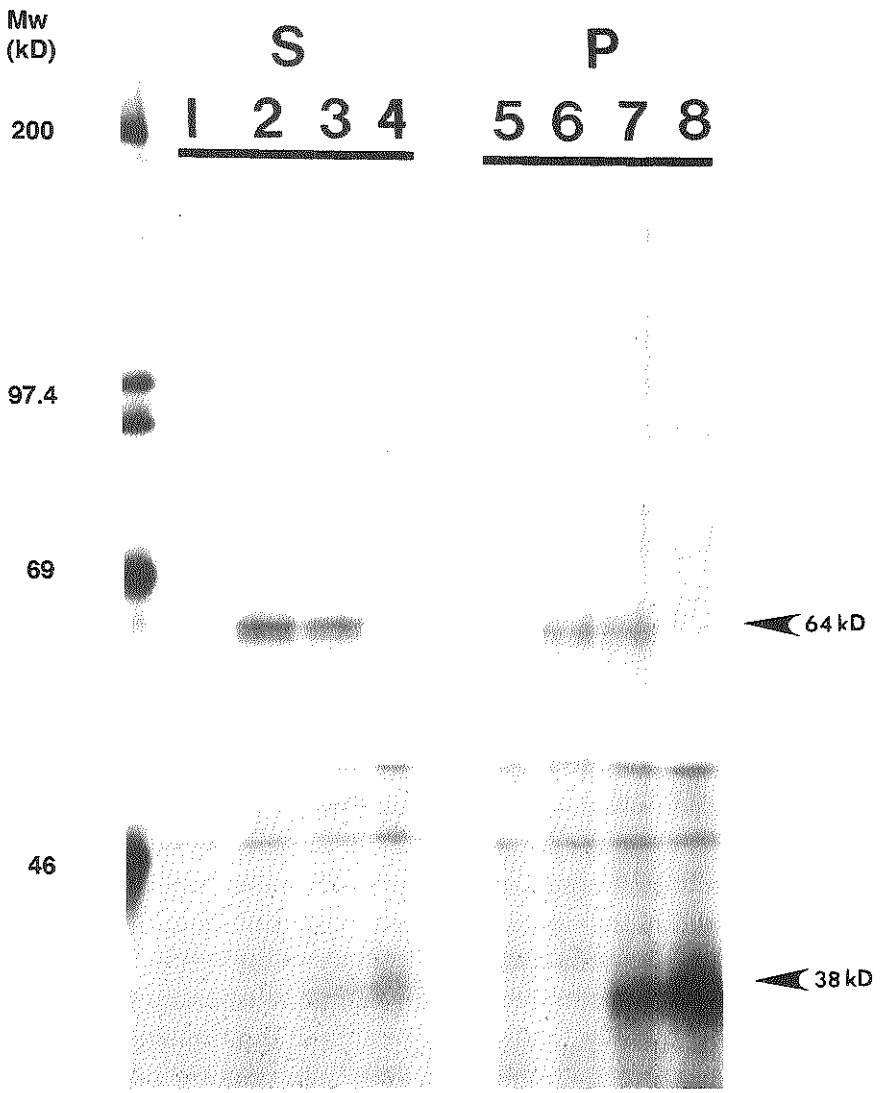
One of the major goals of this thesis was the identification of the 64kD antigen in type 1 diabetes. At the beginning of the experimental work described in this thesis the following data were available on this antigen.

Autoantibodies to a 64kD antigen were first described in 1982 by Bækkeskov and colleagues (350). Sera from type 1 diabetic patients were able to immunoprecipitate proteins from metabolically labeled (^{35}S-methionine) human islets of Langerhans. Using SDS-PAGE analysis, followed by fluorography, two proteins can be detected that were specifically immunoprecipitated by diabetic sera, a 38kD - and a 64kD protein (Figure 1.3). The immunoprecipitation assay is however technically demanding and time consuming, limiting its applicability for large series. Similar results were obtained when rat islets, which are less difficult to obtain, were used. The 64kD protein is not an abundant protein. It has been estimated that it represents at most 0.01% of total membrane bound proteins in the islet cells (351). This is in line with data obtained from two dimensional gel electrophoresis. Taking advantage of the fact that the molecule is partly membrane bound, Triton X-114 solubilization and temperature induced phase separation allowed to achieve a 1000 fold purification (352). The 64kD autoantibodies are of the IgG isotype (353).

The 64kD protein was found to be present in several animal species (350, 353, 354). In an analysis of rat tissues it was found to be islet specific (355), but the analysis did not include brain tissue, since neurons are difficult to label metabolically by ^{35}S-methionine. Islets were digested to single cells and using FACS sorting of these cells, what yields separate ß-cell and non-ß-cell populations, it was possible to show ß-cell specificity of the 64kD protein(355). The protein was found to be present in 2 forms, a membrane bound and a hydrophilic form (352). Which

FIGURE 1.3: Sera from Diabetes Patients Precipitate a 64kD and a 38kD protein.

Fluorogram of SDS-PAGE after immunoprecipitation of ^{35}S-methionine labeled rat islet hydrophilic (S) and amphiphilic (P) protein fractions with control sera (lane 1,5) and sera from newly diagnosed diabetes patients (lanes 2,3,4,6,7,8). Some diabetic sera precipitate only the 64kD protein (visible in both S and P fractions in lanes 2,,6), some precipitate both the 64kD and 38kD protein (lane 3) and some precipitate only the 38kD protein (lane 8), which is primarily present in the P fraction.



subcellular membrane or organelle bound the 64kD protein was not clear (355, 356). Glucose concentrations were found to stimulate 64kD expression (357). In addition, Coxsackie B4 infections in mice increased the ß-cell expression of 64kD (358).

The prevalence of 64kD antibodies at onset of the disease was tested in 3 labs (351, 354, 359, 360). Cumulative data on 134 newly diagnosed patients showed 108 (81%) positives. In this first series 2 of 111 controls (2%) were found positive. ICA's were available in 74 of these sera, of which 59 (80%) were positive. Sera from patients with other autoimmune diseases were tested (351) indicating the specificity for type 1 diabetes.

Of even more importance is the possibility of detecting 64kD antibodies in prediabetic individuals. In the first study on prediabetic individuals, 64kD autoantibodies were detected up to 8 years before clinical diagnosis in 11/14 individuals followed from 4-91 months prior to diagnosis. The 64kD antibodies were sometimes detected prior to ICA, but never later (361). In an other study, 23 of 28 prediabetic individuals, also primarily from a family study, had 64kD autoantibodies 2 - 75 months before diagnosis. Ten of the 23 were IAA negative and 5 ICA negative (359). It should be noted that prior to onset, the response to the 64kD antigen (or a diabetic antigen in general), may already have been diminished and patients negative at onset might still have experienced 64kD directed humoral autoimmunity (362). The few studies performed before 1990 were hampered by limited samples per individual and a relative short period before the clinical manifestation of the disease. A high incidence of 64kD autoantibodies has also been documented in the BB--rat (363) and the NOD-mouse (364).

After an antigenic trigger fades out, the immune response will usually limit itself. Antibodies will gradually disappear and only low, often undetectable levels remain. The decline in 64kD antibody titers has been reported to be present but was slow. Christie and colleagues detected 64kD autoantibodies 6-7 years after clinical onset in patients where ß-cell depletion was complete as judged from undetectable basal and stimulated C-peptide levels (365). No explanation for this has been provided yet. Continuation of humoral autoimmunity to GAD in these cases is likely to be maintained by a source of GAD other than ß-cells.

In sum, the 64kD autoantibodies were the earliest detected signs of humoral autoimmunity in type 1 diabetes. Autoantibodies against 64kD can be used to detect ongoing ß-cell destruction. The protein is ß-cell specific, but is a minor, mem-

brane bound or soluble, constituent of ß-cells The 64kD antigen was found to be present in several species, including animal models of type 1 diabetes.

1.5.9.4 Autoantibodies to a 38kD Protein.

Bækkeskov and colleagues described immunoprecipitation of a 38kD proteins, from islets of DR3 positive donors, with sera from newly diagnosed diabetic patients (350). Immunoprecipitation experiments provide stringent conditions for antigen recognition. The antigen is present in its native form. Antibodies that recognize conformational epitopes on a molecule can not be detected in Western blots, but can by immunoprecipitation. Using these conditions in one and two dimensional gel electrophoresis, virtually no other proteins were precipitated by diabetic sera than the 64kD and the 38kD as shown in figure 1.3. Both proteins could not be detected in Western blots of islet cell proteins stained with diabetic sera. The 38kD protein was recognized in about 20% of newly diagnosed diabetic sera and seemed to be completely distinct from the 64kD autoantibody reactivity. No 38kD antibodies were found in controls. A few patient sera only recognized the 38kD, while several others recognized both the 38kD and 64kD molecules (351).

Other groups also reported activity against a 38kD target molecule. Yoon and colleagues found 38kD autoantibodies after an infection of human islets with Cytomegalo virus (200). In BB-rats, autoantibodies to a 38kD islet cell protein precede the onset of diabetes. These antibodies were detectable in a Western blot method, but only after the blots were renaturated (366). The antibodies were present before clinical onset and predictive for diabetes development. Finally, Roep and colleagues found (human) T-cell reactivity against a 38kD islet protein in newly diagnosed patients (270, 271). It is not known if the 38kD proteins mentioned in these studies are identical and further analysis is required.

1.5.9.5 Other Autoantibodies in Type 1 Diabetes Mellitus.

A number of other targets for autoantibodies have been identified, primarily in newly diagnosed patients. The numbers of patients studied is still small in most cases.

By screening a cDNA library of islets with diabetic sera, Castano and colleagues isolated several clones of potential antigens. One clone was found to

represent carboxypeptidase H, an insulin processing enzyme (367). Another molecule, presumably ß-cell specific was identified by similar techniques (368). This protein is 69kD in size and presumably ß-cell specific. No large studies on T-cell proliferative capacity or autoantibodies to this p69 have been performed yet. A portion of the 69kD peptide, shows resemblance to a fragment of bovine albumin. Autoantibodies to bovine serum albumin (BSA) have been implicated in the etiology of type 1 diabetes. Epidemiological evidence was reported, that early introduction of cow's milk as well as early introduction of solid food was related to a higher incidence of type 1 diabetes ((369, 370) and references therein). Although the data do support some linkage, no proof can be provided from such studies. A (PCFIA) particle concentration fluorescence immunoassay showed that children with type 1 diabetes have autoantibodies against BSA. The diabetic children distinguished themselves from other children with BSA antibodies by the fact that their antibodies recognized a particular 17 amino acid epitope of the BSA, called ABBOS. Some evidence was provided that a Mw 69,000 islet cell protein showed mimicry with the ABBOS fragment (371). No T-cell involvement has been shown yet. The assay has not yet been reproduced by other labs. Thus, a role for ABBOS and BSA in the pathogenesis of type 1 diabetes remains to be established (211)

One report describes that the 64kD autoantigen is the heat shock protein HSP65. Using recombinant mycobacterial Hsp65 and the human counterpart Hsp60, it was shown that the 64kD is a different protein with a lower mobility (372).

In NOD-mice, autoantibodies to a 58kD protein were detected. This protein was identified as peripherin, a structural, intermediate filament, protein present in mouse and rat ß-cells, neurons and human neuroblastoma cells. The NOD-mice were the only species so far to develop autoantibodies to peripherin. Peripherin is cross-reactive with NOD class II molecules, suggesting that it may contribute to defective self-tolerance of islet ß-cells in the NOD-mouse (373).

Diabetic patients often develop autoantibodies to other endocrine organs, such as adrenal and thyroid. Moreover, antibodies to parietal cells as well as non-organ specific antibodies can be found. Although in some cases this is related to polyendocrine diseases, several patients have these autoantibodies without further clinical problems (374). It seems that the susceptibility for an autoimmune disease is linked to diminished tolerance for other antigens. A similar observation can be made in NOD-mice, where besides the islet infiltration several additional autoimmune effects can be observed (375).

Type 1 Diabetes Mellitus has an autoimmune basis. Although this introduction has discussed different mechanisms and hypotheses, no evidence is at present available to explain the disease at the molecular level. None of the antigens discovered is found to be causal in the early events where the immune system loses tolerance of recognizes a ß-cell component as a non-self molecule. In the next chapters, the experimental work of this thesis, aimed at two antigens is presented and discussed.

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Chapter 2

THE 64kD AUTOANTIGEN IN TYPE 1 DIABETES IS THE GABA SYNTHESIZING ENZYME GLUTAMIC ACID DECARBOXYLASE (GAD):

Identification, Characterization and Implications of GAD in type 1 Diabetes Mellitus and Stiff-man Syndrome.

2.1

Identification of the 64K autoantigen in insulin dependent diabetes as the GABA synthesizing enzyme glutamic acid decarboxylase.

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Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase

Steinunn Baekkeskov*, Henk-Jan Aanstoot*, Stephan Christgau*, Annette Reetz†, Michele Solimena†, Marilia Cascalho*, Franco Folli†‡, Hanne Richter-Olesen‖ & Pietro-De Camilli†

* Departments of Microbiology/Immunology, Medicine, and Hormone Research Institute, University of California San Francisco School of Medicine, San Francisco, California 94143, USA
† Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510, USA
‡ Present address: Department of Medicine, Istituto Scientifico S. Raffaele, Milano, Italy
‖ Hagedorn Research Laboratory, DK2820 Gentofte, Denmark

The pancreatic islet β-cell autoantigen of relative molecular mass 64,000 (64K), which is a major target of autoantibodies associated with the development of insulin-dependent diabetes mellitus (IDDM) has been identified as glutamic acid decarboxylase, the biosynthesizing enzyme of the inhibitory neurotransmitter GABA (γ-aminobutyric acid). Pancreatic β cells and a subpopulation of central nervous system neurons express high levels of this enzyme. Autoantibodies against glutamic acid decarboxylase with a higher titre and increased epitope recognition compared with those usually associated with IDDM are found in stiff-man syndrome, a rare neurological disorder characterized by a high coincidence with IDDM.

THE cell-specific destruction of pancreatic β cells, which precedes the clinical onset of insulin-dependent diabetes mellitus is believed to be mediated by autoimmune mechanisms[1]. The autoimmune phenomena associated with the disease include massive lymphocytic infiltration of islets[2] and circulating autoantibodies to β cells[3]. A 64K β-cell autoantigen is a target of autoantibodies in this disease[4]. The 64K autoantibodies are present in $\geq$80% of newly diagnosed IDDM patients and have been detected up to several years before clinical onset of IDDM concomitant with a gradual loss of β cells[5-7]. The 64K antigen was found to be β cell-specific in an analysis of several tissues which did not include the brain[8]. The 64K autoantigen in β cells is detected as a hydrophilic soluble 65K form and a 64K hydrophobic form which can be both membrane bound and soluble (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). The 64K forms can be resolved into two components, α and β (ref. 9). The function of the 64K protein in the β cell has remained elusive.

Most patients with a rare but severe neurological disease called stiff-man syndrome (SMS) have autoantibodies to GABA-secreting neurons. Glutamic acid decarboxylase (GAD), the enzyme that synthesizes GABA from glutamic acid, is the predominant autoantigen[10,11]. Surprisingly, almost all patients positive for the autoantibody to GABA-secreting neurons are also positive for islet cell cytoplasmic antibodies, as demonstrated by immunofluorescence of pancreatic sections, and a significant fraction have IDDM[11]. GAD is selectively expressed in GABA-secreting neurons in the central nervous system (CNS)[12]. Outside neurons, GAD is found at high levels in pancreatic β cells[13-15]. There are at least two isomers of GAD in brain, which are resolved by differences in their mobility on SDS-polyacrylamide gel electrophoresis; their molecular weights have been described as 59–66K (ref. 16).

Because IDDM is the autoimmune disease that is most often associated with SMS and because some of the characteristics of GAD and the 64K autoantigen are similar, we hypothesized that they were the same protein. Here we demonstrate that the 64K autoantigen in IDDM is the same as GAD in pancreatic β cells.

Immunoprecipitation of 64K autoantigen

We first assessed whether serum S3, a sheep antiserum raised against purified rat brain GAD[17], and sera from SMS patients positive for GAD antibodies[10,11], could immunoprecipitate the 64K autoantigen from rat islets. We used [^{35}S]methionine-labelled rat islet cell fractions partially enriched for the 64K antigen (S-100 DP, Fig. 1). The sera positive for GAD antibody (anti-GAD sera) immunoprecipitated a doublet of [^{35}S]methionine-labelled proteins, which on SDS-PAGE have the same mobility as the 64K α/β autoantigen immunoprecipitated by IDDM sera[7] (Fig. 1*a*, lanes 4–9).

To assess whether the proteins recognized by the anti-64K-sera and by anti-GAD sera were the same, supernatants resulting from immunoprecipitation with anti-GAD sera were subsequently reprecipitated with anti-64K IDDM sera. Similarly, supernatants resulting from immunoprecipitation with anti-64K IDDM sera were reprecipitated with anti-GAD sera. Results from those experiments showed that anti-GAD sera, and anti-64K sera each quantitatively removed the protein recognized by the other group of sera, demonstrating complete cross-reactivity between anti-GAD sera and anti-64K sera, and strongly suggesting that the 64K protein is the same as GAD in rat islets (Fig. 1*a*, lanes 10–20).

Trypsin digestion of [^{35}S]methionine-labelled islet cell extracts followed by immunoprecipitation showed that a 55K immunoreactive fragment was formed from both the 64K protein and GAD, further verifying their common identity (Fig. 1*b*). Furthermore, two-dimensional analyses of GAD immunoprecipitated from [^{35}S]methionine-labelled islets with the S3 antiserum revealed an identical pattern to that described for the 64K protein (H. Schierbeck, L. Aagaard and S.B., manuscript submitted; and ref. 9) (results not shown). This pattern was also the same as the two-dimensional pattern of pancreatic and brain GAD shown by western blotting of two-dimensional gels with the S3 serum (see Fig. 5*a*).

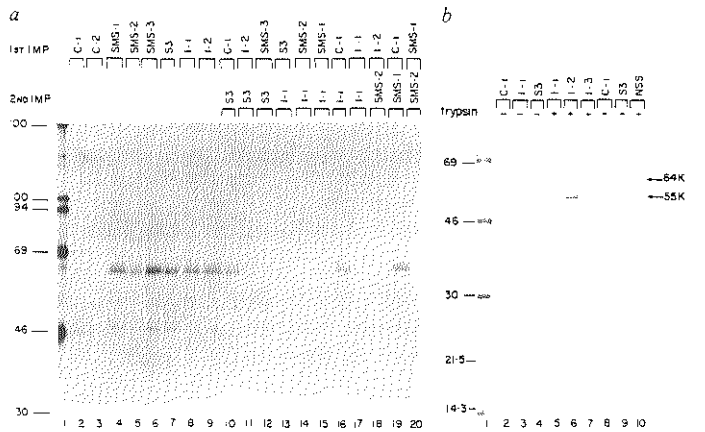


FIG. 1 Anti-GAD sera and anti-64K IDDM sera recognize the same protein in rat islets. *a*, Fluorogram of an SDS-PAGE showing immunoprecipitation of Triton X-114 detergent phase cytosolic fraction from [^{35}S]methionine-labelled rat islets (S-100 DP) with anti-GAD sera, anti-64K IDDM sera and control sera. Lanes 2–9, samples from a single immunoprecipitation with sera indicated at the top of each lane; lanes 10–20, samples from a second immunoprecipitation of supernatants remaining after a first immunoprecipitation. Sera used for the first and second immunoprecipitations are indicated at the top of each lane. Sera used for the immunoprecipitation were: C, sera from healthy individuals: SMS, sera of SMS patients previously shown to be GAD antibody positive[11]; I, sera from newly diagnosed anti-64K-positive IDDM patients[7] (numbers indicate patient code); S3, a sheep antiserum raised to purified rat brain GAD[17]. Relative molecular mass markers are shown in lane 1 ($M_r \times 10^{-3}$). The anti-GAD sera immunoprecipitate GAD from supernatants after immunoprecipitation with control serum (lanes 10 and 19), but not from supernatants after immunoprecipitation with anti-64K sera (lanes 11 and 18). The anti-64K sera immunoprecipitate the 64K protein from supernatants after immunoprecipitation with control serum (lane 16), but not from supernatants after immunoprecipitation with anti-GAD sera (lanes 13–15). The triple band represents the 65K form and 64K α and β forms of the 64K autoantigen (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). *b*, Fluorogram showing immunoprecipitation of the 64K protein and GAD from S-100 DP of [^{35}S]methionine-labelled rat islets before (lanes 2–4) and after (lanes 4–10) trypsin digestion. Serum codes are as in legend for *a*. NSS is a preimmune sheep serum. Trypsin digestion results in a 55K immunoreactive fragment that is recognized by both anti-64K sera and the anti-GAD serum S3.

METHODS. Neonatal rat islets were isolated and labelled with [^{35}S]methionine as described[8]. Islets were swollen on ice for 10 min in 10 mM HEPES, pH 7.4, 1 mM $MgCl_2$ and 1 mM EGTA (HME buffer) and then homogenized by 20 strokes in a glass homogenizer. The homogenate was centrifuged at 2,000*g* to remove cell debris and the postnuclear supernatant centrifuged at 100,000*g* for 1 h to obtain a cytosol (S-100) and a particulate (P-100) fraction. Amphiphilic proteins were purified from the S-100 fraction by a modification of the method described by Bordier[29] for Triton X-114 (TX-114) phase separation. S-100 fraction was made 1% in TX-114, warmed at 37 °C for 2 min to induce TX-114 phase transition, and centrifuged at 15,000*g* for 2 min to separate the aqueous and detergent phases. The detergent phase was diluted in 20 mM Tris buffer, pH 7.4, 150 mM NaCl (TBS) and immunoprecipitated as described[9] using DP from 300 islets for each immunoprecipitate. Immunoprecipitates were analysed by SDS-PAGE (10%) and processed for fluorography[24]. For the trypsin digestion, S-100 DP from 4,000 islets was diluted to 200 μl in TBS. Trypsin (0.74 units) was added and the sample was incubated for 1 h at 25 °C. The reaction was stopped by addition of 10 μl 10 mM HEPES, 5 mM EDTA, 5 mM pyrophosphate, 5 mM benzamidine–HCl, pH 7.5. Digested and undigested material was immunoprecipitated and the immunoprecipitates analysed by SDS-PAGE (15%). Anti-64K sera were from three newly diagnosed IDDM patients[7], control sera were from two healthy individuals. SMS sera were from three anti-GAD-positive individuals[11]. The S3 antiserum was a gift from I. J. Kopin.

Immunoprecipitation of GAD

We next analysed whether anti-64K sera could immunoprecipitate GAD from brain and islets. Membrane and soluble fractions were prepared from brain and islets and immunoprecipitated with serum S3, anti-GAD SMS sera, anti-64K IDDM sera, and control sera. Presence of GAD in the immunoprecipitates was analysed by western blotting. The blots were probed with serum S3 or serum 7673, a rabbit serum raised to a synthetic 17-amino-acid peptide corresponding to the carboxyl terminus of the larger rat brain GAD isoform (ref. 18; and D. Gottlieb, personal communication) (M.S. and A.R., unpublished data). Both sera gave identical results. Figure 2 shows a western blot containing some of such immunoprecipitates probed with serum S3. As expected, an immunoreactive band with the electrophoretic mobility of GAD was detected in immunoprecipitates obtained with anti-GAD sera (Fig. 2, lanes 11, 12, 16). A band of identical mobility was visualized in all immunoprecipitates obtained with anti-64K sera (7/7) (Fig. 2, lanes 1–7, 14), but not in those obtained with control sera (Fig. 2, lanes 8–10, 13, 15, 17). These results demonstrate that the protein immunoprecipitated from brain and islets by anti-64K sera is indistinguishable from GAD.

64K protein has GAD enzyme activity

If the 64K autoantigen is GAD, then the 64K protein should have the enzymatic properties of GAD. We therefore investigated whether GAD enzyme activity could be removed from islet and brain cell lysates by immunoprecipitation with an anti-64K IDDM serum, and whether GAD activity could then be measured in the immunoprecipitates. Brain and islet cell fractions were immunoprecipitated with increasing amounts of an anti-64K IDDM serum, and the GAD enzyme activity

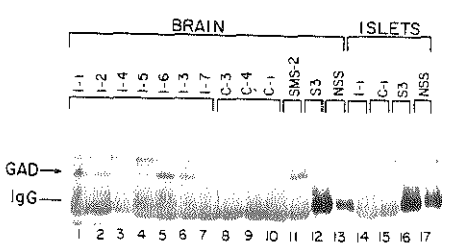


FIG. 2 Anti-64K antibodies immunoprecipitate GAD from brain and islets. Western blot of immunoprecipitates of rat brain (S-100 DP) and islet cell (P-100 DP) fractions obtained with anti-64K sera and anti-GAD sera. The blot was probed with the S3 serum. Sera used for the immunoprecipitation are indicated at the top of each lane by the same codes as in Fig. 1. The 64K protein immunoprecipitated from both brain and islets is immunostained by anti-GAD antibodies. In the gel shown GAD migrated as a single band. METHODS. Neonatal rat brain was homogenized at 4 °C in seven volumes of HME buffer followed by centrifugation at 100,000*g* for 1 h to obtain S-100 and P-100 fractions. S-100 DP was prepared and aliquots (1/13 brain per lane) immunoprecipitated as described in the legend to Fig. 1. P-100 was prepared from neonatal rat islets and extracted in 200 ml TBS with 1% Triton X-114 for 2 h at 4 °C (ref. 9). P-100 DP was prepared as described for S-100 DP and aliquots (1,500 islets per lane) immunoprecipitated. Immunoprecipitates were subjected to SDS-PAGE followed by electroblotting to a PVDF membrane (Immobilon)[30], probing with the S3 serum and visualizing by alkaline phosphatase-conjugated rabbit anti-sheep IgG.

measured after immunoprecipitation in both supernatants and pellets (Fig. 3*a*, *b*). Immunoprecipitation with increasing amounts of anti-64K serum but not with control serum removed the GAD activity from both brain and islet cell lysates in a dose-dependent manner, and, in parallel, increasing amounts of GAD activity appeared in the immunoprecipitates. The GAD activity recovered in the immunoprecipitates did not account for all the activity lost from the supernatants, probably owing to an inhibiting effect of antibodies on enzyme activity. For islet cell extracts from [^{35}S]methionine-labelled islets, SDS-PAGE revealed that the only islet cell protein specifically detected in the immunoprecipitates obtained with the anti-64K IDDM serum was the 64K autoantigen (see Fig. 1). Thus the GAD enzyme activity measured in immunoprecipitates obtained with the anti-64K IDDM serum is a property of the 64K autoantigen.

Brain and β-cell GAD

Analysis of GAD enzyme activity in neonatal and adult rat tissues showed that the expression of GAD is high in brain and islet cells and is either absent or low in a variety of other tissues (results not shown), confirming previous reports[19]. In islets, double immunostaining with a monoclonal antibody to GAD and either glucagon or somatostatin confirmed the localization of GAD to the β-cell core, and the absence of GAD in the other endocrine cells, which are localized to the islet periphery (Fig. 4). GAD in brain and islets was found to have identical mobility on SDS-PAGE (Fig. 2) and by two-dimensional gel electrophoresis using isoelectric focusing/SDS-PAGE (Fig. 5*a*). We compared the immunoreactive trypsin fragments generated from brain and islet GAD. Trypsin generated a 55K immunoreactive fragment from both islet and brain GAD (Fig. 5*b*; see also Fig. 1). In both tissues GAD was found in a soluble hydrophobic form as well as a membrane-bound hydrophobic form (Fig. 5*b*) as described for the 64K islet cell autoantigen (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). The 65K component of the 64K protein in islets (H. Schierbeck, L. Aagaard and S.B., manuscript submitted) was detected in brain and islet cells in some (Figs 1*a*, 5*A*, *b*, *c*, 5*B* and 6*A*) but not in other analyses (Figs 1*b*, 2). Furthermore, the 64K α/β doublet was detected in some analyses (Figs 1*a*, 6*A*). The immunochemical and biochemical properties of the brain and islet GAD therefore indicate that they are very similar.

Antibodies to GAD in SMS and IDDM sera

The GAD reactivity in SMS sera has been demonstrated by western blotting and by immunocytochemical staining of fixed tissue sections[10,11], that is, assays that involve complete or partial denaturation of the antigen. The standard assay for 64K antibodies in IDDM sera has been immunoprecipitation from islet cell lysates prepared in nondenaturing conditions[4–9]. We selected sera from the individuals who had the highest immunoreactivity to the 64K autoantigen in immunoprecipitation experiments in a survey of 112 IDDM patients and prediabetic individuals without SMS[7] and tested them for immunoreactivity to the brain GAD protein on western blots (five sera) and for immunostaining of GABA-secreting neurons (7 sera). The results were compared with those for SMS sera. In contrast to the SMS sera, none of the IDDM sera detected the denatured GAD protein on western blots (Fig. 6*A*), and only one was able to weakly immunostain GABA-secreting neurons (Fig. 6*B*, *c*). Titration of IDDM and SMS sera in immunoprecipitation experiments furthermore showed that the titre of anti-GAD antibodies in SMS sera was 10–200 times that in IDDM sera (results not shown). In another survey of 74 IDDM patients, only three were positive for antibodies to GABA-secreting neurons (Fig. 6*B*, *b*) including the only two which were positive by western blotting (ref. 11, and results not shown). By contrast, all SMS sera that were positive by immunocytochemistry and/or western blotting were also positive by immunoprecipitation (results not shown). So anti-GAD antibodies in SMS patients are generally present at higher titres and recognize more distinct

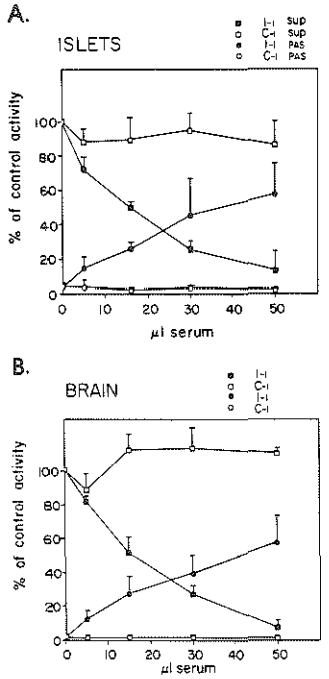


FIG. 3 Precipitation of GAD activity from brain and islets with anti-64K autoantibodies. *a*, Aliquots (700 islets per sample) of S-100 DP from neonatal rat islets were immunoprecipitated with increasing amounts of the anti-64K IDDM serum I-1 (closed symbols) or the control serum C-1 (open symbols). GAD activity was measured in supernatants after immunoprecipitation (squares) and in pellets (circles). The activity in the supernatants was calculated as percentage of the activity in non-immunoprecipitated samples incubated with the same amount of control serum. The activity in the immunoprecipitates was calculated as percentage of the activity in samples incubated without serum. Values are mean of three experiments ± s.d. *b*, As *a*, except that aliquots of S-100 DP from neonatal rat brain were used instead of islet cell material.

METHODS. S-100 DP was prepared from islets and brain as described in the legends to Figs 1 and 2, except that buffers were supplemented with 1 mM aminoethylisothiouronium bromide hydrobromide (AET) and 0.2 mM pyridoxal 5'-phosphate (PLP). S-100 DP was diluted 10 times in 50 mM potassium phosphate pH 6.8, 1 mM AET, 0.2 mM PLP (buffer A) and incubated with the indicated amounts of sera in a total volume of 150 ml for 7 h at 4 °C. Immunocomplexes were absorbed to 150 μl protein A–Sepharose beads (PAS Pharmacia) and isolated by centrifugation. The supernatants were collected and centrifuged three times to remove traces of PAS. PAS pellets were washed five times by centrifugation in buffer A. Enzyme activity in the PAS pellets and the supernatants was measured using a modified version of the assay first described by Albers and O'Brady[31]. Both PAS pellets and supernatants were transferred to 1.5-ml screw cap tubes; 20 μl 5 mM L-glutamate in buffer A and 0.4 μCi [1-^{14}C]L-glutamate (59 mCi mmol^{-1}, Amersham) were added. The tubes were closed immediately with a cap containing Whatman filter paper soaked in 50 μl 1 M hyamine hydroxide in methanol and incubated for 2 h at 37 °C. The filter paper was then removed and the absorbed $^{14}CO_2$ measured in a scintillation counter. The specific activity of GAD in homogenates of neonatal brain and islet cell material was similar (~40–70 mU per g protein).

epitopes than anti-GAD antibodies in IDDM patients. However, in rare cases, anti-GAD antibodies in IDDM patients can recognize denatured GAD. Whereas the 7673 and S3 antisera recognize both the 65K form and the 64K α and β isoforms on western blots (Fig. 6*A*, lanes 2, 4, 5), human GAD autoantibodies as well as the monoclonal antibody GAD6 preferentially recognize the smaller 64K α and β isoforms (Fig. 2, lanes 7–11). The GAD6 antibody is specific for the smaller GAD isoform in brain[16].

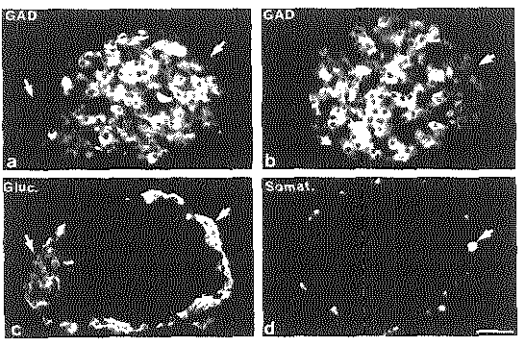


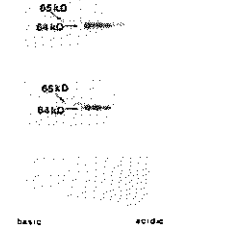
FIG. 4 Immunofluorescence staining of pancreatic islets with GAD, glucagon and somatostatin antibodies. Immunofluorescence micrograph showing pancreatic islets. The islet in *a* and *c* was double-labelled for GAD and glucagon. The islet in *b* and *d* was double-labelled for GAD and somatostatin. Arrows point to corresponding cells in the two pairs of panels. The β cells in the central core of the islet are brightly stained with the GAD antibody, whereas the cells positive for glucagon and somatostatin do not stain with the same antibody.
METHODS. Formaldehyde-fixed frozen sections of rat pancreas were first rhodamine-labelled for GAD using a mouse monoclonal GAD6, raised against purified rat brain GAD[16], and then fluorescein-labelled for glucagon or somatostatin using rabbit polyclonal antibodies to either hormone and using methods described[32]. GAD6 was a gift from D. I. Gottlieb, University of Washington. Scale bar, 25 mm.

Discussion

The 64K autoantigen is the enzyme glutamic acid decarboxylase. We have demonstrated that the 64K autoantigen in IDDM is the enzyme GAD in pancreatic β cells. GAD, its product GABA, and the GABA-metabolizing enzyme GABA transaminase are present at high levels in pancreatic β cells as well as in GABA-secreting neurons[19]. GABA may act as an 'endocrine transmitter' in islets[20-23] and this may explain why GAD is expressed in β cells. The β cell-specific expression of GAD in islets is consistent with its being an autoantigen associated with the selective destruction of pancreatic β cells in IDDM. GAD is a major autoantigen in SMS, a disease in which GABA-secreting neurons are thought to be affected[10,11]. Thus pancreatic β cells and GABA-secreting neurons share a protein that is unusually susceptible to becoming an autoantigen, an observation that will surely motivate studies not only into the pathogenesis of IDDM and SMS, but also into the mechanisms of generation of self-tolerance by the immune system, and its failures.

Differences in humoral autoimmunity to GAD in SMS and IDDM. The humoral autoimmunity towards GAD in the two diseases in which GAD-expressing cells are either destroyed (IDDM) or thought to be affected (SMS), suggests a direct relationship between GAD autoantibodies and the diseases. Not all SMS patients with GAD antibodies develop IDDM, and only a small proportion of IDDM patients develop SMS, in spite of their having GAD autoantibodies for several years. This suggests that there are other components to both diseases. The development of SMS seems to involve a stronger antibody response to GAD and includes epitope recognition which is usually absent in IDDM, perhaps as a result of different characteristics of pancreatic β cells and GABA-secreting neurons. For example, the microenvironment of the CNS is protected by the blood–brain barrier and the antigenic requirements to initiate an autoimmune response across this barrier may be distinct. Pancreatic β cells express major histocompatibility complex (MHC) class I molecules[24] which are thought to present self-peptides to the immune system, whereas CNS neurons normally do not[25].

Subcellular localization of GAD in brain and islets and its role as an autoantigen. Synaptic-like vesicles have recently been identified in endocrine cells[26] and may be the storage sites of GABA in β cells, similar to synaptic vesicles in brain. Immunofluorescence studies have shown colocalization of GAD and membrane markers of these synaptic-like microvesicles (A.R., M.S. and



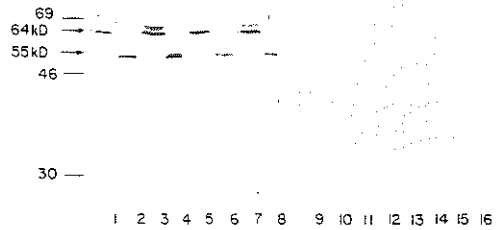


FIG. 5 GAD in brain and islets have similar properties. *A*, Two-dimensional gel electrophoresis of neonatal rat and islet cell GAD/64K antigen. *a*, *b*, Western blots of two-dimensional gels of a neonatal islet particulate fraction (*a*) and a brain fraction (*b*) probed with the GAD antiserum S3. *c*, *d*, Fluorograms of two-dimensional gels of immunoprecipitates of a [^{35}S]methionine-labelled rat islets extract (S-100 DP) with the anti-64K IDDM serum I-1 (*c*) and *a* with a control serum C-1 (*d*). The soluble fractions of brain and islets (*b* and *c*) contain both the 65K pI 7,1 component and the 64K 6,7 α component, whereas the particulate fraction contains only the 64K pI 6,7 α component (H. Schierbeck, L. Aagaard and S.B., manuscript submitted). Both the 65K and the 64K component display charge heterogeneity previously described for the 64K autoantigen in islets[9]. The panels both demonstrate the identical behaviour of the 64K protein (*c*) and GAD (*a* and *b*), further proving they are the same protein, and show the similarities of brain and islet GAD with regard to both charge and size. *B*, Western blot of soluble and particulate GAD from neonatal rat brain and islets before and after trypsin digestion. Lanes 1–8, probing with S3 serum; lanes 9–16, probing with normal (preimmune) sheet serum. Trypsin digestion of both brain and islet GAD results in a 55K immunoreactive GAD fragment.
METHODS. A particulate fraction was prepared from neonatal rat islet homogenates by centrifugation at 36,000*g* (*a*). A low-speed synaptosomal supernatant was prepared from brain as described[33] (*b*). S-100 DP was prepared from neonatal rat islets and immunoprecipitated as described in the legend to Fig. 1 (*c* and *d*). Two-dimensional gel electrophoresis was performed as described by O'Farrell[34] and modified by Ames and Nikaido[35]. Immunoblotting was according to Towbin *et al.*[30]. GAD in *a* and *b* was visualized by probing with the anti-GAD serum S3, followed by rabbit anti-sheep IgG serum and ^{125}I-labelled protein A and autoradiography. For *B*, S-100 DP and P-100 DP from islets and brain were prepared as described in legends to Figs 1 and 2 and digested with trypsin as described in legend to Fig. 1. Brain and islet cell fractions were subjected to SDS-PAGE using 15% polyacrylamide gel. Western blotting and staining procedures were as described in legend to Fig. 2.

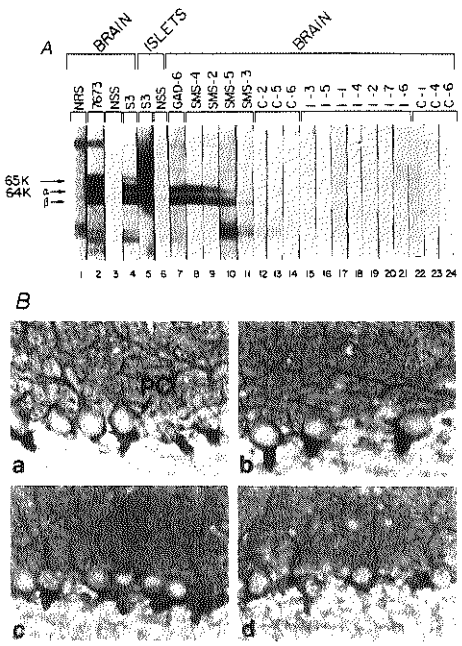


FIG. 6 Comparison of GAD immunoreactivity in SMS and IDDM sera. *A*, Western blots of S-100 DP from neonatal rat brain (lanes 1–4 and 7–24) and islets (lanes 5 and 6) probed with anti-GAD SMS sera and sera from IDDM patients and prediabetic individuals with high immunoreactivity to the 64K/GAD protein in immunoprecipitation experiments. Sera are indicated at the top of each lane by the same codes as in Fig. 1. The S3 serum, 7673 serum, GAD6 serum and anti-GAD SMS sera recognize the denatured form of GAD in brain and islets, whereas the anti-64K IDDM sera do not. The S3 and 7673 sera react with both the 65K form and the 64K α and β forms, whereas GAD6 (previously shown to react selectively with the smaller brain GAD isoform[16]) and the SMS sera only react with the 64K α and β components. *B*, Bright-field light microscopy micrographs showing immunostaining of GABA-secreting nerve terminals in the rat cerebellar cortex with SMS and IDDM sera. *a*, SMS-3 serum; *b*, IDDM serum I-8; *c*, IDDM serum I-2; *d*, IDDM serum I-1. All sera, except I-1, show the typical stain of GABA-secreting nerve terminals. Note the accumulation of immunoreactivity at the base of the Purkinje cells, where the GABA-secreting nerve endings of basket cells terminate.

METHODS. S-100 DP aliquots from rat brain and rat islets were subjected to SDS-PAGE, electroblotted and immunostained as described in the legend to Fig. 2. Dilutions for immunostaining of western blots were 1/200 for GAD6, 1/250 for IDDM sera, control human sera, SMS sera 2 and 5, the 7673 serum and normal rabbit serum, 1/500 for SMS sera 3 and 4 and 1/2,000 for S3 and normal (preimmune) sheep serum. For *B*, methods as described in ref. 11.

P.D.C., unpublished results). We found that GAD in both brain and islets behave similarly with regard to hydrophobicity and compartmentalization, that is, both were detected in a soluble hydrophobic and a membrane-bound hydrophobic form. Although it cannot be excluded that GAD is expressed at the surface of β cells, it is more likely that the protein remains confined to the cytoplasmic space. Thus autoantibodies to GAD are unlikely to see the intact antigen on the surface of normal β cells. But peptides derived from the GAD molecule may be expressed at the cell surface in the cleft of MHC class I antigens, and so be recognized by pathogenic T cells (ref. 27, and refs therein).

The identification of the 64K antigen as the enzyme GAD is of relevance in elucidating the role of this antigen in the development of IDDM. If the GAD autoantigen is shown to be critical for the initiation of β-cell destruction, then an approach to therapy might be to develop ways of preventing or reversing the autoimmune response towards GAD in β cells of susceptible individuals. This would be similar to the successful prevention of autoimmune disease in experimental allergic encephalomyelitis[28]. □

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2.2

Stiff-man Syndrome and Insulin Dependent Diabetes Mellitus: Similarities and Differences in Autoimmune Reactions.

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Stiff-Man Syndrome and Insulin-Dependent Diabetes Mellitus: Similarities and Differences in Autoimmune Reactions

H. Aanstoot, A. Michaels, S. Christgau, Y. Shi, J. Kim, and S. Baekkeskov

Hormone Research Institute and the Departments of Microbiology/Immunology and Medicine, University of California, San Francisco, California 94143-0534

Since the first description of stiff-man syndrome (SMS) in 1956, researchers have noted an association between this disease and type 1 or insulin-dependent diabetes mellitus (IDDM) (1). In particular, roughly 30% of SMS patients also develop IDDM. In 1966, Young suggested that autoimmune processes may be involved in the etiology of SMS. Subsequent research efforts revealed a link between SMS and some additional autoimmune diseases, and organ-specific autoantibodies were found in SMS patients (2,3). The recent discovery that the biosynthesizing enzyme for the inhibitory neurotransmitter γ-aminobutyric acid (GABA), glutamic acid decarboxylase (GAD), is the major target of autoantibodies in both SMS (4) and IDDM (5) shows that both diseases are characterized by a breakdown of tolerance to this antigen and suggests that they share some important etiological features. The two major sites of GAD expression are pancreatic β-cells, which are destroyed in IDDM, and GABAergic neurons, which are believed to be affected in SMS (5).

Despite the fact that SMS and IDDM share a common autoantigen, the clinical presentation of patients with these diseases is dramatically different. Furthermore, the prevalence of diabetes among SMS patients is markedly higher than the prevalence of SMS in diabetics. This chapter evaluates the specific features of autoimmunity to GAD in SMS and IDDM. Although GAD autoantibodies appear in both SMS and IDDM, there are major differences in the titer and epitope recognition between the two diseases. Moreover, the microenvironment of the sites of GAD expression in brain and pancreas may provide significantly different conditions for autoimmune reactions. On the basis of analysis of autoimmune features in SMS and IDDM, we propose that SMS is essentially an antibody-mediated disease whereas IDDM mainly

results from T-cell–mediated autoimmunity. Further elucidation of the autoimmune mechanisms involved in the etiology of SMS and IDDM will guide the development of diagnostic and possibly preventative and therapeutic strategies in managing these conditions.

AUTOIMMUNE MECHANISMS IN STIFF-MAN SYNDROME

Autoimmune Phenomena in SMS

Most case histories describing SMS patients have reported the presence of autoantibodies and autoimmune diseases, particularly islet-cell antibodies (ICA) and IDDM. McEvoy (6) reviewed 98 patients who fulfilled most or all of the diagnostic criteria for SMS. Approximately 30% of these patients had IDDM and 40% were diagnosed with an additional organ-specific autoimmune disease, including Hashimoto's thyroiditis, Graves' disease, myasthenia gravis, adrenal or ovarian failure, and/or pernicious anemia (6). In another survey of 33 SMS patients, 24 (73%) had organ-specific autoantibodies and 9 of these (27%) had clinical signs of autoimmune disease (4). Thus, although the majority of SMS patients have signs of autoimmune disorders, a significant fraction do not present these characteristics.

GABAergic Neurons and SMS

Clinical observations and neurophysiologic studies indicate that SMS is a disease of the CNS (7). In addition, neuropharmacologic investigations suggest that the disorder is associated with a dysfunction of the GABAergic system (8). Benzodiazepines and other agonists of GABAergic neurotransmission significantly relieve the muscle spasms and stiffness in SMS patients (9). These observations have focused attention on the GABAergic neurons as the site of dysfunction in SMS.

GAD Is an Autoantigen in a Subgroup of SMS Patients

Two studies by Solimena and colleagues (3,4) suggested a link between autoimmunity and pathology of GABAergic neurons in SMS. In a 1988 case report (3), the authors described a SMS patient who developed IDDM and had antibodies in serum and cerebrospinal fluid (CSF) that intensely stained GABAergic neurons in sections of rat cerebellum. The staining patterns were identical to that of an antiserum raised against GAD. Furthermore, Western blotting suggested that the serum and CSF contained antibodies to GAD. In subsequent work, it was found that 20 of 33 SMS patients tested positive for antibodies to GABAergic neurons (4). In these 20 subjects, GAD was

identified by Western blotting as the principal autoantigen. Interestingly, 18 of 19 GABAergic antibody-positive patients tested also had islet-cell cytoplasmic antibodies (ICCA) as measured by indirect immunofluorescence on frozen pancreas sections. Furthermore, 6 of the 20 GABAergic antibody-positive patients had IDDM. In contrast, in the 13 SMS patients negative for antibodies against GABAergic neurons, neither ICCA nor IDDM was reported (4).

Interestingly, when Solimena and colleagues tested 57 newly diagnosed IDDM patients without SMS for GAD antibodies, only 1 was found to be positive. However, the GAD antibody-positive diabetic patient was unusual in also having cerebellar ataxia. As discussed below, the reason for the negative results in this group of diabetic patients is that GAD antibodies associated with IDDM, in contrast to SMS, usually recognize only conformational epitopes in the molecule and were therefore not detected in the assays of Solimena and colleagues, which used partly or fully denatured protein for their analysis (4).

The results of autoantibody analysis in the SMS patients (4) appear to indicate that at least two groups of patients can be distinguished. One group has autoantibodies against GAD, often shows signs of organ-specific autoimmunity, and has a high incidence of IDDM. The second group does not have GAD autoantibodies or other signs of autoimmunity and has no increased risk for diabetes. It is possible that the lack of autoantibodies reflects the depletion of the autoantigen over time. Alternatively, SMS could represent at least two distinct etiologic processes. Analogous to IDDM, we propose to designate the autoimmune-associated group as type 1 SMS and the apparently non–autoimmune-associated group as type 2 SMS. Table 1 lists some observed differences that require further characterization to establish the two groups as separate entities. An additional example of SMS is the dominantly inherited congenital SMS described by Klein et al. (10) and Sander et al. (11). The two families described in these studies, as well as sporadic juvenile-onset SMS (12–14), require further analysis for autoimmune phenomena to determine whether they can be classified as either type 1 SMS or type 2

TABLE 1. *Possible differences between type 1 and type 2 stiff-man syndrome*

Clinical differences
Prodromal symptoms?
Objective differences in clinical examination?
Differences in age of onset?
Laboratory differences
No GAD- or organ-specific antibodies in type 2?
Time course of antibodies in type 1?
HLA haplotype differences?
Electrophysiological differences?
Pharmacological differences
Differences in response to drugs used in the treatment of SMS

SMS. Therefore, the pathophysiology of the GABAergic system and the identification of GAD as a principal autoantigen support the hypothesis that autoimmune processes are involved in the pathogenesis of at least a subgroup of SMS patients. The etiology of type 2 SMS, however, remains obscure.

AUTOIMMUNE MECHANISMS IN IDDM

IDDM Is an Autoimmune Disease

The most common autoimmune disease associated with SMS is IDDM. The components of the autoimmune processes in IDDM are fairly well established and involve both cellular and humoral immunity (15). Because GAD is an autoantigen in both type 1 SMS and IDDM, elucidation of the autoimmune mechanisms involved in pancreatic β-cell destruction may also lead to a clearer understanding of the etiology of SMS. In IDDM, the pancreatic β-cells are destroyed by an autoimmune process that extends over a period of time. The clinical symptoms develop only when ≥80% of the β-cells have been eradicated (16).

Genetic Susceptibility in IDDM

Several autoimmune diseases are associated with particular human major histocompatibility complex (MHC) (HLA) haplotypes. Examples of MHC–disease associations include HLA-DQw6 in pemphigus vulgaris (17), HLA-DR2 in multiple sclerosis (18), and HLA-DR3 and -DR4 in IDDM (19). Among Caucasian individuals with IDDM, 95% express HLA-DR3 and/or -DR4, compared with 40% of the general population (20). Furthermore, antigens expressed by the HLA-DQ locus demonstrate an even stronger association to either IDDM susceptibility or protection than HLA-DR (21).

Two mechanisms can be suggested to explain the strong association between IDDM and certain HLA types. In the first, T-lymphocyte receptors (TCR) recognize antigens in conjunction with HLA molecules. Therefore, certain HLA molecules may present an antigenic peptide to T-cells in such a way that an autoimmune reaction results (22). In contrast, the presentation of the antigen by other protective HLA molecules may result in deletion of autoimmune clones (23,24). An alternative explanation is that other genes closely linked to HLA genes may be the actual genes that predispose for IDDM. Candidate genes include the proteasome and peptide transporter genes recently located in the HLA class II region (25,26). These genes encode proteins that are likely to be involved in antigen processing and translocation of peptides into the endoplasmic reticulum, where they associate with HLA class I molecules (25,26).

Although specific HLA haplotypes are essential for the development of

IDDM, they are not in themselves sufficient. The most striking evidence comes from twin studies demonstrating a concordance rate of only 36% in monozygotic twins (27).

Environmental Factors in IDDM

Several lines of evidence suggest that environmental factors are important in the etiology of IDDM. There are differences in disease incidence between several countries that cannot be attributed solely to genetic factors (28). Thus, for example, studies in migrant populations show differences in incidence between the immigrant group and the native country (29). In addition, several areas in the world show a linear increase of IDDM over the past 15 to 20 years without major genetic drifts (30). Furthermore, seasonal variation strengthens the idea that environmental factors are important (31). The long preclinical phase of IDDM makes it difficult, however, to identify which environmental factors are critical in the initial events.

Viral infections, such as rubella and coxsackie B virus, are the strongest examples of environmental factors contributing to the etiology of IDDM. Diabetes is found in 20% of patients with congenital rubella syndrome, with a median onset age of 13 years. There is a strong association with the HLA haplotype (32). Evidence also argues for a possible role of coxsackie B viruses (33). The mechanisms involved are not clear. Molecular mimicry between a viral antigen and an autoantigen has been suggested as a possible mechanism initiating β-cell destruction and resulting in IDDM. A viral antigen with a homology to a self-antigen can stimulate T- and B-cells that cross-react and home to the site of expression of the autoantigen. For example, in a transgenic mouse model the targeting of a LCMV viral transgene to pancreatic β-cells did not result in autoimmunity and β-cell destruction despite low levels of tolerance to this protein. However, infection with the LCMV virus resulted in β-cell autoimmunity and destruction and the development of IDDM (34,35). The results of these transgenic experiments demonstrate that viral infections can recruit components of the immune system (e.g., cytotoxic T-cells) that can react with an otherwise ignored self-protein.

In conclusion, current evidence suggests that environmental events are involved in the etiology of IDDM. Despite a strong association between IDDM and several viral infections, specific mechanisms have not yet been identified.

Autoantibodies in IDDM and Identification of the 64-kDa Autoantigen

Among the most thoroughly studied autoimmune abnormalities associated with IDDM is the high incidence of circulating islet-specific autoantibodies. Autoantibodies can be identified at the time of onset but also years before

the clinical diagnosis of the disease. Autoantibody titers can be used as a predictive marker, but the specificity and sensitivity of most tests are not yet optimal.

In 1974, Bottazzo et al. (36) first described (ICCA) in IDDM using an indirect immunofluorescence assay and frozen sections of human pancreas. Insulin autoantibodies (IAA) have been identified in patients with IDDM both during the preclinical phase and at diagnosis (37,38). The frequency of IAA is age-dependent. In young children frequencies up to 40% can be found in newly diagnosed patients (39). In contrast, the incidence is less than 10% in older patients (40). ICCA are often present in sera from SMS patients (6), but no data are available on IAA in this disease.

In 1982, Baekkeskov and colleagues (41) identified autoantibodies to a 64-kDa protein by immunoprecipitation of detergent lysates of [^{35}S] methionine-labeled human islets. The 64-kDa antigen was found to be β-cell-specific in an analysis of several tissues in which neurons could not be included because of their poor biosynthetic labelling in vitro (42). The antigen was shown to have the properties of an amphiphilic membrane protein and to have an isoelectric point (pI) of 6.7 (43). Autoantibodies to the 64-kDa protein are present in 82 to 90% of newly diagnosed IDDM patients and in a majority of prediabetic individuals (see 44–47 for review). The presence of 64-kDa autoantibodies has a higher predictive value than ICCA or IAA (Aanstoot et al., unpublished results). Moreover, 64-kDa autoantibodies have been detected earlier but never later than ICCA or IAA in the preclinical phase of IDDM (45,46). The high incidence and very early appearance of 64-kDa autoantibodies suggest that the protein may represent the primary target protein of the initial autoimmune reaction against β-cells. The exact role of the 64-kDa protein in the β-cell destruction is, however, still unclear.

The characterization of the 64-kDa protein finally led to its identification as the enzyme GAD (5). Using the sensitive techniques developed for immunoprecipitation of the 64-kDa protein in its native configuration, we showed that there was complete immunological crossreactivity between the 64-kDa protein and GAD. The unequivocal identification of the 64-kDa protein as GAD furthermore involved demonstration of identical size and pI (6.7–6.9) of GAD forms in brain and the 64-kDa antigen forms in islets, identical trypsin-generated fragments of the two proteins, and finally, and perhaps most important, that the 64-kDa protein had GAD enzyme activity (5).

GAD AS A COMMON AUTOANTIGEN IN SMS AND IDDM

GAD in Neurons and β-Cells

GAD is the synthesizing enzyme for the inhibitory neurotransmitter GABA. Two distinct GAD genes located on different chromosomes have

been identified in neurons (48). One gene encodes a 67-kDa protein, GAD_{67}, while the other gene encodes a slightly smaller form, GAD_{65} (48). Immunohistochemical analyses of GAD in neurons suggest that GAD_{67} is predominantly present in the cell body of the neuron, whereas GAD_{65} is more concentrated in nerve endings (49). Immunoelectron microscopy (EM) has localized GAD in nerve endings in close proximity to the synaptic vesicles (50,51). Recent work with postembedding immuno-EM suggests that GAD is localized to the membrane of small synaptic vesicles (SSV) (52).

GAD in β-cells is expressed at levels comparable to those of GAD in the CNS (53). Pancreatic β-cells express two forms of GAD (5,54). The larger protein, which is expressed at low levels in rat islets, is hydrophilic and soluble throughout its lifetime and was shown to be identical to GAD_{67} in neurons (54). The major GAD protein in β-cells, alias the 64-kDa antigen, has been shown to be identical to GAD_{65} in neurons with regard to antigenic epitopes, size, and charge (54). A comparison of the primary structure of GAD_{65} in islets (55) and brain (56) shows that they are identical. GAD_{65} is also synthesized as a hydrophilic and soluble molecule but matures into a firmly membrane-anchored protein in a process that seems to involve two steps (54). The first step results in a hydrophobic molecule which is soluble or has a low membrane avidity. The second step results in a firmly membrane-anchored molecule. The exact subcellular localization of the membrane-anchored GAD_{65} in β-cells is not known, but EM analysis suggests that GAD_{65} is localized in the membrane of synaptic-like microvesicles (SLMV) containing GABA (52). Similar to neurons, β-cells contain other components of the GABAergic system, including GABA and the GABA-metabolizing enzyme GABA transaminase (GABA-T) (57). Although the function of GABA in islets is unclear, the identification of $GABA_A$ receptors on glucagon-secreting α-cells suggests that GABA has a paracrine role in islets (58).

Presentation of GAD to the Immune System

There are at least two mechanisms by which GAD could become visible to the immune system as a target for autoimmunity. First, peptide fragments generated from the GAD molecule during its natural turnover can be expressed at the cell surface in conjunction with MHC class I antigens and can thus be recognized by cytotoxic T-lymphocytes. Second, if membrane-bound GAD_{65} protrudes into the lumen of the SLMV the antigen may become exposed at the cell surface after fusion of the SLMV with the plasma membrane during GABA secretion and be recognized by GAD autoantibodies. The first mechanism would be applicable to both the GAD_{65} and GAD_{67} proteins, whereas the second mechanism would be applicable only to the membrane-bound form of GAD_{65} (54).

DIFFERENCES IN AUTOIMMUNE REACTIONS IN TYPE 1 SMS AND IDDM

The identification of a common antigen in type 1 SMS and IDDM suggests that the diseases may share common features. However, if GAD is a major target antigen in both type 1 SMS and IDDM, why do not all SMS patients with GAD antibodies develop IDDM and why do IDDM patients almost never develop SMS? Also, why in type 1 SMS patients do the clinical symptoms of diabetes usually develop after the onset of the SMS symptoms (6)?

It can be suggested that differences between the forms of GAD in brain and β-cells could account for differences in the etiology of SMS and IDDM. As described above, this possibility seems to have been excluded, although differences in posttranslational modifications of GAD proteins in brain and β-cells may still exist. There are some striking differences, however, in the character of GAD autoantibodies and in the target cell environment in the two diseases. These factors require further consideration and may play a significant role in disease etiology.

Differences in Antibody Reactions

Two major differences have been observed between GAD autoantibodies in type 1 SMS and IDDM (5). First, GAD autoantibodies in SMS are usually of a significantly higher titer than antibodies in IDDM. Second, autoantibodies in IDDM recognize only conformational and not primary epitopes on both GAD_{65} and GAD_{67}. In type 1 SMS, autoantibodies to GAD also recognize conformation epitopes in both GAD_{65} and GAD_{67}. However, in contrast to IDDM, SMS antibodies also recognize a primary epitope (or epitopes) in GAD_{65} which is absent in GAD_{67} (5).

Although our analyses suggest that type 1 SMS sera usually recognize denatured GAD_{65}, one report (59) describes serum from an SMS patient that recognized only conformational epitopes and not denatured forms of GAD in brain. Therefore, primary GAD_{65} epitope recognition may not be characteristic of all SMS patients. Serum from this individual stained GABAergic neurons and β-cells. The characterization of autoantibodies in type 1 SMS and IDDM is summarized in Table 2.

Differences in Target Cells

Neurons and β-cells differ significantly in their visibility to the immune system. First, the blood–brain barrier protects the microenvironment of the CNS. In addition, β-cells express MHC class I molecules (60) which can present GAD epitopes to cytotoxic T-cells. Under normal conditions, neurons do not express MHC class I antigens (61).

TABLE 2. *Comparison of GAD antibodies in IDDM and in type 1 and type 2 SMS*

	IDDM	Type 1 SMS	Type 2 SMS
Titers	1–10[a]	10–100[a]	N/D[c]
Primary epitope recognition (4,5)	No	Yes[b]	N/D[c]
Conformational epitope recognition (5)	Yes	Yes	N/D[c]
Frequency of GAD antibodies at onset (4,41,44,47)	80–90%	90–100%	N/D[c]
Before onset (45–47)	79–82%	N/A[d]	N/A[d]
Antibodies to GAD involved in pathogenesis	Probably not	Probably	No
Other organ-specific autoantibodies	Yes	Yes	No

[a] Arbitrary units.
[b] Recognition is restricted to primary epitopes in GAD_{65}.
[c] N/D, not detected.
[d] N/A, not available.

The destruction of β-cells in IDDM is believed to be T-cell–mediated (62). In SMS, few reports are available that describe the pathologic features of the neurons involved. Most studies found no histologic abnormalities and, in particular, there was no evidence of mononuclear cell infiltration (63,64). Nakamura and colleagues (65) have described an SMS patient with gliosis of the spinal cord, a feature that could be the result of a chronic inflammation. Unfortunately, histology of the brain was not interpretable. Complete destruction of GABAergic neurons due to an infiltration of mononuclear cells would be extremely devastating for several brain functions and would most likely be incompatible with life. Although the symptoms in SMS can be extremely severe and debilitating, the damage to GABAergic neurons seems to be more or less limited to a subgroup of GABAergic neurons in the motor neuron system. SMS symptoms can be influenced pharmacologically, suggesting a functional impairment rather than a complete destruction of GABAergic neurons.

HYPOTHESIS

If GAD is the primary target of pathogenic autoimmune processes in both diseases, we propose the following hypothesis for the distinct etiologies of type 1 SMS and IDDM based on differences in autoantigen presentation, characteristics of autoantibodies, and microenvironments of target cells. In IDDM, cytotoxic T-lymphocytes directed to or cross-reacting with GAD recognize GAD fragments in conjunction with MHC class I on the surface of β-cells and mediate β-cell destruction, resulting in disease. Antibodies to GAD, although a marker of the disease, play only a secondary role in the pathogenesis. Neurons are not affected because they are protected by the blood–brain barrier and do not express MHC class I antigens. Furthermore, the regulation of the immune response in the CNS is complex. Necessary

costimulatory mechanisms for T-cells may be lacking (66), and resistance or susceptibility to autoimmune processes may be related to the ability of brain endothelial and glial cells to express sufficient levels of MHC molecules to induce autoreactive T-cells (67). In type 1 SMS, however, autoantibodies of high titer develop which somehow pass the blood–brain barrier and recognize specific GAD epitopes on the surface of GABAergic neurons. These antibodies may mediate functional impairment of GABAergic neurons, perhaps by blocking the membrane-associated GAD_{65} form at the surface after exocytosis of GABA. Other examples of autoimmune diseases in which autoantibodies inhibit function of their target molecules include pernicious anemia (68), pemphigus vulgaris (69), and systemic autoimmune diseases such as scleroderma (70). The antibodies involved usually recognize antigenic epitopes that are highly conserved in evolution and often represent functionally important domains of the antigen (70). A pathogenic role of the antibodies in SMS cannot explain the high coincidence of IDDM. Rather, it can be proposed that diabetes develops in SMS patients when the antibody response is accompanied by cytotoxic T-cells to GAD which can home to the pancreas and destroy β-cells (71). In summary, we propose that if GAD is the primary target antigen in both type 1 SMS and IDDM, the first disease may be primarily mediated by humoral response to the protein, whereas cytotoxic T-cells predominate in the pathology of the second disease.

MOLECULAR MECHANISMS AND ROLE OF GAD AUTOIMMUNITY IN TYPE 1 SMS AND IDDM

Although GAD is identified as a major target of autoantibodies associated with β-cell loss and impairment of GABAergic neurons, some major questions remain to be answered. The first question is whether type 1 SMS is an autoimmune disease and, if so, whether it is associated with the same MHC haplotypes as IDDM. Although neurons lack MHC class I antigen expression under normal conditions, MHC class I antigen expression by neurons in vivo is induced during viral and bacterial infections of the CNS (72). Furthermore, the initial presentation of GAD to a T-helper cell would still involve MHC class II molecules. In the CNS, MHC class II antigens are expressed in astrocytes and perivascular microglial cells which, for example, can present myelin basic protein, the primary target autoantigen in the animal model of multiple sclerosis experimental autoimmune encephalomyelitis (73).

A few reports are available that describe the HLA typing of SMS patients (3,74,75). The DR3/DR4 haplotype has been found in some patients, and others are described as positive for the HLA-B44 allele which is in linkage disequilibrium with DR4. Thus, the limited data presently available suggest that the MHC haplotypes associated with IDDM may also be found in SMS patients.

A correlation between autoimmunity and infectious disease is established in some cases and serves as an example of environmental factors involved in disease. For example, in rheumatic fever, molecular mimicry between a streptococcal antigen and heart valve components results in an autoimmune destruction of the valves (76). The low incidence of SMS makes it difficult to assess the role of predisposing environmental factors such as infection. One report has described the development of a partial SMS after bacterial infection with *Borrelia burgdorferi*, the causative agent of Lyme disease (77). *B. burgdorferi* is associated with chronic neurologic dysfunction (78) but has been related to SMS only in this one case. Its role in SMS therefore remains obscure. Associations with viral infections have not been described for SMS.

If GAD autoantibodies in type 1 SMS are pathogenic, it can be expected that therapies that lower antibody levels will prove beneficial. One such method, plasmapheresis, has been tried with variable success in SMS patients (74,79). Brashear and Phillips (80) described one patient with progressive SMS who was treated with plasmapheresis. Serum antibody titers against GABAergic neurons were decreased by this treatment, and the patient improved. It is of note that this patient did not have GAD antibodies in the CSF. It is likely that high titers of GAD antibodies in the CSF are less amenable to plasmapheresis and that the benefit of this treatment is limited to patients with antibodies restricted to the blood circulation.

A second major question is whether the autoimmune responses to GAD are the consequence rather than the cause of β-cell destruction and neuronal degeneration. It is of note that in several neurologic diseases, neurons die and their contents become exposed to the immune system without initiating an autoimmune response to GAD (4,71). Furthermore, it can be argued that the release of the rare protein GAD from damaged β-cells or neurons is accompanied by the release of all other cell components, including both rare and abundant proteins. The appearance of IgG antibodies of a very high specificity and affinity to GAD but not those other components therefore strongly suggests that the immune system has been specifically triggered into activity towards GAD and that GAD autoimmunity plays a role in disease pathogenesis rather than being merely a consequence of cell death.

Animal models of GAD autoimmunity are presently being developed and will allow studies of the role of GAD in both β-cell destruction and impairment of GABAergic neurons.

CONCLUSIONS

The identification of GAD as the major target for humoral autoimmunity in type 1 SMS and IDDM allows a number of questions to be addressed for the first time. In IDDM, the development of rapid and accurate tests for GAD antibodies will help in prediction and diagnosis. In SMS, the analysis

of GAD antibodies will help in the diagnosis and further definition of different groups of patients.

The pathogenic mechanisms of both SMS and diabetes remain to be elucidated. The evaluation of the role of GAD in T-cell–mediated immunity in both diseases is a major goal. The differences in autoantigenic epitopes detected in type 1 SMS and IDDM are important and form the basis for further studies aimed at elucidation of the molecular mechanisms of β-cell destruction and impairment of GABAergic neurons. It is believed that the accumulation of knowledge in this area will eventually open up avenues for preventing or blocking the autoimmune responses to both β-cells and neurons.

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2.3. Studies on the characterization of GAD in ß-cells and neurons.

2.3.1

Pancreatic ß-cells Express Two Autoantigenic Forms of Glutamic Acid Decarboxylase, a 65-kDa Hydrophilic Form and a 64kDa Amphiphilic Form Which Can Be Both Membrane-bound and Soluble.

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Pancreatic β Cells Express Two Autoantigenic Forms of Glutamic Acid Decarboxylase, a 65-kDa Hydrophilic Form and a 64-kDa Amphiphilic Form Which Can Be Both Membrane-bound and Soluble*

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Stephen Christgau‡§¶, Helen Schierbeck§, Henk-Jan Aanstoot‡, Lissi Aagaard§, Kathi Begley‡, Hans Kofod§, Kim Hejnaes§, and Steinunn Baekkeskov‡§¶‖

From the ‡Department of Microbiology/Immunology, Department of Medicine, and Hormone Research Institute, University of California San Francisco, San Francisco, California 94143-0534 and the §Hagedorn Research Laboratory, Gentofte, Denmark

The 64-kDa pancreatic β-cell autoantigen, which is a target of autoantibodies associated with early as well as progressive stages of β-cell destruction, resulting in insulin-dependent diabetes (IDDM) in humans, has been identified as the γ-aminobutyric acid-synthesizing enzyme glutamic acid decarboxylase. We have identified two autoantigenic forms of this protein in rat pancreatic β-cells, a M_r 65,000 (GAD_{65}) hydrophilic and soluble form of pI 6.9–7.1 and a M_r 64,000 (GAD_{64}) component of pI 6.7. GAD_{64} is more abundant than GAD_{65} and has three distinct forms with regard to cellular compartment and hydrophobicity. A major portion of GAD_{64} is hydrophobic and firmly membrane-anchored and can only be released from membrane fractions by detergent. A second portion is hydrophobic but soluble or of a low membrane avidity, and a third minor portion is soluble and hydrophilic. All the GAD_{64} forms have identical pI and mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Results of pulse-chase labeling with [^{35}S]methionine are consistent with GAD_{64} being synthesized as a soluble protein that is processed into a firmly membrane-anchored form in a process which involves increases in hydrophobicity but no detectable changes in size or charge. All the GAD_{64} forms can be resolved into two isoforms, α and β, which differ by approximately 1 kDa in mobility on sodium dodecyl sulfate-polyacrylamide gel electrophoresis but are identical with regard to all other parameters analyzed in this study. GAD_{65} has a shorter half-life than the GAD_{64} forms, remains hydrophilic and soluble, and does not resolve into isomers. Comparative analysis of the brain and β-cell forms of GAD show that GAD_{65} and GAD_{64} in pancreatic β-cells correspond to the larger and smaller forms of GAD in brain, respectively. The expression of different forms and the flexibility in subcellular localization of the GAD autoantigen in β-cells may have implications for both its function and autoantigenicity.

Insulin dependent diabetes mellitus (IDDM)[1] is characterized by a selective loss of the insulin producing β-cells in a process which can span several years, and is characterized by clear indications of an autoimmune response, that includes circulating islet cell antibodies (Castano and Eisenbarth, 1990). We have used the circulating antibodies present in IDDM sera to identify a M_r 64,000 (64 kD) target human islet cell autoantigen by immunoprecipitation of [^{35}S]methionine-labeled human islets (Baekkeskov *et al.*, 1982). Antibodies to the human islet cell 64-kD protein and its rat islet counterpart are present in approximately 80% of newly diagnosed IDDM patients and have been detected up to several years before the clinical onset of the disease, concomitantly with a decrease of β-cell function (Sigurdsson and Baekkeskov, 1990 for review). The 64-kD protein was recently identified as the GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) in pancreatic β-cells (Baekkeskov *et al.*, 1990). The presence of GAD and its product GABA in islet β-cells (Garry *et al.*, 1986) and the presence of $GABA_A$ receptors on islet α- and δ-cells (Rorsman *et al.*, 1989; Reusens-Billen *et al.*, 1984) suggests a role of GABA in paracrine signalling between the β-cell and the other endocrine islet cells. GAD is expressed in high concentrations in GABA-ergic neurons in the central nervous system (Mugnaini and Oertel, 1985) and in the oviduct (Erdo *et al.*, 1989). Two major forms of GAD have been detected in the brain and their molecular masses have been described as 59–67 kDa (Kaufman *et al.*, 1991; Legay *et al.*, 1987; Chang and Gottlieb, 1988). The larger brain form has been cloned and sequenced from cat (Kaufman *et al.*, 1986; Kobayashi *et al.*, 1987), rat (Julien *et al.*, 1990; Wyborski *et al.*, 1990), and mouse (Katarova *et al.*, 1990). The smaller GAD form in rat brain has recently been cloned, sequenced, and shown to be a product of a different gene (Erlander *et al.*, 1991).

Both the human (Baekkeskov *et al.*, 1987; Baekkeskov *et al.*, 1989) and rat (Christie *et al.*, 1988, 1990) β-cell 64-kD GAD autoantigen have been shown to have amphiphilic membrane protein properties, and this characteristic has been used in most analyses to partially purify the protein. The amphiphilic human β-cell 64-kD GAD autoantigen (GAD_{64}) was detected as two isomers α and β, which were identical in charge but differed in molecular mass by approximately 1 kDa (Baekkeskov *et al.*, 1989). In the present study, we have

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¶ Current address: Hormone Research Inst., University of California, San Francisco, CA 94143-0534.

‖ To whom correspondance should be addressed.

[1] The abbreviations used are: IDDM, insulin-dependent diabetes mellitus; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; P-100, crude particulate fraction; S1-100, first cytosol fraction; S2-100, second cytosol fraction; TX-100, Triton X-100; TX-114, Triton X-114; WP-100, washed particulate fraction; Hepes, 4-(2-piperazine)-1-ethanesulfonic acid; 2D, two-dimensional; NEPHGE, nonequilibrium pH-gradient gel electrophoresis; IEF, isoelectric focusing; kb, kilobases.

analyzed autoantigenic forms of GAD in both soluble and membrane compartments of rat islets and characterized those with regard to size, charge, hydrophobicity, and half-life. We show that islets of Langerhans express a less abundant 65-kDa soluble hydrophilic form of GAD (GAD_{65}) in addition to GAD_{64} α and β, and provide evidence that only GAD_{64} becomes membrane bound in a process which involves modification with small noncharged hydrophobic residue(s). We also show that GAD_{65} and GAD_{64} are homologous to the larger and smaller brain forms of GAD, respectively.

MATERIALS AND METHODS

Isolation of Islets and Biosynthetic Labeling—Isolation of rat islets, maintenance in culture and radioactive labeling with [^{35}S]methionine for 4 h was carried out as described (Baekkeskov *et al.*, 1989). In pulse-chase labeling experiments, rat islets were starved in methionine-free RPMI 1640 medium at 37 °C for 30 min and then given a pulse of [^{35}S]methionine (specific activity, >1000 Ci/mmol, Amersham) for 30 or 40 min followed by chase periods in medium containing 5 × the normal content of nonradioactive methionine for 0–72 h before harvesting. Labeled islets were harvested by centrifugation, washed twice in nonradioactive medium and once in 20 mM Hepes, pH 7.4, 150 mM NaCl and 10 mM benzamidine/HCl, and then either immediately processed for homogenization and isolation of soluble and membrane compartments or snap frozen and stored in aliquots at −80 °C.

Analysis of Compartmentalization of the GAD Autoantigen—Islets were homogenized in an isotonic sucrose buffer, 10 mM Hepes/NaOH, pH 7.4, 0.25 M sucrose, 10 mM benzamidine/HCl, 0.1 mM *p*-chloromercuribenzenesulfonic acid and 0.25% aprotinin (Novo-Nordisk, Bagsvaerd, Denmark) (homogenization buffer) at 4 °C and separated into crude particulate (P-100) and cytosol fractions (S1-100) by ultracentrifugation at 100,000 × *g* for 1 h as described (Baekkeskov *et al.*, 1989). The crude P-100 fraction was washed at 4 °C by resuspension in 10 mM Hepes/NaOH (pH 7.4), 150 mM NaCl, 10 mM benzamidine-HCl, 0.25% Trasylol, 0.1 mM p-chloromercuribenzenesulfonic acid and 0.1 mM Na_3VO_4 (Hepes buffer A), followed by ultracentrifugation at 100,000 × *g* for 1 h resulting in a new S100 fraction (S2-100) and a washed P-100 (WP-100) fraction. The WP-100 fraction was extracted in Hepes buffer A containing 2% of either Triton X-100 (TX-100) or Triton X-114 (TX-114) (extraction buffer) at 4 °C for 2 h followed by ultracentrifugation as above.

For quantitative analysis of GAD, the resulting pellet was subjected to repeated extractions and ultracentrifugations. Usually all detergent releasable GAD was recovered in the two first extracts of the WP-100 fraction. The S1-100 and S2-100 fractions and the WP-100 extracts were immunoprecipitated quantitatively with GAD antisera (Christie *et al.*, 1988) either directly or after preclearance with control sera as described (Baekkeskov *et al.*, 1987). In brief fractions were incubated with an excess of serum for 16 h at 4 °C to form immunocomplexes, which were then isolated by adsorbtion to excess amounts of protein A-Sepharose (Pharmacia LKB Technology Inc., Uppsala, Sweden). Analysis of supernatants after immunoprecipitation by reimmunoprecipitation with antisera and protein A-Sepharose revealed that GAD was depleted quantitatively by the first immunoprecipitation. Immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. Densitometric scanning of fluorograms was carried out as described (Christie *et al.*, 1988). The results of density measurement of GAD in each fraction were expressed as percent of the sum of density values for GAD in all three fractions. Distribution of total protein into the three fractions was expressed as a percentage of trichloroacetic acid-precipitable counts. WP-100 values were expressed as the sum of GAD recovered in repeated extracts of the WP-100 fraction.

For salt and EDTA washing experiments identical aliquots of the WP-100 fraction were taken up in either Hepes buffer A or the same buffer supplemented with one of the following components: 0.5 M NaCl, 0.2 M Na_2CO_3, 0.05 M $Na_4P_2O_7$, 0.2 M $MgCl_2$, 0.05 M EDTA, or 2% TX-100 and incubated at 4 °C for 1 h. The samples were then ultracentrifuged at 100,000 × *g* and the resulting supernatants immunoprecipitated and subjected to SDS-PAGE and fluorography.

TX-114 Partitioning Assays—TX-114 detergent phase separations of detergent extracts of the WP-100 fraction or of soluble fractions following addition of TX-114 were performed as described (Baekkeskov *et al.*, 1989). For comparative analysis of TX-114 detergent phase partition of GAD in soluble and particulate fractions, S1-100, S2-100, and a WP-100 extract were each diluted with either extraction buffer or homogenization buffer, supplemented with TX-114 to obtain an identical buffer composition in each fraction during phase separation. Following TX-114 phase separation (Bordier, 1981; Baekkeskov *et al.*, 1987) the aqueous (a) and detergent (d) phases of each fraction were immunoprecipitated and analyzed by SDS-PAGE and fluorography. Distribution of GAD into a and d in each fraction was measured by densitometric scanning of fluorograms and expressed as a percentage of the sum of the values in both fractions.

Gel Electrophoretic Analysis—One-dimensional SDS-PAGE was performed using uniform 8.5 or 10% SDS-polyacrylamide gels and either the buffer system of Laemmli (1970) (0.055 M Tris base, 0.192 M glycine, and 0.1% SDS) or a modified Laemmli buffer system (0.025 M Tris base, 0.192 M glycine, 0.05% SDS) (Fey *et al.*, 1984). The GAD_{64} α and β components usually only resolved in the latter buffer system.

Two types of two-dimensional (2D) gel electrophoresis experiments were performed. The first involved NEPHGE (O'Farrell *et al.*, 1977) in the first dimension as described earlier (Baekkeskov *et al.*, 1989). This method has the advantage of applying the sample at the acidic end (anode) which results in less aggregates of GAD at the top of the gel than in the isoelectric focusing gels where proteins are applied at the basic end (cathode). However, this method does not allow determination of true isoelectric points. For this purpose equilibrium IEF was carried out in the first dimension in tube gels (170 × 1.55 mm (id)) as described by O'Farrell (1975) consisting of 5.8% v/v ampholines (pH 5–7) and 6.7% ampholines (pH 3.5–10) (Pharmacia). Following prefocusing at 1200 V for 4 h, the samples were subjected to electrophoresis for 20 h using 0.02 M NaOH at the anode and 0.01 M H_3PO_4 at the cathode. The pH gradient was determined by elution of the ampholines from 1-cm slices of gel and measuring the pH. The second dimension for both the NEPHGE and IEF gels was SDS-PAGE on 10% slab gels using modified Laemmli buffers (Fey *et al.*, 1984).

^{14}C-Methylated β—phosphorylase(molecular weight 94,000 and 100,000) bovine serum albumin (M_r 69,000), ovalbumin (M_r 45,000) and carbonic anhydrase (M_r 30,000) were used as M_r markers in the gels. Gels were stained and processed for fluorography as described (Baekkeskov *et al.*, 1987). Fluorograms were prepared using Kodak XAR-5 film.

Protein Determinations—Protein quantification was carried out according to Bradford (1976).

Immunoblotting—SDS gels were electroblotted to polyvinylidene difluoride membranes (Immobilon, Millipore). Unreacted binding sites were blocked by incubation for 60 min in 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20 containing 3% skim milk. The blots were incubated for 2 h with the primary antibody diluted in the same buffer (sera 1266 and normal rabbit serum, 1:1000; K2, 1:2000; GAD6 ascites 1:500) followed by visualization with alkaline phosphatase-labeled second antibodies.

Antisera—The antisera used in this study included three IDDM patient sera which were strongly positive for GAD autoantibodies and three control sera from healthy individuals; a polyclonal rabbit antiserum (1266) generated against a synthetic peptide prepared according to described methods (Atar *et al.*, 1989) and containing the C-terminal sequence of the larger rat brain form of GAD (Cys-Trp-Ser-Ser-Arg-Thr-Gln-Leu-Leu-His-Ser-Pro-Ile-Leu-Thr-Ser-Ser-Ser-Arg-Arg) (Julien *et al.*, 1990; Wyborski *et al.*, 1990) coupled to keyhole limpet hemocyanin (a gift from Dr. J. S. Petersen, Hagedorn Research Laboratory, Gentofte Denmark); GAD6, a mouse monoclonal antibody (ascites), which was raised against immunoaffinity purified rat brain GAD and which specifically recognizes the smaller GAD form in brain on Western blots (Chang and Gottlieb, 1988) (a gift from Dr. D. Gottlieb, Univ. of Washington, St. Louis), and K2, a rabbit antiserum raised to the larger form of cat GAD produced in a bacterial expression system and which almost exclusively reacts with the larger brain form on Western blots (Kaufman *et al.*, 1991) (a gift from Dr. Allan Tobin, UCLA).

Transient Expression of the Larger Form of Brain GAD in COS Cells—The eukaryotic expression vector 91023B (Wong *et al.*, 1985) (a gift by Dr. Randall Kaufman, Genetics Institute, Boston) was modified at the *Eco*RI cloning site with synthetic linkers to produce a *Spe*I site. This plasmid is called pEXP130. A plasmid containing a full-length cDNA clone encoding the larger rat brain form of GAD (a gift from Dr. A. Tobin, UCLA) was digested with *Xba*I to isolate a 3-kb fragment corresponding to nucleotides −250 to 2750. The 5′ 200 base pairs are intronic (an artifact in cDNA synthesis) but contain

no translational start sequence. The *Xba*I fragment was ligated to the *Spe*I site of pEXP130 and transformed using standard methods. Sense and antisense conformations were screened and named pGAD17 and pGAD16, respectively.

COS7 cells (American Type Culture Collection) were transfected using a lipofectin reagent (BRL) according to the manufacturer's protocol. 60 μg of lipofectin and 30 μg of either pGAD16 or pGAD17 were added to 100-mm dishes of COS7 cells at 80% confluency in GIBCO optimum medium supplemented with 20% FCS. Following 5-h incubation at 37 °C, an equal volume of Dulbecco's modified Eagle's medium with 20% fetal bovine serum was added. Cells were harvested after 48 h, snap frozen, and stored in aliquots at −80°C.

RESULTS

Analysis of Amphiphilic GAD$_{64}$ α and β in Membrane Fractions of Rat Islets—We have previously identified an amphiphilic 64-kD autoantigenic form of GAD (GAD_{64}) in human islets and showed that this form can resolve into two isomers α and β which have an identical pI of 6.7 and differ in molecular mass by about 1 kDa. The frequent inavailability of fresh human material has hampered further analyses and motivated the identification of alternative sources of islets for the characterization of this protein in pancreatic β-cells. Rat islets have been shown to express amphiphilic GAD_{64} in particulate fractions (Christie *et al.*, 1988, Baekkeskov *et al.*, 1989, 1990). To analyze this form in more detail we immunoprecipitated GAD_{64} from TX-114 detergent phase-purified extracts of crude particulate fraction (P-100) of a large number ($n = 88$) of rat islet preparations using GAD_{64} antibody-positive IDDM sera. GAD_{64} in particulate rat islet fractions was also detected as two isoforms, α and β, which differ in molecular mass by about 1 kDa (Fig. 1*A*) and have a pI of 6.7 (Fig. 1*B*), suggesting a significant homology between the human and rat GAD_{64} autoantigen. The α/β ratio differed among the preparations (Fig. 1*C*) and the β isoform was practically absent in approximately 25% of the preparations. The β-isoform did not differ from the α isoform in any discernible properties, except mobility on SDS-PAGE. Thus the α and β isoforms behaved identically with regard to subcellular distribution, hydrophobicity, membrane anchoring, and turnover time (see below). The β isoform may be derived from the α isoform or *vice versa*, either *de novo* or during isolation of GAD. For this reason we refer to the 64-kD pI 6.7 α/β doublet as one entity, GAD_{64}, unless otherwise indicated.

Detection of Amphiphilic and Hydrophilic GAD$_{64}$ but Only Hydrophilic GAD$_{65}$ in Soluble Fractions of Rat Islets—To analyze the distribution of the GAD autoantigen into soluble and membrane compartments, islets were homogenized and subjected to ultracentrifugation to prepare a primary cytosol (S1-100) fraction. The crude particulate fraction (P-100) was resuspended in an isotonic Hepes buffer to remove cytosolic proteins and proteins loosely associated to membranes, followed by ultracentrifugation to prepare a secondary cytosol fraction (S2-100) and a washed particulate fraction (WP-100). GAD was immunoprecipitated from the different fractions using GAD antibody-positive IDDM sera and analyzed by SDS-PAGE and fluorography.

Analyses of the soluble fractions of S1-100 and S2-100 showed that the expression of GAD in rat islets was not restricted to membrane bound compartments. Thus SDS-PAGE (Fig. 2, *A* and *B*) and 2D gel electrophoresis (Fig. 2*C*) showed the presence of the GAD_{64} form of pI 6.7 in both the S1-100 and S2-100 cytosol fractions in addition to the particulate fractions. No difference was detected in the mobility of GAD_{64} in the different compartments (Fig. 1*C*; Fig. 2). The analysis of the soluble and particulate fractions furthermore revealed an additional form of GAD of M_r 65,000 and pI 6.9–7.1 (GAD_{65}) in the S1-100 and S2-100 fractions (Fig. 2*A*, *lane 2*; Fig. 2*C*, *a* and *c*). GAD_{65} was absent in the WP-100 fraction (Fig. 2*C*, *d*). Electron microscopic analysis of the S1-100 and S2-100 fractions did not reveal the presence of small membrane vesicles or fragments (data not shown), suggesting that the GAD_{64} and GAD_{65} forms detected in these fractions were indeed soluble.

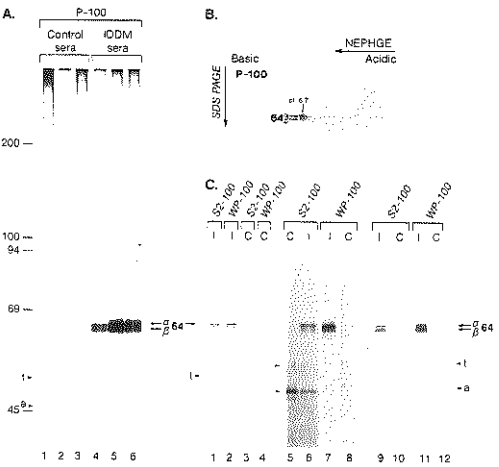

FIG. 1. **Analysis of the amphiphilic GAD$_{64}$ α/β.** *A* SDS-PAGE analysis of particulate amphiphilic GAD_{64} immunoprecipitated from rat islets by GAD antibody-positive sera from three IDDM patients (*lanes 4–6*). Immunoprecipitation with sera from three healthy control individuals is shown in *lanes 1–3*. [^{35}S]Methionine-labeled rat islets were homogenized in an isotonic Hepes/sucrose buffer and the homogenate centrifuged at 100,000 × *g* for 1 h. The pellet (P-100) was extracted in Triton X-114 and the lysate subjected to temperature-induced phase separation. The detergent-enriched phase was precleared with normal human serum and then immunoprecipitated. In addition to the positions of GAD_{64} α/β, the positions of actin (*a*) and tubulin (*t*), which were present as background in all immunoprecipitates, are indicated. The sera used in *lanes 4* and *5* were used in all subsequent analyses of GAD and sera in *lanes 1* and *2* as control sera. *B*, NEPHGE/SDS-PAGE analysis of particulate amphiphilic GAD_{64} prepared as in *A*. The pI of the α and β components was determined by a separate analysis using IEF/SDS-PAGE (not shown). *C*, SDS-PAGE analysis of amphiphilic GAD_{64} immunoprecipitated from either soluble (S2-100) or particulate (WP-100) fractions of three different rat islet cell preparations. The crude particulate fraction (P-100) from each islet preparation was washed by resuspension in Hepes buffer A, followed by centrifugation at 100,000 × *g* for 1 h to prepare a supernatant (S2-100) and a washed particulate fraction (WP-100). TX-114 detergent phase-purified material from each fraction was immunoprecipitated with either a control serum (*C*) or a GAD antibody-positive IDDM serum (*I*). The GAD_{64} α/β ratio varies between the three preparations shown in *lanes 1–4*, *5–8*, and *9–12*, respectively, but is similar for S2-100 and WP-100 in each preparation. Note that samples from S2-100 (*lanes 5* and *6*) and WP-100 (*lanes 7* and *8*) of one islet preparation were analyzed on two separate gels.

To analyze the hydrophobicity of GAD_{65} and GAD_{64} in the different fractions, the soluble fractions S1-100 and S2-100 and the particulate fractions were subjected to TX-114 phase separation. The distribution of GAD_{65} and GAD_{64} into the detergent and aqueous phases was analyzed by immunoprecipitation. This analysis showed that GAD_{65} was only present in the aqueous phase and never partitioned into the detergent phase demonstrating that this form is hydrophilic (Fig. 2, *A* and *B*). However, whereas GAD_{64} in both the S2-100, P-100, and WP-100 fractions partitioned into the TX-114 detergent phase (Fig. 2, *A* and *B*), GAD_{64} in the S1-100 fraction was

detected predominantly in the aqueous phase (see below), suggesting heterogeneity in hydrophilic/hydrophobic properties of GAD_{64} in the different compartments.

We considered that the difference in mobility between the α and β components of GAD_{64} might represent the membrane anchor, and therefore that the distribution of those components might differ between the soluble and particulate compartments. The α/β ratio was however identical in each compartment (Fig. 1*C*; Fig. 2*B* and results not shown). In the preparations where the β isoform was not evident, the α isoform was still found in the different compartments (Fig. 2, *A* and *C*). Thus the α and β isoforms of GAD_{64} show identical behavior with regard to compartmentalization.

As described earlier (Baekkeskov *et al.*, 1988, 1989) TX-114 detergent phase purification of the amphiphilic form of GAD_{64} efficiently eliminates background proteins in immunoprecipitates (Figs. 1 and 2). In contrast, several background proteins can be detected in immunoprecipitates of crude cellular fractions and in particular in the aqueous phases using either IDDM or control sera (Fig. 2*A*, *lanes 2–5*; Fig. 2*B*, *lanes 3,4, 6,7*). As shown elsewhere (Baekkeskov *et al.*, 1989) the background proteins represent a minor fraction of abundant cellular proteins carried nonspecifically through the immunoprecipitation procedure. Since GAD is a very rare protein in the crude extracts, a small fraction of an abundant cellular protein may be detected on a fluorogram similarly to the total amount of GAD present in this fraction, which is being specifically immunoprecipitated by IDDM sera.

In addition to GAD_{65} and GAD_{64}, a component of M_r 55,000 was sometimes specifically detected in immunoprecipitates with IDDM sera (Fig. 2*A*, *lanes 2* and *4*; Fig. 2*B*, *lanes 3* and *7*). This component was only detected in the aqueous phases following phase separation. Mild proteolytic digestion of both GAD_{65} and GAD_{64} with trypsin and other proteases results in a hydrophilic fragment of this size (Baekkeskov *et al.*, 1990).[2] Furthermore we have occasionally detected a 55-kDa GAD fragment in islet cell extracts which have not been treated with proteases. Thus GAD seems to be unusually susceptible to degradation resulting in a 55-kDa fragment.[3] It is therefore likely that the 55-kDa component detected in some immunoprecipitates with IDDM sera in this study represents a hydrophilic fragment of either GAD_{65} or GAD_{64} generated by proteolytic cleavage either *in vivo* or during preparation of immunoprecipitates.

GAD_{64} and GAD_{65} were quantitatively immunoprecipitated from the S1-100, S2-100, and WP-100 fractions in six independent experiments, and the relative distribution estimated by densitometric scanning of fluorograms and compared to the distribution of total proteins (Fig. 3). The fraction of GAD_{64} detected in S1-100 (~10%) was 4.5-fold lower than the fraction of total [^{35}S]methionine-labeled proteins in this fraction. In contrast, the fraction of GAD_{64} detected in the S2-100 fraction (~30%) was about 1.7-fold higher than the fraction of total [^{35}S]methionine-labeled proteins. This difference in fractionation suggests that GAD_{64} in the S2-100 fraction consisted not only of a truly soluble protein entrapped in membrane vesicles, but also of protein directly released from the membranes. The washed particulate (WP-100) fraction contained approximately 60% of the [^{35}S]methionine-labeled GAD_{64}, consistent with a membrane anchoring. Quantitative determination of GAD_{65} was more difficult due to an interfering background band in this area in some preparations of the particulate fraction. The values presented in Fig. 3, ~55% in S1-100, ~40 in S2-100, and 5% in WP-100 are therefore a

[2] K. Hejnaes and S. Baekkeskov, unpublished results.
[3] S. Christgau and S. Baekkeskov, unpublished observation.

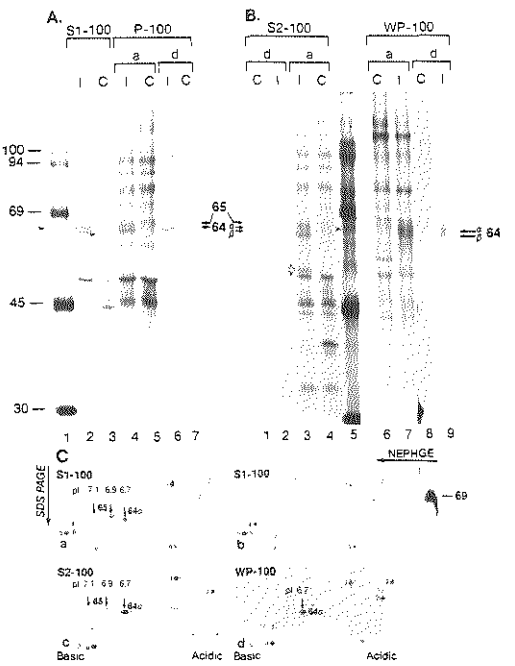

FIG. 2. **One- and two-dimensional gel electrophoretic analysis of GAD in membrane bound and soluble compartments.** *A*, SDS-PAGE analysis of GAD immunoprecipitated from S1-100 and P-100 fractions. Primary cytosol (S1-100) and a crude particulate (P-100) fraction were prepared and detergent phase (*d*) and aqueous phase (*a*) derived from the P-100 extract as described in Fig. 1, followed by immunoprecipitation with control (*C*) and IDDM (*I*) sera, SDS-PAGE analysis using modified Laemmli buffer, and fluorography. The immunoprecipitates in *lanes 2* and *3* represent S1-100 prepared from 3000 rat islets and the immunoprecipitates in *lanes 4–7* represent P-100 prepared from 1000 islets to obtain comparable signal intensities between the soluble S1-100 and particulate P-100 fractions. In addition to GAD_{64} α, the IDDM serum specifically immunoprecipitates a 65-kDa band (GAD_{65}) from S1-100 (*lane 2*). This band is also present in the aqueous phase of the P-100 fraction (*lane 4*), although it is not clearly distinguished from the background. Only GAD_{64} α is present in the detergent phase of the particulate fraction (*lane 6*). M_r markers at the positions indicated are shown in *lane 1*. The M_r marker with mobility between the GAD_{65} and GAD_{64} α (indicated with an *arrowhead* in *lane 1*) and the background band indicated with an *arrowhead* in *lane 3*, which has been shown to have mobility in between the GAD_{64} α and β components, were used together with the TX-114 phase distribution pattern to identify the two bands as GAD_{65} and GAD_{64} α as opposed to GAD_{64} α and β. 2D analyses confirmed that this preparation of islets lacked the GAD_{64} β (not shown). The background bands in immunoprecipitates of S1-100 and the aqueous phases represent a small fraction of major cellular proteins carried nonspecifically through the immunoprecipitation procedure. IDDM sera may, however, specifically recognize a weak 55-kDa band in *lanes 2* and *4* (see legend to Fig. 2*B*); *B*, SDS-PAGE analysis of GAD immunoprecipitated from aqueous and detergent phases prepared from S2-100 and WP-100 fractions as described in the legend to Fig. 1*B*. M_r markers are shown in *lane 5*. GAD_{65} is absent in WP-100 (*lanes 7* and *9*) but present in S2-100 aqueous phase (*lane 3*). GAD_{65} does not partition into the TX-114 detergent phase (*lane 2*). In addition to GAD_{65} and GAD_{64}, the IDDM serum specifically immunoprecipitates a 55-kDa band (*open arrow*) from the aqueous phases (*lanes 3* and *7*). This protein may represent a proteolytic fragment of GAD_{65} or GAD_{64}. *C*, NEPHGE/SDS-PAGE analysis of immunoprecipitates of S1-100 with IDDM serum (*a*) and a control serum (*b*) and of S2-100 (*c*) and WP-100 (*d*) with IDDM serum. The exact pIs of the GAD_{65} and GAD_{64} α/β components were

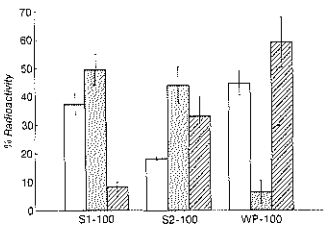

FIG. 3. **Quantitative analysis of the distribution of total cellular proteins, GAD_{65}, and GAD_{64} into soluble and membrane-bound islet cell compartments.** Portions of 2000 [^{35}S] methionine-labeled rat islets were subjected to homogenization followed by ultracentrifugation at 100,000 × *g* to fractionate S1-100 (300 μl) and P-100 fractions. The crude P-100 was resuspended in 300 μl of HEPES buffer A and ultracentrifuged to yield S2-100 and WP-100 fractions. WP-100 was extracted twice in 150 μl of extraction buffer containing 2% TX-100. S1-100, S2-100, and P-100 extracts were precleared twice by immunoprecipitation with normal serum and then divided in two aliquots and immunoprecipitated with GAD antibody-positive IDDM serum and a negative control serum, followed by resolution on SDS-gels in modified Laemmli buffers and fluorography. The distribution of GAD_{65} (*dark-shaded bars*), GAD_{64} (*shaded bars*), and total cellular proteins (*open bars*) into the three fractions was assessed as described under "Materials and Methods" and mean ±S.D. calculated from six experiments.

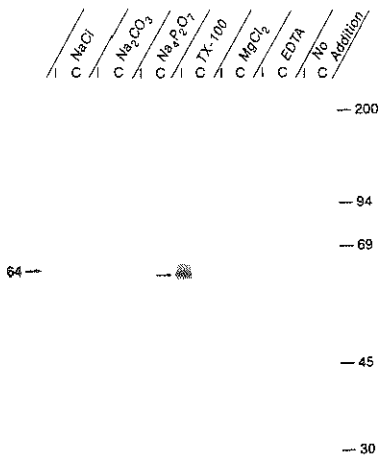

FIG. 4. **SDS-PAGE analysis of GAD_{64} released from membrane fractions in different conditions.** [^{35}S]Methionine-labeled rat islets were homogenized in homogenization buffer and separated into S1-100 and a crude P-100. The crude P-100 fraction was resuspended in Hepes washing buffer, aliquoted in seven equal portions and ultracentrifuged. Each WP-100 aliquot was extracted in Hepes washing buffer supplemented as indicated with salts, detergent, EDTA, or nothing, and the extracts were immunoprecipitated with GAD antibody-positive IDDM serum (*I*) and a negative control serum (C) followed by analysis of GAD_{64} in immunoprecipitates by SDS-PAGE in standard Laemmli buffers and fluorography.

rough estimate. Nevertheless they demonstrate that GAD_{65} is soluble and not membrane-anchored. Thus rat islets express two forms of GAD recognized by autoantibodies in IDDM, a soluble and hydrophilic form, GAD_{65}, of pI 6.9–7.1 and a second form, GAD_{64}, of pI 6.7 which is heterogeneous with regard to both subcellular localization and hydrophobicity.

A Major Portion of GAD_{64} Is Firmly Membrane-anchored—The results described above demonstrate that a subpopulation of GAD_{64} behaves as a soluble protein during traditional cell fractionation, and suggested that membrane-bound GAD_{64} might not be an integral membrane protein, but rather associated with the periphery of the membrane, such that it can be released during washing of the particulate fraction. To address this possibility, compounds which release peripheral membrane proteins were tested for their ability to release GAD_{64}. Aliquots of WP-100 fractions were incubated in Hepes buffer A supplemented with one of the following compounds: 0.5 M NaCl; 0.2 M Na_2CO_3 (pH 10.9); 0.5 M $Na_4P_2O_7$; 0.2 M $MgCl_2$; or 50 mM EDTA. In addition, the detergent TX-100 was used to release integral membrane proteins. After ultracentrifugation to remove insoluble material, the supernatants were analyzed for released proteins by immunoprecipitation and SDS-PAGE (Fig. 4). GAD_{64} in the WP-100 fraction was only released from the membranes in a significant manner by detergent, but not by any of the agents known to release peripheral membrane proteins. This result indicates that the hydrophobic GAD_{64} exists in two different forms with regard to cellular compartments. One form, found in the S2-100 fraction, is soluble or has a low membrane avidity. The other form, found in the WP-100 fraction, is firmly membrane-anchored and, in the presence of enzyme inhibitors (Hepes buffer A), can only be released from the membrane by detergent. Time-dependent spontaneous release of membrane anchored GAD_{64} from the WP-100 fraction can, however, take place in buffer compositions without enzyme inhibitors and may signify an endogenous enzyme capable of removing the membrane anchor of GAD_{64} and resulting in the amphiphilic but soluble S2-100 form.[3]

Differential Hydrophobicity of the Membrane-bound and Soluble Forms of GAD_{64}—A portion of GAD_{64} consistently remained in the aqueous phase even after repeated detergent extractions and phase separations. This distribution pattern might either be due to its size precluding quantitative partitioning into the detergent phase (Bjerrum *et al.*, 1983), or to a heterogeneity in the protein with regard to hydrophobic properties. As shown above, a large proportion of GAD_{64} in both the S2-100 and WP-100 fractions partitioned into the TX-114 detergent phase. To investigate whether the soluble and membrane-bound fractions of GAD_{64} differed in their amphiphilic properties, we analyzed their distribution between the two phases. The S1-100, S2-100, and WP-100 fraction were each subjected to a phase separation using identical buffer compositions in the three fractions. The distribution of GAD_{64} in the aqueous phase and detergent phase was analyzed by quantitative immunoprecipitation and SDS-PAGE. The relative quantity of GAD_{64} in the different phases was estimated by densitometric scanning of fluorograms from nine experiments and compared to the distribution of total cell proteins (Fig. 5). In all the experiments, no differences in the ratio between the α and β forms of GAD_{64} were detected between the aqueous and detergent phases. In the nine independent experiments, total cellular proteins in the S1-100 and S2-100 fractions behaved similarly, with ~10% and ~11% distributing into the detergent phase, respectively. As expected, a much higher proportion, ~55% of the total cellular proteins in the WP-100 fraction separated into the detergent phase. The corresponding figures for GAD_{64} in the detergent phases, as assessed after immunoprecipitation, were ~8% in the S1-100, and ~60% in the WP-100 fractions, respectively. The results show that the membrane-anchored and S2-100

determined by IEF/SDS-PAGE analysis in separate experiments (not shown). Background spots are labeled with small numerals to enable comparison between different panels.

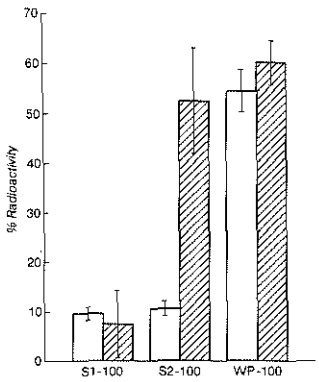

FIG. 5. **Quantitative analysis of the hydrophobicity of GAD_{64} in soluble and membrane-bound islet cell compartments by TX-114 partitioning.** S1-100, S2-100, and WP-100 fractions were prepared from portions of 2000 [^{35}S]methionine-labeled rat islets and subjected to phase separation as described under "Materials and Methods." The aqueous (*a*) and detergent (*d*) phases were subjected to quantitative immunoprecipitation with a GAD antibody-positive IDDM serum and a control serum followed by resolution of immunoprecipitates by SDS-PAGE in modified Laemmli buffers and fluorography. The density of bands on fluorograms was measured by densitometry. The *bars* show the percentage distribution of total cellular proteins (*open bars*) and the GAD_{64} autoantigen (*shaded bars*) into the detergent phase of S1-100, S2-100, and WP-100 fractions. Mean ±S.D. was calculated from the results of nine experiments.

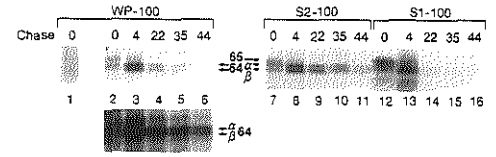

FIG. 6. **Turnover of GAD_{65} and GAD_{64} α/β in soluble and membrane-bound compartments.** Portions of 2000 islets were starved for 30 min in 2-ml methionine-free medium, the medium was decreased to 600 μl and the islets were labeled for 40 min with 1 mCi of [^{35}S]methionine. Islets were chased in medium containing five times the normal methionine content for the indicated number of hours and then washed and fractionated. S-100, S2-100, and an extract of WP-100 were immunoprecipitated and then analyzed by SDS-PAGE in modified Laemmli buffers and fluorography. *Lane 1* shows an immunoprecipitate with control serum, *lanes 2–16* with IDDM serum. The gel was fluorographed for 1 month to obtain an exposure suitable for densitometric analysis of GAD in the WP-100 fraction (*top panel*) and then refluorographed for 3.5 months to obtain an appropriate exposure of GAD in the S1-100 and S2-100 fractions. The WP-100 fraction in the long exposure is shown in the *lower panel* for comparison.

forms of GAD_{64} are more hydrophobic than GAD_{64} found in the S1-100 cytosol fraction. Furthermore, the membrane-anchored GAD_{64} and the S1-100 GAD_{64} each follow the general pattern of TX-114 partition characteristics in their respective compartments. The hydrophobic characteristics of GAD_{64} in the S2-100 fraction were, however, anomalous for that fraction, in that ~50% partitioned into the detergent phase. GAD_{64} residing in this fraction is either cytosolic or has been released from the membrane, and yet displays a TX-114 binding pattern resembling that of the protein in the particulate fraction. The data are compatible with the existence of three populations of GAD_{64} which differ with regard to compartment and hydrophobicity. One form is localized to the S1-100 fraction and is mainly hydrophilic. The second form, which is most concentrated in S2-100, has a significantly increased hydrophobicity compared to the S1-100 form and seems to have a low membrane avidity. The third form, which predominates in the WP-100 fraction, has similar hydrophobicity as the S2-100 form but differs in being tightly membrane-bound.

Pulse-chase Analysis of GAD_{65} and GAD_{64}—Islets were labeled for short periods and then subjected to chase periods of 1–48 h to assess the half-life of GAD_{65} and GAD_{64} in soluble and membrane-bound compartments. The results of one such experiment are shown in Fig. 6. Based on densitometric scanning of the autoradiogram shown in Fig. 6 and of three other experiments it was concluded that α and β of GAD_{64} had the same rate of turnover in each individual fraction, with the maximum incorporation at 4 h of chase. In contrast, GAD_{65} had a maximum incorporation at 0 h of chase, and had a shorter half-life (≤4 h) than the GAD_{64} α/β doublet. GAD_{65} was hydrophilic throughout the time course in that it did not separate into the detergent phase (data not shown). The half-life of GAD_{64} in the three different fractions S1-100, S2-100, and WP-100 was estimated to be about 6–10 h in the S1-100 fraction, about 22–28 h in the S2-100 fraction, and about 20–30 h in the WP-100 fraction. TX-114 phase separation of pulse-chase-labeled material showed that GAD_{64} in the S1-100 and S2-100 fractions displayed the same amphilicity pattern as demonstrated in the 4-h-labeled islets, *i.e.* the form predominating in S1-100 being hydrophilic and the S2-100 and WP-100 forms being amphiphilic (about 50% distributing into the detergent phase, data not shown). These results suggest that the GAD_{64} autoantigen in the β-cells is synthesized as a hydrophilic soluble form which predominates in the S1-100 fraction and then is processed into the hydrophobic forms seen in the S2-100 and WP-100 fractions by a maturation process that results in membrane anchoring for the WP-100 form.

Comparative Analysis of Brain and β-Cell Forms of GAD—We have shown that brain and β-cell forms of GAD have identical mobility by SDS-PAGE and identical patterns on two-dimensional gels (NEPHGE/SDS-PAGE) Baekkeskov *et al.*, 1990), suggesting that islets and brain express identical forms of GAD and that GAD_{65} and GAD_{64} in islets correspond to the larger and smaller forms of GAD in brain (Chang and Gottlieb, 1988), respectively. The smaller brain form has been cloned and sequenced recently and shown to be encoded by a different gene than the larger form (Erlander *et al.*, 1991). Thus in brain the two forms are clearly different and do not have a precursor-product relationship. To further assess the possible identity between the GAD forms in the two tissues, the brain and β-cell forms of GAD, as well as the larger brain form transfected and expressed in COS cells, were analyzed in parallel by immunoblotting using a set of distinctive antibodies: 1) An antibody (1266) raised against a C-terminal peptide in the larger brain form, which recognizes both forms of GAD in brain (Fig. 7, *lane 5*), in agreement with the homology between the two forms at the C terminus (Erlander *et al.*, 1991); 2) an antibody which preferably recognizes the larger brain form (the K2 antibody, Kaufman *et al.*, 1991); or 3) an antibody which is specific for the smaller brain form (the GAD6 antibody, Chang and Gottlieb, 1988). As shown previously (Baekkeskov *et al.*, 1990) the mobility of GAD_{65} and GAD_{64} in islet cells on SDS-PAGE was identical to that of the larger and smaller brain forms of GAD respectively (Fig. 7). The 1266 antibody recognized both GAD_{65} and GAD_{64} in β-cells (Fig. 7, *lane 6*). The K2 antibody specifically stained GAD_{65} in islets (Fig. 7, *lane 3*) in analogy with its staining of

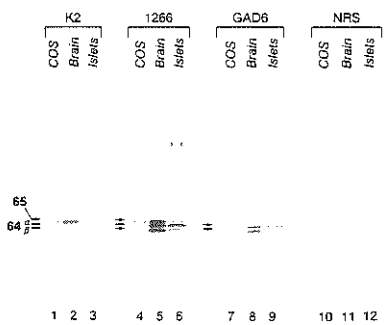

FIG. 7. **Comparison of islet and brain forms of GAD.** Western blot of a soluble fraction (S1-100 + S2-100) prepared from COS cells expressing the larger brain form of GAD (*lanes 1,4,7,* and *10*, 15 μg of protein per *lane*), from rat brain (*lanes 2,5,8,* and *11*, 25 μg of protein per *lane*), and from rat islets (*lanes 3,6,9,* and *12*, 25 μg of protein per *lane*). *Lanes 1–3* were probed with the K2 antiserum, which recognizes almost exclusively the larger form of GAD in brain (Kaufman *et al.*, 1991, *lanes 1* and *2*). This antibody specifically recognizes GAD_{65} and does not show detectable staining of GAD_{64} in islets (*lane 3*). *Lanes 4–6* were probed with the 1266 antiserum, which recognizes both forms of GAD in brain (*lanes 4* and *5*) and in islets (*lane 6*) and furthermore stains some background bands. *Lanes 7–9* were probed with the GAD6 ascites, which only recognizes the smaller form of GAD in brain (Chang and Gottlieb, 1988, *lane 8*). This antibody specifically binds GAD_{64} and does not react with GAD_{65} in islets (*lane 9*).

the larger brain form of GAD in both the COS expression system and brain tissue (Fig. 7, *lanes 1* and *2*). In contrast GAD_{64} and the smaller brain form were specifically recognized by the GAD6 antibody which did not stain GAD_{65} or the larger brain form (Fig. 7, *lanes 7–9*). Those results are consistent with GAD_{65} and GAD_{64} representing two distinct forms of GAD in pancreatic β-cells which are identical in size and antigenicity to the larger and smaller brain forms of GAD, respectively.

DISCUSSION

The 64-kDa autoantigen, which is a major target of autoantibodies associated with insulin dependent diabetes has been identified as the GABA-synthesizing enzyme glutamic acid decarboxylase in pancreatic β-cells (Baekkeskov *et al.*, 1990). In the present report we have used human autoantibodies to identify and characterize the different autoantigenic forms of this enzyme in membrane-bound and soluble fractions of pancreatic β-cells in steady state and pulse-chase-labeling experiments. Although GAD has been detected in both membrane bound and soluble compartments of brain (Chang and Gottlieb, 1988; Baekkeskov *et al.*, 1990) and β-cells (Baekkeskov *et al.*, 1990), this study represents the first detailed characterization of membrane bound and soluble forms of this enzyme.

The results show that pancreatic β-cells express two distinct forms of GAD, a larger form of M_r, approximately 65,000 (GAD_{65}), which is hydrophilic and soluble and has a pI of 6.9–7.1, and a smaller form of M_r, approximately 64,000, and pI 6.7 (GAD_{64}), which partitions between soluble and membrane bound compartments and is heterogeneous with regard to amphiphilicity. The pulse-chase analysis suggests that both GAD_{65} and GAD_{64} are synthesized as hydrophilic soluble molecules, which are predominantly found in the S1-100 fraction, and that only GAD_{64} is posttranslationally modified to become an amphiphilic molecule which can either be soluble or firmly membrane anchored. It is conceivable that GAD_{64} is modified by hydrophobic residues in a two-step process, which results in first a hydrophobic form which is either soluble or of a low membrane avidity and which is predominantly found in the S2-100 fraction and second, a firmly membrane-anchored form found in the WP-100 fraction. The second step may be reversible.[3] The modification of GAD_{64} is not accompanied by detectable changes in size or charge, suggesting that the modification is mediated by small hydrophobic noncharged residues. Based on those results we propose that the membrane anchoring is mediated by a small lipid or fatty acid(s).

2D gel electrophoretic analysis of the GAD forms in rat brain and islets showed that both tissues have a larger and a smaller GAD form of similar size and pI (Baekkeskov *et al.*, 1990). Based on the 2D gel electrophoretic analysis (Baekkeskov *et al.*, 1990) and the size and immunochemical comparisons of the brain and β-cell forms presented here, we conclude that the 65- and the 64-kDa β-cell form correspond to the larger and smaller brain forms of GAD, respectively. We have recently isolated cDNA spanning the entire amino acid coding region for GAD_{65} in rat islet cells. Sequencing of the cDNA[4] confirmed that the sequence of GAD_{65} in islets is identical to that of the larger GAD form in rat brain (Julien *et al.*, 1990; Wyborski *et al.*, 1990). The amino acid sequence of the GAD_{65} form does not contain membrane anchoring domains (Julien *et al.*, 1990; Wyborski *et al.*, 1990) in agreement with the soluble hydrophilic properties of GAD_{65} demonstrated in the present study. Similarly the amino acid sequence of the smaller rat brain form of GAD does not contain stretches of hydrophobic amino acids (Erlander *et al.*, 1991), in agreement with our results that GAD_{64} in islets is also being synthesized as a hydrophilic soluble molecule. The amphiphilic properties of the more mature GAD_{64} and the ability of this form to become membrane anchored, however, clearly distinguishes it from GAD_{65}, which remains hydrophilic and soluble throughout its lifetime.

What is the subcellular localization of GAD? In brain, electron microscopic studies suggest that GAD is present in proximity to or associated with the membrane of synaptic vesicles which contain GABA (Wood *et al.*, 1976). Immunogold electron microscopic analysis of pancreatic sections suggest that GAD is localized to the membrane of small vesicles in β-cells, which contain GABA and stain for the synaptic vesicle protein synaptophysin (Aanstoot *et al.*, 1991; Reetz *et al.*, 1991). It is thus conceivable that membrane bound GAD may become visible at the surface following fusion of GABA-containing vesicles with the plasma membrane during secretion. However the exact subcellular distribution of the two forms of GAD in brain and β-cells remains to be elucidated. The two forms of mammalian GAD are encoded by two distinct and unlinked genes (Erlander *et al.*, 1991). The reason why two different forms of GAD have evolved is unknown. It is conceivable that the differences in compartmentalization of the two GAD forms demonstrated in this study may affect their enzymatic functions and influence the intracellular routes of transport and secretion of their product, GABA. Furthermore the ability of the smaller β-cell form to be either membrane bound or soluble may reflect the possibility to control its amount in membrane compartments, a characteristic which may influence the visibility of the protein to the immune system.

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[4] A. Covacci and S. Baekkeskov, unpublished results.

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2.3.2
Membrane Anchoring of the Autoantigen GAD_{65} to Microvesicles in Pancreatic ß-cells by Palmitoylation in the NH2-Terminal Domain

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Membrane Anchoring of the Autoantigen GAD_{65} to Microvesicles in Pancreatic β-cells by Palmitoylation in the NH_2-Terminal Domain

Stephen Christgau,* Henk-Jan Aanstoot,* Helen Schierbeck,‡ Kathi Begley,* Søren Tullin,‡ Kim Hejnaes,‡ and Steinunn Baekkeskov*‡

*Hormone Research Institute and the Departments of Microbiology/Immunology and Medicine, University of California, San Francisco, California 94143-0534; and ‡Hagedorn Research Laboratory, DK 2820 Gentofte, Denmark

Abstract. Pancreatic β-cells and γ-aminobutyric acid (GABA)-secreting neurons both express the enzyme glutamic acid decarboxylase (GAD) which is a major target of autoantibodies associated with β-cell destruction and impairment of GABA-ergic neurotransmitter pathways. The predominant form of GAD in pancreatic β-cells, GAD_{65}, is synthesized as a soluble hydrophilic molecule, which is modified to become firmly membrane anchored. Here we show by immunogold electron microscopy that GAD_{65} is localized to the membrane of small vesicles which are identical in size to small synaptic-like microvesicles in pancreatic β-cells. The NH_2-terminal domain of GAD_{65} is the site of a two-step modification, the last of which results in a firm membrane anchoring that involves posttranslational hydroxylamine sensitive palmitoylation. GAD_{65} can be released from the membrane by an apparent enzyme activity in islets, suggesting that the membrane anchoring step is reversible and potentially regulated. The hydrophobic modifications and consequent membrane anchoring of GAD_{65} to microvesicles that store its product GABA may be of functional importance and, moreover, significant for its selective role as an autoantigen.

THE γ-aminobutyric acid (GABA)[1]-synthesizing enzyme glutamic acid decarboxylase (GAD) is expressed at comparable levels in GABA-secreting neurons and in pancreatic β-cells in the islets of Langerhans (Okada et al., 1976). GABA is a major inhibitory neurotransmitter in the CNS, whereas its function in islets remains elusive. The expression of $GABA_A$ receptors on the glucagon producing α-cells in islets (Rorsman et al., 1989) suggests that GABA may play a role in paracrine signaling within the islet. GAD has been identified as the 64-kD autoantigen of the β-cells (Baekkeskov et al., 1990), to which autoantibodies arise concomitant with β-cell destruction and the development of insulin-dependent diabetes mellitus (IDDM) (Baekkeskov et al., 1982, 1987; Atkinson et al., 1990). Furthermore, GAD in neurons is an autoantigen in stiff-man syndrome, a rare neurological disease characterized by a high coincidence with IDDM (Solimena et al., 1990). The reasons for the unusual susceptibility of the GAD molecule to becoming an autoantigen are unclear, and their elucidation is a major goal. Neurons express two GAD proteins encoded by two distinct genes. The proteins differ mainly in the first 100 amino acids, but share extensive homology in the remainder of the molecules (Erlander et al., 1991).

We have identified two GAD proteins in pancreatic β-cells (Baekkeskov et al., 1990; Christgau et al., 1991). The larger 65-kD protein, which is expressed at low levels in rat islets, is hydrophilic and soluble throughout its lifetime and was shown to be identical to the larger GAD protein in brain (Christgau et al., 1991). This protein is now called GAD_{67} (also called GAD-1 and previously GAD_{65}). The smaller 64-kD protein, which is the major GAD protein in β-cells, was shown to be identical to the smaller GAD protein in brain, with regard to antigenic epitopes, size and charge (Christgau et al., 1991). A comparison of the primary structure of the smaller GAD protein in islets (Karlsen et al., 1991) and the smaller GAD protein in brain (Bu et al., 1992) shows that they are identical. This protein is now called GAD_{65} (also called GAD-2 and previously GAD_{64}). GAD_{65} is also synthesized as a hydrophilic and soluble molecule but matures into a firmly membrane anchored protein in a process that seems to involve two steps (Christgau et al., 1991). The first step results in a hydrophobic molecule which is soluble or has a low membrane avidity. The second step results in membrane anchoring. Thus three distinct forms of

Steinunn Baekkeskov's current address is Hormone Research Institute and the Departments of Microbiology/Immunology and Medicine, University of California, San Francisco, CA 94143-0534. Correspondence should be addressed to Steinunn Baekkeskov.

1. *Abbreviations used in this paper*: GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; IDDM, insulin dependent diabetes mellitus; PIG, phospatidyl inositol glycan; PIPLC, phosphatidyl inositol–specific phospholipase C; RP-HPLC, reverse phase high pressure liquid chromatography; S1, First cytosol fraction; S2, second cytosol fraction; SMS, stiff-man syndrome; TX-114, Triton X-114; WP, washed particle fraction.

GAD_{65} can be detected in pancreatic β-cells, a hydrophilic soluble form, a hydrophobic form that is soluble or has a low membrane avidity, and a firmly membrane-bound form that can only be released from membranes by detergent, and thus has the characteristics of an intrinsic membrane protein. The maturation of GAD_{65} does not involve detectable changes in size or charge, which suggests that membrane anchoring may be mediated by a small lipid (Christgau et al., 1991). Although antibodies in many diabetic and stiff-man syndrome patients recognize both GAD_{67} and GAD_{65} in their native configuration, the incidence of antibodies to GAD_{65} is significantly higher than to GAD_{67} (our unpublished results). Furthermore, GAD_{65}, but not GAD_{67}, possesses a primary structure epitope or epitopes that are recognized by stiff-man syndrome antibodies but not by IDDM antibodies (Baekkeskov et al., 1990). Thus GAD_{65} has autoantigenic properties that clearly set it aside from GAD_{67} and that may be related to its subcellular targeting to membrane compartments. GAD is an intracellular enzyme, but its exact subcellular localization is not known. Immunohistochemical staining and confocal microscopy analysis of neurons and β-cells showed colocalization of GAD with GABA and synaptophysin, a marker protein for synaptic vesicles (Reetz et al., 1991). Such studies have also demonstrated the localization of GAD and GABA to a vesicular compartment, which is clearly distinct from the insulin secretory granules in β-cells (Sorenson et al., 1991; Reetz et al., 1991) and which was tentatively identified as synaptic vesicles in neurons and synaptic like microvesicles in β-cells (Reetz et al., 1991). Immunoperoxidase EM of brain sections and immunogold EM of brain and islet cell homogenates using GAD antibodies however did not show GAD immunoreactivity in synaptic vesicles but rather localized GAD to the cytoplasm and in the proximity of synaptic vesicles in neurons and around membranes of pleomorphic microvesicles of tubular and cisternal elements, as well as throughout the cytoplasm in pancreatic β-cells (Wood et al., 1976; Reetz et al., 1991). Furthermore, GAD could not be recovered on synaptic vesicles or synaptic-like microvesicles nor in other membrane compartments after subcellular fractionation (Reetz et al., 1991). The EM analysis and the subcellular fractionation data are consistent with the localization of GAD_{67} in the cytoplasm, but raise questions about the subcellular localization of the membrane bound GAD_{65}.

In this report we have analyzed the subcellular localization of GAD_{65} in intact islets by immunogold EM. Furthermore, we have characterized the hydrophobic membrane anchoring domain in the GAD_{65} molecule and addressed the question of whether GAD_{65} is modified by one or more of the lipid moieties which are known to mediate membrane anchoring of proteins, i.e., phosphatidylinositolglycan (PIG) polyisoprenyl groups, and/or fatty acids (Sefton and Buss 1987; Hancock et al., 1989; Casey et al., 1989). We present evidence that GAD_{65} is anchored to the membrane of vesicles, which are likely to be the synaptic-like microvesicles in β-cells, and that membrane anchoring is achieved by a post-translational hydroxylamine-sensitive palmitoylation in the NH_2-terminal region of the molecule where GAD_{65} differs significantly from GAD_{67}. The membrane anchoring is reversible, which may reflect the possibility of controlling the amount of the protein in membrane compartments.

Materials and Methods

Antibodies

S3, a sheep antiserum raised to purified rat brain GAD (Oertel et al., 1980), was a gift from I. Kopin (National Institutes of Health). The GAD6 mAb (Chang and Gottlieb, 1988) was a gift from D. Gottlieb (Washington University, St. Louis, MO). A polyclonal rabbit antibody (1266) raised against a synthetic peptide containing the carboxyl-terminal sequence of rat GAD_{67} (Cys-Thr-Gln-Ser-Asp-Ile-Asp-Phe-Leu-Ile-Glu-Glu-Ile-Glu-Arg-Leu-Gly-Gln-Asp-Leu) (Wyborski et al., 1990) was a gift from J. S. Petersen (Hagedorn Research Laboratory, Gentofte, Denmark). Sera from IDDM patients with high titers of GAD autoantibodies have been described earlier (Baekkeskov et al., 1989).

Isolation of Islets and Biosynthetic Labeling

Rat islets were used in all experiments described in this study except that biosynthetic labeling with ^{3}H-fatty acids was also carried out using human islets. Isolation of human and rat islets, maintenance in culture and radioactive labeling with [^{35}S]methionine for 4 h was carried out as described (Baekkeskov et al., 1989). For biosynthetic labeling with fatty acids, islets were washed twice by centrifugation in DME containing 16 mM glucose and supplemented with 5 mM Na-pyruvate, 5 mg/ml fatty acid free BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and nonessential amino acids (FA medium). Islets were labeled for 4 h at 37°C in the same medium containing either (9, 10) ^{3}H-palmitic acid or (9, 10) ^{3}H-myristic acid (50–60 Ci/mmol, Amersham Corp., Arlington Heights, IL) at 800 μCi/ml. In experiments to analyze effect of emetine in labeling experiments, islets were preincubated with 10 μM emetine followed by [^{35}S]methionine labeling for 4 h in the presence of 10 μM emetine. Similarly in experiments with mevinolin (a kind gift from Dr. A. W. Alberts, Merck, Sharp, and Dohme Research Laboratories, Rahway, NJ), islets were preincubated for 1 h with 10, 20, 30, 40, or 50 μM mevinolin and then labeled for 4 h with [^{35}S]methionine in the presence of the same concentrations of mevinolin. For biosynthetic labeling with ^{3}H-mevalonic acid, islets were preincubated for 1 h with 50 μM mevinolin in FA medium and labeled with ^{3}H-mevalono lactone (36 Ci/mmol, New England Nuclear DuPont Co., Wilmington, DE) at 250 μCi/ml for 4 h. Islets were also labeled with ^{3}H-myoinositol (90.5 Ci/mmol, Amersham Corp.), ^{3}H-ethanolamine (40 Ci/mmol, Amersham Corp.) and ^{3}H-glucosamine (32 Ci/mmol, Amersham Corp.) for 6 h in RPMI 1640 containing 16 mM glucose and supplemented with 1% FCS, 0.5% human serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and isotope at 200 μCi/ml. Labeled islets were harvested by centrifugation, washed twice in nonradioactive medium and once in 20 mM Hepes, pH 7.4, 150 mM NaCl and 10 mM benzamidine/HCl, and then either immediately processed for homogenization and isolation of soluble and particulate fractions or snap frozen and stored in aliquots at −80°C.

Expression of GAD_{65} in Sf9 Insect Cells

Recombinant GAD_{65} baculovirus vectors were constructed by ligating a 2.4-kb EcoRI fragment of rat GAD_{65} cDNA clone pGAD-92 (a kind gift from Dr. A. Tobin, UCLA) into the EcoRI site of the baculovirus transfer vector pVL1392 (Invitrogen, San Diego, CA). Clone pGAD-515 containing the sense orientation for GAD_{65} was used in a calcium phosphate cotransfection with wild-type Autographa californica nuclear polyhedrosis virus. Recombinant viruses were then isolated as described (Summers and Smith, 1987). For radiolabeling of GAD_{65} in insect cells, *Spodoptera frugiperda* Sf9 cells were infected with recombinant baculovirus. 48 h later the cells were labeled for 4 h with either ^{3}H-palmitate, ^{3}H-myristate, ^{3}H-mevalonolactone at 800 μCi/ml, or ^{35}S-methionine at 80 μCi/ml as described above for islets, except that labeling was in Grace medium.

Fractionation of Islets and Sf9 Cells

Separation of soluble and membrane-bound fractions was carried out by two methods described in detail previously (Christgau et al., 1991; Baekkeskov et al., 1990). The first method involves homogenization in an isotonic Hepes/sucrose buffer (10 mM Hepes/NaOH, pH 7.4, 0.25 M sucrose, 10 mM benzamidine/HCl, 0.1 mM p-chloromercuribenzene sulfonic acid and 0.25% Trasylol) (Christgau et al., 1991) followed by ultracentrifugation at

100,000 *g* or higher for 1 h to separate a crude particulate and a cytosol (S1) fraction. The crude particulate fraction is then resuspended in Hepes buffer A (10 mM Hepes/NaOH, pH 7.4, 150 mM NaCl, 10 mM benzamidine/HCl, 0.25% Trasylol, 0.1 mM P-chloromercuribenzene sulfonic acid and 0.1 mM Na_3VO_4) followed by ultracentrifugation at 100,000 *g* or higher for 1 h resulting in a new cytosol fraction, S2, and a washed particulate fraction, WP. The S1 fraction prepared by this method contains mainly GAD_{67} and the hydrophilic soluble form of GAD_{65}. The crude particulate fraction contains both a hydrophobic firmly membrane anchored GAD_{65} and hydrophobic GAD_{65} which is either soluble or has a low membrane avidity. The two hydrophobic GAD_{65} forms separate into the WP and S2 fractions, respectively (Christgau et al., 1991). The second method uses homogenization in a hypotonic Hepes buffer (10 mM Hepes/NaOH, pH 7.4, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM aminoethylisothiouronium bromide hydrobromide, and 0.2 mM pyridoxal 5'-phosphate (Hepes buffer B) (Baekkeskov et al., 1990), followed by ultracentrifugation at 100,000 *g* or higher. The hypotonic conditions result in release of most of the hydrophobic, soluble, or low membrane avidity GAD_{65} form into the cytosol and combine in one fraction the soluble forms of GAD_{65}. The membrane fraction contains the firmly membrane-anchored hydrophobic GAD_{65} form which can only be released by detergents.

Proteins were extracted from the particulate fractions with 1% Triton X-114 (TX-114) in either Hepes buffer A or B, as described (Baekkeskov et al., 1990; Christgau et al., 1991).

TX-114 Partitioning Assays

TX-114 detergent phase separations of detergent extracts of particulate fractions or of soluble fractions after addition of TX-114 were performed according to Bordier (1981) (Baekkeskov et al., 1989). For comparative analyses of the amphiphilic properties of GAD_{65} in different fractions, buffer compositions were adjusted to obtain identical conditions in each fraction.

Experiments with Phosphatidyl Inositol-specific Phospholipase C

The phosphatidyl inositol-specific phospholipase C (PIPLC) used for these experiments was either from *Bacillus thuringiensis* (a kind gift from Dr. M. Low, The Rockefeller University, New York) or *Bacillus cereus* (Boehringer Mannheim Corp., Indianapolis, IN). Islets were homogenized in isotonic Hepes buffer and the P-100 fraction washed twice by resuspension and ultracentrifugation followed by resuspension and incubation for 30, 60, or 120 min at 0, 13, 24, and 37°C in the presence or absence of PIPLC at concentrations ranging from 125 mU/ml to 5 U/ml. Experiments were performed using three different buffer compositions for washings of the P-100 fraction and the incubations with and without PIPLC. Hepes buffer C was 10 mM Hepes/NaOH, pH 7.4, supplemented with 0.25% Trasylol. Hepes buffer D was 10 mM Hepes/NaOH, pH 7.4, supplemented with 10 μg/ml pepstatin A, 20 μg/ml leupeptin, 10 μg/ml antipain, and 0.01% Trasylol. Buffer E was 10 mM Tris/HCl, pH 7.5, 0.25 M sucrose, 5 mM PMSF, 5 mM iodoacetate, 1 mM leupeptin, and 100 μM *N*-α-p-tosyl-L-lysine chloromethyl ketone. Hepes buffer A was used for control incubations.

Immunochemical Analysis and Gelelectrophoresis

SDS-PAGE, immunoprecipitation, and immunoblotting were performed as described (Baekkeskov et al., 1989, 1990; Christgau et al., 1991).

Chemical Analysis of the Fatty Acid Group

For reverse phase high pressure liquid chromotography (RP-HPLC) analysis of covalently bound fatty acids, ^{3}H-palmitate-labeled GAD_{65} was excised from SDS-gels and the gel pieces were incubated for 2 h at 30°C in 1.5 M NaOH in 20% methanol to release fatty acids followed by pentane extraction to remove neutral lipids. The reaction mixture was acidified with HCl and fatty acids extracted into pentane. The free fatty acids isolated from GAD_{65} and cold standards were derivatized to their corresponding phenacyl esters according to Wood and Lee (1983), and analyzed on an Altex C-18 ultrasphere reverse phase HPLC column. Bound material was eluted with a linear gradient of 75–100% acetonitrile. Absorbance at 242 nm and cpms were measured in the eluted fractions. For RP-HPLC analysis of total fatty acids in Sf9 cells, the membrane fraction was extracted with chloroform methanol, hydrolyzed, and analyzed as described above. Peaks were identified by comparison with the retention time for cold fatty acid standards carried along through the derivatization procedure.

Tryptic Cleavage of GAD

TX-114 detergent phase purified particulate and soluble fractions prepared in hypotonic Hepes buffer were diluted to 1% TX-114 and incubated with and without trypsin as described (Baekkeskov et al., 1990) followed by a renewed phase separation and analysis of aqueous and detergent phases by immunoblotting. To analyze the ability of trypsin to release GAD_{65} from membranes, aliquots of P-100 were prepared, washed two times in Hepes buffer B, resuspended in 200 μl of the same buffer, and incubated for 1 h at 25°C in the presence or absence of 4.0 U trypsin (Sigma Chem. Co., St. Louis, MO). The trypsin digestion was terminated by placing the samples on ice and adding 1/10 vol of 10 mM Hepes/NaOH, pH 7.5, 5 mM benzamidine/HCl, 5 mM EDTA, 5 mM $Na_4P_2O_7$, and 1/10 vol of 1 mg/ml soya bean trypsin inhibitor in water. Particulate material was sedimented by ultracentrifugation at 265,000 *g*. Released material and particulate extracts were subjected to TX-114 phase separation followed by Western blot analysis of GAD in the different fractions.

Spontaneous Release of GAD_{65} from Membrane Fractions

Islets were homogenized in 10 mM Hepes, 150 mM NaCl, pH 7.4 (Hepes buffer F). The P-100 particulate fraction was washed twice in the same buffer and separated into aliquots. Membrane aliquots were resuspended in either Hepes buffer F or in the same buffer supplemented with 0.1 mM PCMBS, 5 mM EDTA, 0.25% trasylol, 5 mM NaF, 10 mM benzamidine/HCl, 0.1 mM Na_3VO_4, pH 7.4, and incubated for 5, 15, 45, and 75 min at 25°C, respectively, followed by centrifugation at 265,000 *g* for 30 min and analysis of released material by Western blotting.

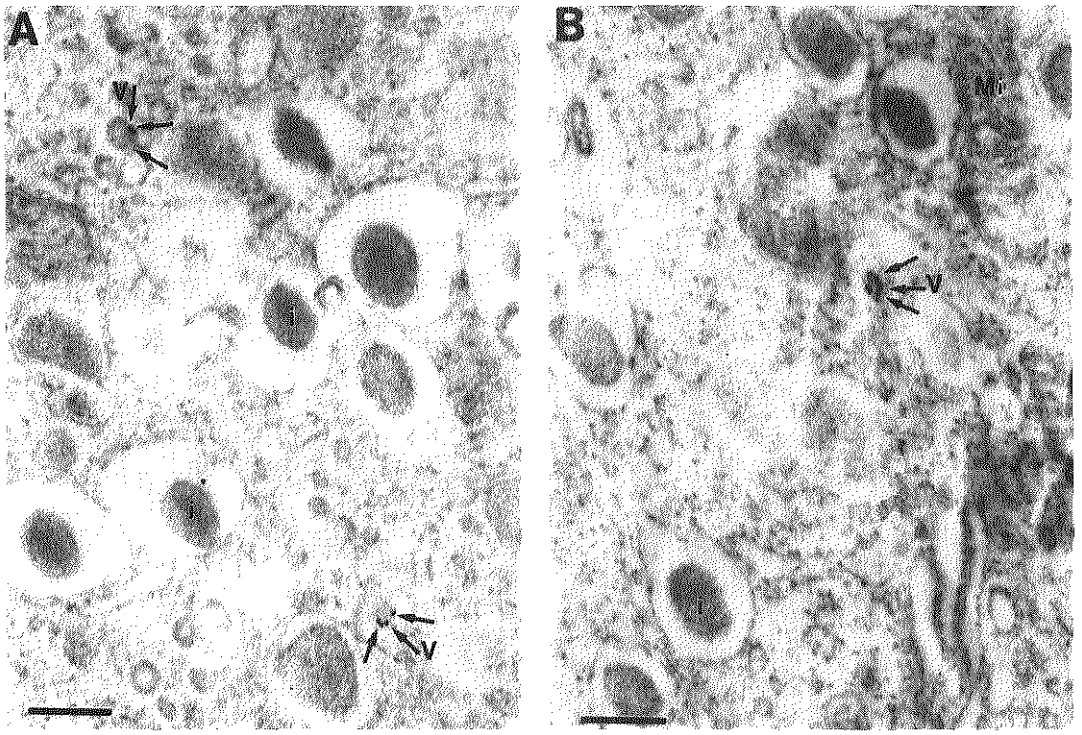
Electron Microscopy

Immunogold electronmicroscopy was performed on neonatal rat islets, which had been maintained in culture for 3–5 d. Islets were fixed for 6 h in 4% paraformaldehyde, 0.1% glutaraldehyde in 100 mM cacodylate buffer containing 0.2 mM $CaCl_2$, and 4% sucrose. Islets were postfixed for 15 min in 1% OsO_4, 1.5% ferricyanide in 0.1 M 2,4,6-trimethylpyridine and for 30 min in 2% aqueous uranyl acetate. Dehydration and embedding in LR White resin (medium hardness; Polysciences Inc., Warrington, PA) was performed as described (Newman et al., 1983). 50-nm sections were cut on a Ultracut E microtome (Reichard Jung, Deerfield, IL) and placed on grids. Antibody incubation was for 18 h with the GAD antiserum 1266 or S3 (dilution 1:100) followed by incubation with appropriate 10-nm gold-conjugated secondary antibodies (E-Y Labs., San Mateo, CA). Control grids were stained with normal sheep or rabbit serum followed by the secondary antiserum or with secondary antiserum only. Grids were coated with formvar and viewed on a JECL-100C transmission electron microscope at 60 kV. β-cells as well as non-β-cells (α-cells, exocrine cells) were viewed on each grid and subjected to quantitative analysis by counting the gold particles. α-cells and exocrine cells did not differ significantly in total gold particle counts and α-cells were selected as control cells for further subcellular analysis. Gold particles were counted in 19 β-cells on 19 different grids representing 4 different islet isolations and in 7 adjacent α-cells stained with the 1266 antibody. 1821 gold particles were detected in the β-cells, giving 95.8 ± 17 (mean ± SD) particles per β-cell. The α-cell counts revealed 226 particles or 32.3 ± 4.7 particles per α-cell. As a control six β-cells (227 particles, 41.3 ± 2.5 per β-cell) and five adjacent α-cells (165 particles, 33 ± 4.7 per α-cell) were counted on six grids stained with normal rabbit serum. Cells were divided into the following subcellular areas during the quantitative analysis: small vesicles of diameter 30–60 nm (49.6 ± 17.6, mean ± SD), ER/Golgi, other membranes (including plasma membranes and unidentified membranes), cytoplasma, nuclei, large dense core vesicles (insulin or glucagon secretory vesicles), mitochondria/other (other = unidentified subcellular structures). The mean values for each fraction obtained with the 1266 serum were corrected by subtraction of mean values obtained with the nonimmune serum and the pooled standard deviations calculated.

Results

GAD_{65} Is Localized to the Membrane of Small Vesicles

To analyze the subcellular localization of GAD_{65}, sections of fixed and embedded rat islets were sectioned and sub-



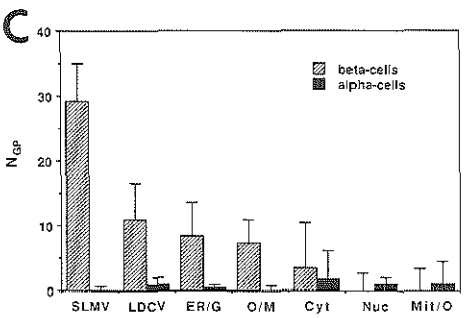


Figure 1. Electron micrographic analysis of GAD expression in pancreatic islet cells. (*A* and *B*) Micrographs of β-cell areas showing the localization of GAD by an immunogold labeling technique. The sections shown in *A* and *B* were stained with the S3 antiserum to GAD. Some microvesicles with a clear core (*V*) show GAD staining in the membrane. Insulin-containing secretory granules (*i*) are characterized by a typical halo around a dense granule core. *Mi*, mitochondrion. (*C*) Quantitative analysis of the localization of GAD in different subcellular areas of β (*black bars*) and α-cells (*shaded bars*) on electronmicrographs subjected to immunogold labeling using the 1266 antiserum to GAD. N_{GP} is the corrected number of gold particles obtained by subtracting nonimmune serum values from those produced with the GAD specific 1266 antiserum as described in Materials and Methods. SD is indicated on the bars. *SMLV*, small vesicles 30–60 nm in diam; *LDCV*, large dense core vesicles which secrete insulin or glucagon; *ER/G*, ER and Golgi membranes; *O/M*, other nonidentified membranes and plasma membranes; *Cyt*, Cytoplasm; *Nuc*, Nuclei; *Mit/O* Mitochondria and nonidentified subcellular structures. Bars, 240 nm.

jected to immunogold staining using both the S3 antiserum, which has a high immunoreactivity to GAD_{65} but reacts only weakly with GAD_{67} in immunoprecipitation experiments and on Western blots (results not shown), and the 1266 antiserum, which recognizes both forms (Christgau et al., 1991). The specificity of EM immunogold labeling of fixed and embedded tissue with both antisera was high and the sensitivity low (Fig. 1). The predominant immunolabeling for GAD with either antiserum was in the membrane of small vesicles (30–60 nm in diam) in β-cells (Fig. 1, *A–C*). The specificity of this localization is illustrated by the number of gold particles detected in this compartment in β-cells immunolabeled with the 1266 antiserum (29.7 ± 6.6 particles/cell, mean ± SD) which was ~60-fold higher than the labeling of comparable small vesicles in α-cells (0.4 ± 0.7 particles/cell), and 60–75-fold higher than nonspecific labeling of small vesicles in β- and α-cells produced with a nonimmune serum (0.5 ± 0.8 particles/cell and 0.4 ± 0.5 particles/cell, respectively). The gold particles in β-cells labeled with nonimmune serum and in α-cells labeled with

either GAD antiserum or nonimmune serum were concentrated in the cytoplasm, mitochondria, and nuclear fractions. The results of a quantitative analysis of electronmicrographs immunolabeled with the 1266 antiserum and corrected for background immunolabeling with nonimmune serum (Fig. 1 *C*) indicate that ~50% of the specific immunolabeling for GAD in β-cells is localized to the membrane of the small vesicles, 18% is found in insulin secretory granules, 14% in ER/Golgi membranes, 12% in other membranes (unidentified membranes and plasma membranes), and 6% in the cytoplasm. The low level of specific GAD immunolabeling in the cytoplasm of β-cells is consistent with the low level of cytosolic GAD_{67} protein in these cells and with the majority of the GAD_{65} protein being membrane associated (Christgau et al., 1991). The specific GAD immunolabeling in insulin granules was detected in the perigranular space but not in the dense insulin core. The gold particles sometimes formed characteristic circular structures of 30–60 nm in diameter just inside the insulin secretory granule membrane (results not shown) suggesting that they represent small vesicles that have fused with the insulin granule. This result, which merits further investigation, suggests that the small GAD-positive vesicles can fuse with and perhaps release GABA into the insulin secretory granules. The specific GAD immunolabeling in all compartments was restricted to β-cells. Thus the number of gold particles in α-cells (Fig. 1 *C*) and exocrine cells (results not shown) was not significantly different from background immunolabeling with nonspecific serum, which is consistent with the β-cell specific expression of GAD (Okada et al., 1976; Baekkeskov et al., 1990; Sorensen et al., 1991). The results provide evidence that GAD_{65} is predominantly localized to the membrane of microvesicles in the pancreatic β-cell. The small vesicles are clearly distinct from the insulin granules in those cells (Fig. 1) and are likely to be the synaptic-like microvesicles containing GABA which have been described in β-cells (Reetz et al., 1991). This result raises questions of the mechanism by which this subcellular localization is achieved. The following sections characterize the membrane anchoring of GAD_{65}.

Palmitoylation Distinguishes Membrane-bound and Soluble Forms of GAD_{65}

Depending on the conditions by which islets of Langerhans are homogenized and separated into soluble and particulate fractions, hydrophilic soluble GAD_{65} (S1-form) and hydrophobic soluble or low membrane avidity GAD_{65} (S2-form) can either be released simultaneously (in hypotonic Hepes homogenization buffer), or sequentially (in isotonic Hepes/sucrose homogenization buffer followed by resuspension of a crude particulate fraction in an isotonic Hepes buffer and resedimentation of membranes to form WP). The hydrophobic membrane-anchored GAD_{65} (WP-form) can, in both conditions, only be released from the particulate fraction by detergent (Christgau et al., 1991 and results not shown). Two-dimensional gel electrophoretic analyses of the membrane-anchored and soluble GAD_{65} forms has not revealed any differences in size or charge between the different forms (Christgau et al., 1991), suggesting that the hydrophobic modification and membrane anchoring is mediated by a fatty acid or a small neutral lipid.

To assess the possibility that GAD_{65} contains fatty acids either covalently linked to the protein backbone or as a part of a larger lipid structure, human and rat islets were labeled for 4 h with either ^{3}H-palmitic acid or ^{3}H-myristic acid at the same specific activity. Islets were homogenized in isotonic conditions and the two cytosolic fractions, S1 and S2, and the washed WP were prepared. GAD_{65} was isolated by immunoprecipitation and analyzed by SDS-PAGE and fluorography (Fig. 2). The incorporation of radioactivity was detected in GAD_{65} immunoprecipitated from detergent lysates of whole human islets (results not shown) and only in the washed membrane fractions but not the cytosolic S1 and S2 fractions of rat islets labeled with ^{3}H-palmitic acid (Fig. 2). Incorporation of radioactivity into GAD_{65} was not detected in experiments with ^{3}H-myristic acid (results not shown). The absence of label in background bands and in soluble forms of GAD_{65} in the immunoprecipitates from ^{3}H-palmitic acid–labeled material (Fig. 2) suggested that the incorporation of tritium into membrane-bound GAD_{65} was not due to conversion of label to radioactive amino acids. Thus the results suggest that palmitic acid itself or a lipid derivative of palmitate is covalently linked to the membrane anchored but not the soluble forms of GAD_{65} and is involved in the membrane anchoring of the enzyme.

Palmitoylation of GAD_{65} Is a Posttranslational Event

Palmitoylation of some cellular proteins is greatly reduced when protein synthesis is inhibited, indicating that acylation in these cases is cotranslational (Magee and Courtneidge, 1985; Olson et al., 1985). In contrast, acylation of other proteins, including p21 N-ras (Magee et al., 1987), GAP 43 (Skene and Virag, 1989), and others (Olson and Spizz, 1986), is unaffected by these inhibitors, showing that acylation of these proteins is posttranslational. To assess whether the incorporation of radioactivity into membrane-anchored GAD_{65} in the presence of ^{3}H-palmitic acid is dependent on protein synthesis, we analyzed the effect of emetine, an inhibitor of protein biosynthesis (Fig. 2 *B*). The presence of 10 μM emetine did not inhibit incorporation of radioactivity into membrane-anchored GAD_{65} in labeling experiments with ^{3}H-palmitic acid (Fig. 2 *B*, compare lanes *4* and *7*), despite inhibiting [^{35}S]methionine incorporation >90%. This result shows that the labeling of the membrane-anchored GAD_{65} in the presence of ^{3}H-palmitic acid is a posttranslational event independent of protein synthesis and excludes the possibility that the protein is labeled by biosynthetic incorporation of ^{3}H amino acids derived from palmitate. The data do not, however, exclude the possibility that GAD_{65} is modified posttranslationally by a lipid derivative of palmitate.

GAD Is Palmitoylated in Sf9 Insect Cells

Sf9 insect cells have been shown to correctly modify expressed proteins with both palmityl (Lanford, 1988), myristyl (Gheysen et al., 1989), and isoprenyl moieties (Khosravi-Far et al., 1991). Since GAD_{65} is a rare protein in pancreatic β-cells (Baekkeskov et al., 1989), and since the autoradiographic detection of ^{3}H is very insensitive, we assessed whether recombinant GAD_{65} expressed at high levels in Sf9 cells could be used to further characterize ^{3}H-palmitate–labeled GAD_{65}. GAD_{65} was distributed in soluble and membrane-bound fractions in Sf9 cells, much as in pancreatic

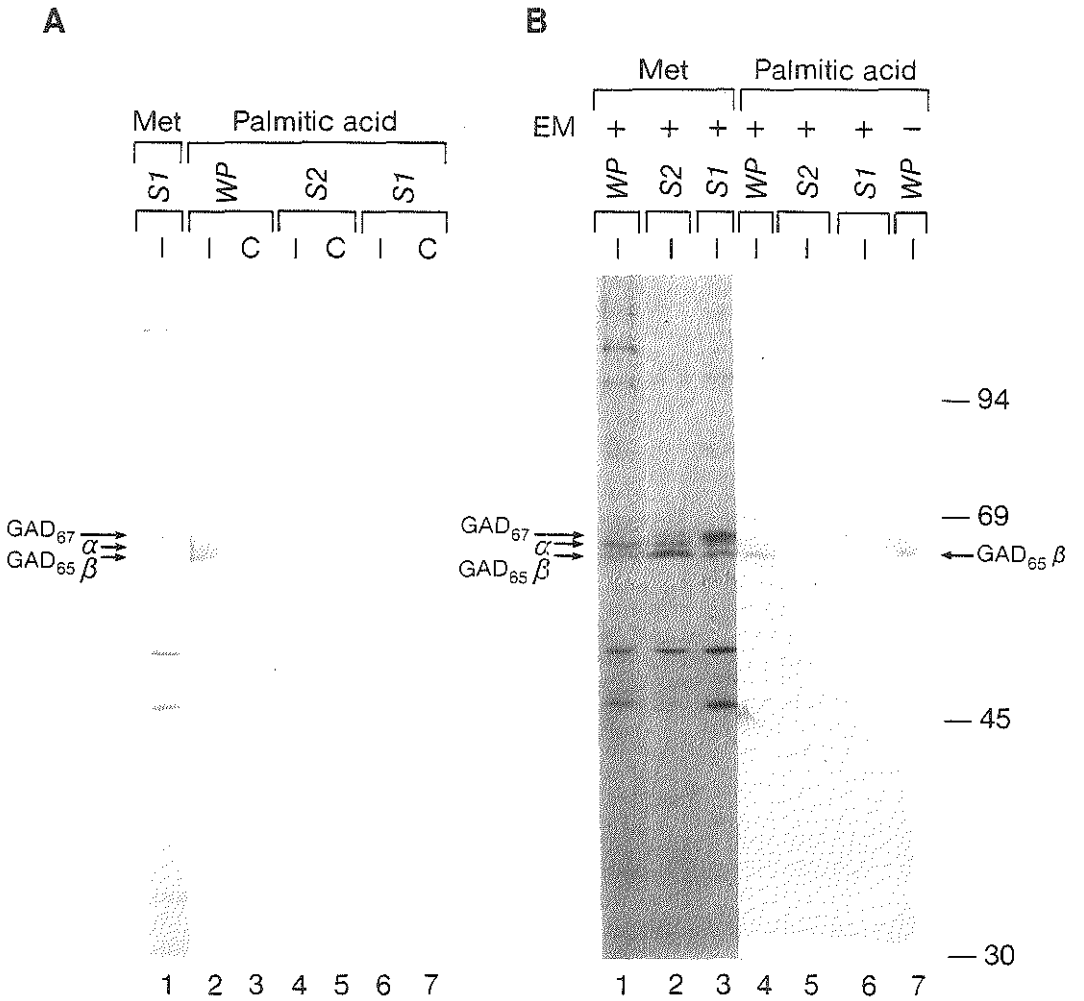


Figure 2. Posttranslational incorporation of palmitic acid into membrane-bound but not soluble forms of GAD_{65}. (*A*) Radioactive labeling of membrane bound GAD_{65} in the presence of ^{3}H-palmitic acid. Rat islets were labeled in the presence of ^{3}H-palmitic acid for 4 h and subjected to homogenization in isotonic conditions to prepare a cytosolic (*S1*) and a crude membrane fraction. The crude membrane fraction was washed by resuspension and centrifugation resulting in a supernatant (*S2*) containing mainly cytosolic proteins and WP fractions. The S1 fraction (lanes *6* and *7*), was concentrated fivefold and subjected to immunoprecipitation together with 2/3 of the S2 (lanes *4* and *5*) and 1/3 of the WP fraction (lanes *6* and *7*) to obtain similar levels of GAD_{65} in each fraction. Immunoprecipitation was performed first with control serum (*C*) (lanes *3*, *5* and *7*) and then with IDDM serum (*I*) (lanes *2*, *4*, and *6*). Immunoprecipitates were analyzed by SDS-PAGE in modified Laemmli buffers followed by fluorography. A short exposure to visualize [^{35}S]methionine-labeled GAD_{67} and GAD_{65} components immunoprecipitated from the S1 fraction of 1,000 islets is shown in lane *1*. Note that GAD_{65} splits into two components α and β, which have been shown to be identical with regard to all parameters analyzed except mobility on SDS-PAGE. (*B*) Effect of emetine on incorporation of radioactivity into membrane-bound GAD_{65} in the presence of palmitic acid. Rat islets were preincubated in FA medium either with or without 10 nM emetine for 1 h and then labeled with ^{3}H-palmitic acid in the same medium for 4 h. Aliquots of rat islets were incubated in parallel with or without emetine and labeled with [^{35}S]methionine. S1, S2, and WP fractions were prepared, immunoprecipitated with IDDM serum (*I*), and analyzed by SDS-PAGE in modified Laemmli buffers and fluorography. Fluorography shown for [^{35}S]methionine-labeled proteins was for 14 d with emetine (lanes *1*, *2*, and *3*), at which time lanes without emetine were completely black (not shown). Fluorography for ^{3}H-labeled proteins with (lanes *4–6*) and without emetine (lane *7*) was for 3 mo. Note that in this experiment the GAD_{65} β component is more pronounced than α in lanes 2, 3, 4, and 7. The position of molecular mass markers is indicated in kilodaltons.

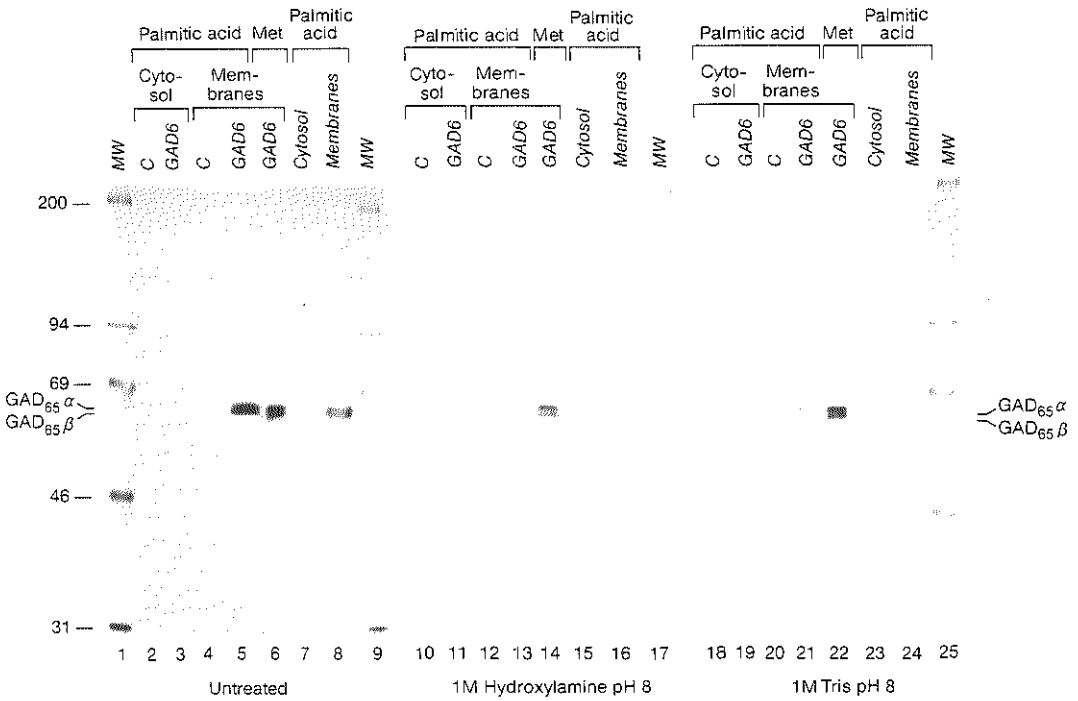


Figure 3. Cellular localization and hydroxylamine sensitivity of ^{3}H palmitate labeled GAD_{65} in Sf9 cells. Sf9 cells were infected with baculovirus containing the rat GAD_{65} cDNA and grown in the presence of either [^{35}S]methionine or ^{3}H-palmitic acid and separated into a cytosolic and a membrane fraction after homogenization in hypotonic conditions. Cytosol and membrane fractions from ^{3}H-palmitic acid–labeled islets were immunoprecipitated with either the GAD6 mAb or an irrelevant mAb (*C*). Immunoprecipitates were divided into three equal size aliquots and analyzed in triplicate by SDS-PAGE (lanes *2–5, 10–13,* and *18–21*) in parallel with an aliquot of nonimmunoprecipitated cytosol and membrane fractions of ^{3}H-palmitate–labeled material (lanes *7, 8, 15, 16, 23,* and *24*) and an immunoprecipitate of a [^{35}S]methionine–labeled membrane fraction (lanes *6, 14* and *22*). The triplicate gels were either untreated or incubated in either 1 M hydroxylamine, pH 8, or 1 M Tris, pH 8, before processing for fluorography. Molecular mass markers indicated in kilodaltons are shown in lanes 1, 9, 17, and 25.

β-cells. Again, as in β-cells, membrane-bound GAD_{65} behaved like an intrinsic membrane protein, which could only be removed by detergent and not by salt, EDTA, or basic pH treatment (results not shown). Consistent with the results in β-cells, incorporation of radioactivity from ^{3}H-palmitate was detected in membrane-bound but not soluble GAD_{65} in Sf9 cells (Fig. 3).

Palmitoylation of GAD_{65} Is Hydroxylamine Sensitive

Hydroxylamine at pH 8.0 is used as a method for a gentle removal of fatty acid from proteins (Omary and Trowbridge, 1981; Magee et al., 1984) and can be used as a diagnostic test of a thioester- or ester-linked fatty acid. Incubations of ^{3}H-palmitic–labeled membrane-bound GAD_{65} on SDS-polyacrylamide gels with hydroxylamine efficiently removed the radioactivity (Fig. 3, lanes *13* and *16*) without loss of [^{35}S]-methionine-labeled GAD_{65} (Fig. 3, lane *14*).

The fatty acid composition of the ^{3}H-palmitate–labeled Sf9 cells was analyzed to assess whether palmitate had been converted to other fatty acids during the labeling period. Fatty acids were extracted from a membrane fraction prepared from the cells which contained 91% of the incorporated label. The fatty acids were derivatized and analyzed by reverse phase high performance liquid chromatography (RP-HPLC). Fatty acid standards were cochromatographed to allow the identification of extracted fatty acids. 51% of the incorporated label in Sf9 cells was found as palmitic acid, 26% was found as myristic acid, 10% was found as stearic acid, and 13% was found as minor unidentified peaks. (Fig. 4 *A*). The label incorporated into GAD_{65} was identified by excising radiolabeled GAD_{65} from gels, followed by hydrolysis under alkaline conditions. The released fatty acids were analyzed by RP-HPLC and identified by cochromatography with standards. Only palmitic acid was detected in GAD_{65} excised from gels (Fig. 4 *B*). Thus the incorporation of radioactivity into membrane-bound GAD_{65} in the presence of ^{3}H palmitic acid represents palmitoylation of the protein and not incorporation of a palmitate-derived fatty acid confirming that the acylation of GAD_{65} is specific for palmitic acid.

The analyses of membrane-bound and soluble forms of GAD_{65} suggested that the hydrophobic soluble and hydrophobic membrane-bound GAD_{65} represent differential stages of a two-step posttranslational modification with hydrophobic residues. Thus, attachment of the first hydrophobic resi-

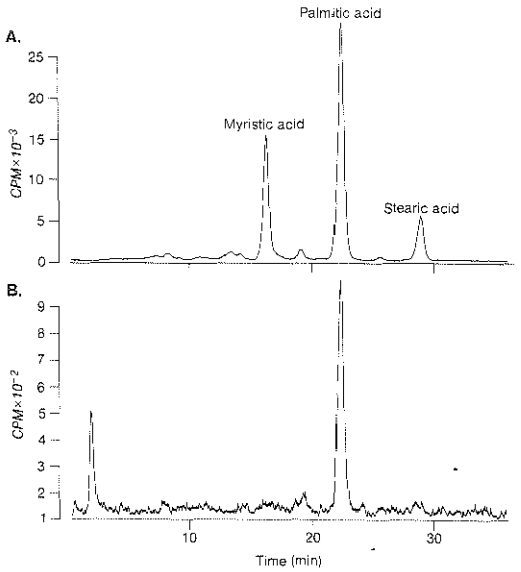


Figure 4. Identification of palmitic acid as the lipid attached to GAD_{65}. (*A*) Reverse phase HPLC analysis of ^{3}H-labeled fatty acids isolated from ^{3}H-palmitic acid–labeled Sf9 cells. The positions of myristate, palmitate, and stearate were identified by cochromatography with standards. (*B*) Analysis of ^{3}H-labeled fatty acids isolated from GAD_{65} purified from ^{3}H-palmitic acid–labeled Sf9 cells. The palmitate peak was identified by cochromatography with standards. The peak in the beginning of the chromatogram is nonderivatized fatty acids.

due would result in soluble hydrophobic GAD_{65} and then palmitoylation of this form would result in the membrane-anchored form.

To examine the possibility that the GAD_{65} autoantigen in β-cells is polyisoprenylated and that this modification is responsible for the hydrophobicity of the S2 form, we tested whether ^{3}H-mevalonate was incorporated into GAD_{65} in either rat islet cells or Sf9 cells. The results of those experiments were negative, suggesting that a polyisoprenyl moiety is not a part of the hydrophobic modification and membrane anchoring of GAD_{65} (data not shown). Mevinolin, an inhibitor of polyisoprenyl synthesis, also inhibited protein synthesis in islet cells, but did not inhibit hydrophobic modification and membrane anchoring of GAD_{65} significantly. The results are consistent with the absence of the known COOH-terminal consensus sequences for polyisoprenylation, CAAX, CC, or CXC (Hancock et al., 1989; Khosravi-Far et al., 1991) in GAD_{65} (Erlander et al., 1991).

Spontaneous and Time-dependent Release of GAD_{65} from Membranes

In the initial phases of this study we had assessed whether GAD_{65} was membrane anchored by a PIG moiety (for review see Ferguson and Williams, 1988). In this type of membrane anchor, the diacylglycerol moiety of the PIG is inserted into the lipid bilayer of membranes, but can be removed by the action of exogenous or endogenous phosphatidyl inositol specific phospholipase C (PIPLC) (for review see Ferguson and Williams, 1988).

Several lines of evidence, however, led to the conclusion that GAD_{65} is not modified by a PIG residue. First, no incorporation of radioactivity into GAD_{65} was detected in the presence of ^{3}H-inositol, ^{3}H-ethanolamine, or ^{3}H-glucosamine which are building blocks of the PIG anchor. Second, if the soluble and membrane-bound forms of GAD_{65} differed by a PIG moiety, they would be expected to have different isoelectric points, which is not the case (Christgau et al., 1991).

Finally, we analyzed the ability of PIPLC to release GAD_{65} from washed islet cell membranes in the presence of buffers with and without components which either inhibit or stimulate the activity of PIPLC. The standard Hepes buffer A, which contains a cocktail of proteolytic and other enzyme inhibitors, was used in control experiments. These experiments revealed that GAD_{65} was released from the membranes in all conditions except in buffer A, but equally with and without the addition of PIPLC. Taken together, the results suggest that an endogenous enzyme, which is inhibited in the conditions of Hepes buffer A, can cleave the membrane anchor and release GAD_{65}. Exogenously added

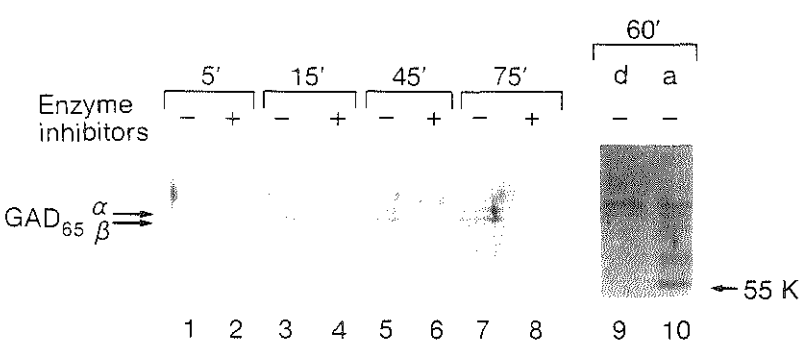


Figure 5. Time-dependent release of GAD_{65} from membranes. Washed membrane fractions prepared from rat islets homogenized in hypotonic conditions were resuspended and incubated in the presence or absence of protease inhibitors for the indicated length of time and then repelleted by ultracentrifugation at 265,000 *g*. The release of GAD_{65} was analyzed by immunoprecipitation of supernatants, SDS-PAGE, and immunoblotting with the 1266 COOH-terminal peptide antibody. GAD_{65} released during a 60-min incubation without enzyme inhibitors was subjected to a TX-114 phase separation to analyze the distribution into detergent (*d*) and aqueous (*a*) phases (lanes *9* and *10*).

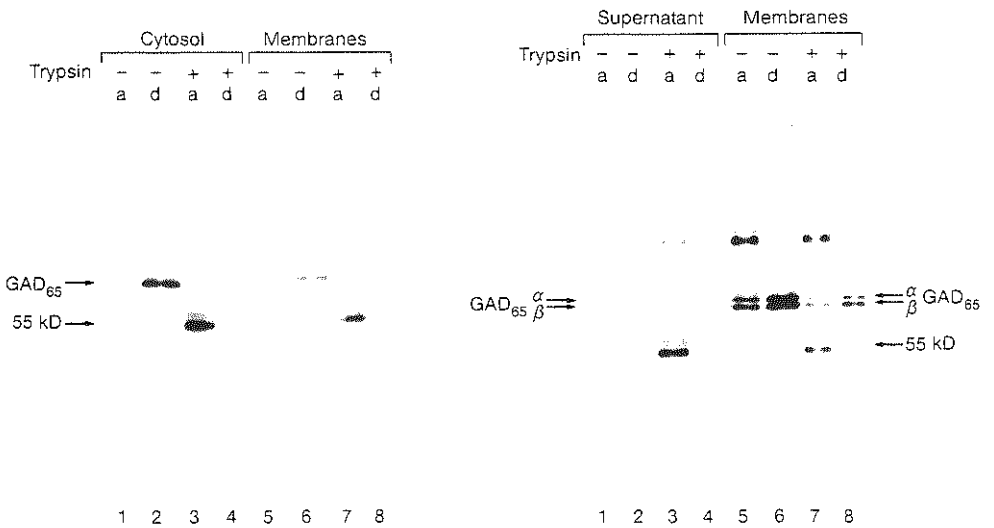


Figure 6. Absence of hydrophobic and membrane anchoring moieties in a 55-kDC-terminal trypsin fragment of GAD_{65}. (*A*) Tx-114 detergent phase was prepared from a cytosol and membrane fraction isolated from rat islets homogenized in hypotonic conditions. The detergent phases were incubated with and without trypsin followed by a new TX-114 phase separation. The detergent phase (*d*) and aqueous phase (*a*) from this last separation were analyzed by SDS-PAGE followed by Western blotting and immunostaining with the COOH-terminal peptide antiserum 1266. (*B*) Washed membranes were prepared from rat islets homogenized in hypotonic conditions resuspended and incubated with and without trypsin followed by ultracentrifugation at 265.000 *g*. The supernatants and TX-114 extracts of residual membranes were subjected to phase separation and aqueous (*a*) and detergent (*d*) phases were analyzed by SDS-PAGE, Western blotting, and immunostaining with the COOH-terminal peptide antiserum 1266.

PIPLC does not, however, affect this release, consistent with the membrane anchor not having the properties of a PIG residue.

To analyze the release of GAD_{65} further, washed membranes prepared from islets, were incubated with and without enzyme inhibitors for different periods of time. No release of GAD_{65} from membranes was detected in the presence of enzyme inhibitors. In the absence of enzyme inhibitors, however, amphiphilic GAD_{65} was released from membranes in a time-dependent fashion (Fig. 5), suggesting that islets contain an enzyme activity which can cleave the GAD_{65} membrane anchor, but not the hydrophobic moiety of the soluble S2 form. GAD_{65} released from membranes of islet cells did partition into the TX-114 detergent phase (Fig. 5, lanes *9* and *10*, see also Fig. 6 *B*), suggesting that the release represents a conversion of the firmly membrane anchored form to the soluble but still hydrophobic S2 form. In addition a 55-kD hydrophilic fragment of GAD_{65} was detected in the fraction released from the membrane in some experiments (Fig. 5, lane *10*), suggesting either that the protein can also be released from the membrane by proteolytic cleavage or that released GAD_{65} is susceptible to proteolytic cleavage.

Localization of the Hydrophobic Membrane Anchoring Region of GAD_{65} to a Fragment at the NH_2 Terminal

We addressed the localization of the hydrophobic residue(s) and membrane anchor in the GAD_{65} protein by analyzing the hydrophobicity of fragment(s) generated by mild trypsin digestion of the hydrophobic soluble and membrane forms of GAD_{65}. Islets were homogenized in a hypotonic buffer and cytosol and membrane fractions prepared. The amphiphilic proteins in both fractions were purified into the detergent phase of TX-114 and incubated with and without trypsin followed by a new TX-114 phase separation. The detergent and aqueous phases from this last phase separation were analyzed by SDS-PAGE and immunoblotting using an antiserum raised against a 19-amino acid COOH-terminal peptide of the larger brain form of GAD (GAD_{67}). Because of an extensive homology at the COOH terminus between GAD_{67} and GAD_{65} (Erlander et al., 1991), this antiserum recognizes both forms equally well. The results are shown in Fig. 6 *A*. Trypsin digestion resulted in a 55-kD fragment in both the soluble and the particulate fraction, which was recognized by the COOH-terminal peptide antibody, and therefore contains the COOH-terminal part of the GAD molecule.

In incubations without trypsin, GAD_{65} from both soluble and particulate fractions partitioned into the TX-114 detergent phase. The 55-kD fragment from both fractions was, however, exclusively detected in the aqueous phase and had thus lost its hydrophobic moiety (Fig. 6 *A*). The results suggest that the hydrophobic residue(s) in both the soluble and membrane anchored form reside in a 9–10-kD fragment at the NH_2-terminus.

We next analyzed whether trypsin was able to release membrane-bound GAD_{65}. The particulate fraction was resuspended and washed three times in TBS to remove loosely associated proteins, and then incubated with and

without trypsin for 1 h. Released proteins were analyzed by western blotting (Fig. 6 *B*). Although a minor fraction of full-length amphiphilic GAD_{65} is released spontaneously from the membranes in those conditions (Fig. 6 *B*, lanes 1 and 2), the majority of GAD_{65} remains membrane-anchored (Figure 6 *B*, lanes *5* and *6*). In experiments containing trypsin, GAD_{65} was released from the membranes as a 55-kD hydrophilic COOH-terminal fragment (Fig. 6 *B*), indicating that the membrane-anchoring moiety resides in a 9–10-kD fragment at the NH_2-terminus. Attempts to detect the NH_2-terminal fragment following trypsin digestion by immunoblotting were unsuccessful. Thus, a small (9–10 kD) fragment was not detected by immunostaining with the polyclonal antibody S3 nor with the monoclonal antibody GAD6. It is conceivable that neither antibody reacts with the NH_2-terminal fragment or that the fragment is further digested into small fragments by trypsin since it contains several basic residues (Erlander et al., 1991). In attempts to detect the NH_2-terminal fragment, the trypsin experiments described above and shown in Fig. 6 *A* and *B* were repeated using ^{3}H-palmitic acid–labeled Sf9 cells and analysis by SDS-PAGE followed by both fluorography of gels as well as immunostaining of Western blots using the COOH-terminal peptide antibody 1266. In those experiments following trypsin treatment, as expected ^{3}H-palmitic acid labeling was only detected in residual particulate full-length GAD_{65} but not in the 55 kD fragment (results not shown). A residual 9–10 kD fragment was detected in fluorograms of total extracts of membranes treated with trypsin but not in membranes from control incubations and may represent the ^{3}H-palmitoylated NH_2-terminal fragment of GAD_{65} (not shown). Consistently this fragment did not stain with the 1266 antibody on Western blots. In sum the experiments with trypsin provide evidence that the hydrophobic modification and membrane anchoring of GAD_{65} takes place in the NH_2-terminal 9–10-kD part of the protein.

Discussion

In pancreatic islets GAD_{65} undergoes membrane anchoring in a process that involves a two-step modification. The first step results in a hydrophobic molecule which is soluble or has a low membrane avidity. The second step results in a firm membrane anchoring. (Christgau et al., 1991). The results described herein show that the second step involves a hydroxylamine-sensitive palmitoylation, and results in a molecule with intrinsic membrane protein properties. GAD_{65} can be released from islet cell membranes in a time-dependent manner, in a process in which enzyme activity is implicated, suggesting that the second step is reversible and that GAD_{65} may be flexible in its membrane-anchoring properties. Immunogold EM analysis with GAD antibodies show clear labeling of the membrane of small vesicles in pancreatic β-cells, which are similar in size to synaptic-like microvesicles (Reetz et al., 1991) and clearly distinct from the insulin-containing secretory vesicles in those cells. The EM results shown here are consistent with data from immunohistochemical analysis of GAD and GABA in β-cells and neurons by confocal microscopy (Sorenson et al., 1991; Reetz et al., 1991), which show localization of both to a vesicular compartment. This compartment was shown to be concentrated in nerve terminals and in the distal portions of neurite-like extensions in β-cells, and was tentatively identified as synaptic vesicles in neurons and synaptic-like microvesicles in β-cells by colocalization with synaptophysin (Reetz et al., 1991). Based on the confocal microscopy data (Sorenson et al., 1991; Reetz et al., 1991), on the electron microscopic analysis shown here, and on the demonstration of the intrinsic membrane protein properties of GAD_{65} (Christgau et al., 1991, this study, and unpublished results) we conclude that the membrane-anchored form of GAD_{65} is localized to the membrane of synaptic-like microvesicles containing GABA in β-cells and synaptic vesicles in neurons. A previous immunogold EM study using embedded brain and islet-cell homogenates (Reetz et al., 1991) has shown concentrations of GAD immunoreactivity in the proximity of synaptic vesicles in brain and synaptic-like microvesicles containing GABA in pancreatic β-cells. However this study did not reveal an association of GAD with those microvesicles. This previous failure to detect GAD in synaptic vesicle membranes by immunogold EM of homogenized islets, and by subcellular fractionation (Reetz et al., 1991) is most likely because of the reversible nature of the membrane anchoring we have now documented. In fact, preparation of membranes in the absence of enzyme inhibitors and at elevated temperatures or at weak alkaline pH can result in the majority of GAD_{65} being recovered as soluble (our unpublished results). Furthermore, even in the absence of trypsin a fraction of GAD is often recovered as a 55-kD soluble and hydrophilic COOH-terminal fragment (Christgau et al., 1991; and this study), suggesting that GAD may also be released from membranes by proteolytic cleavage during homogenization and/or further fractionation of cells.

The characteristics of the posttranslational modifications and the subcellular and amphiphilic properties of the different forms of GAD_{65} resemble in many ways those of the ras proteins (Hancock et al., 1989) and the rab GTP-binding proteins (for review see Balch, 1990), all of which exist both in a soluble hydrophobic and a membrane-bound hydrophobic form, with intrinsic membrane protein properties. The latter group includes the rab 3A and 3B proteins which are also found in the membrane of synaptic vesicles (for review see Südhof and Jahn, 1991). The posttranslational modification and membrane anchoring of the ras and the rab proteins, however, involves isoprenylation and carboxymethylation at the COOH terminus which contains either a CAAX, CXC, or CC motif (Hancock et al., 1989; Farnsworth et al., 1991; Kohsravi-Far et al., 1991). GAD_{65} does not contain an isoprenylation motif at the COOH terminus (Erlander et al., 1991) and does not seem to undergo this modification. Rather, the first modification of GAD_{65} appears to involve a different mechanism. The second modification of GAD_{65}, however, does resemble that of some of the ras proteins in being a palmitoylation that results in firm membrane anchoring. Furthermore, as for ras proteins (Magee et al., 1987), the membrane anchoring is reversible.

Interestingly, GAD_{65} can be posttranslationally modified, palmitoylated, and membrane anchored in insect Sf9 cells and in cos cells (our unpublished results), which apparently do not contain synaptic-like microvesicles. This result suggests that the posttranslational modification of GAD_{65} and its targeting to synaptic vesicles are separate events. Posttranslational modification and membrane anchoring may take place before budding of membrane vesicles from the

Golgi complex. Then perhaps the final targeting of the membrane-anchored form to synaptic-like microvesicles in β-cells and synaptic vesicles in neurons depends on interaction with components of synaptic vesicles which are absent in Sf9 and cos cells.

Relevance for Function

In cerebral cortex, both GABA and glutamate have been shown to be stored in synaptic vesicles. It is however unclear whether those amino acids reside in the same or different vesicles (Burger et al., 1991). Membrane-anchored GAD_{65} may therefore be localized so as to provide GABA directly to the vesicle that secretes it, thereby coordinating enzymatic activity and GABA secretion. An important question is whether GAD_{65} resides in the lumen or at the cytosolic face of the vesicles. In the first case the enzyme would be in the immediate proximity of glutamate and/or GABA. It is notable that GAD_{65} lacks a known signal sequence (Erlander et al., 1991) and thus would require a novel transport mechanism to achieve a luminal localization. If GAD is anchored at the cytoplasmic face, which seems most likely, it could associate with its substrate glutamate and release its product GABA in coordination with their transporters in the membrane of synaptic vesicles (Burger et al., 1991 and references therein).

In rat pancreatic β-cells membrane attachment is the exclusive property of the predominant GAD_{65} form and is not detected in the minor nonallelic protein, GAD_{67}, which remains hydrophilic and soluble throughout its lifespan (Christgau et al., 1991). GAD_{65} and GAD_{67} are most divergent in the first 100 amino acids but share in high degree of homology in the remainder of the molecules (Erlander et al., 1991). Taking advantage of a trypsin sensitive hot spot, we have now shown evidence that the area of hydrophobic modification and membrane anchoring of GAD_{65} resides in the 9–10-kD fragment at the NH_2 terminus where this protein differs most significantly from GAD_{67}.

The reason why two genes encoding distinct versions of this enzyme have developed and why β-cells preferably express GAD_{65} is not known. GAD_{67} binds the coenzyme pyridoxal phosphate more tightly and is less dependent on exogenous coenzyme than GAD_{65}, which is mainly found in the apoenzyme form (Martin et al., 1991). Accordingly GAD_{65} activity is more sensitive to pyridoxal phosphate levels than GAD_{67} (Kaufman et al., 1991). In neurons GAD_{65} has been shown to be predominantly localized to nerve endings (Henry and Tappaz 1991; Kaufman et al., 1991) consistent with its localization in the membrane of synaptic vesicles which concentrate proximal to the synaptic membrane. GAD_{67} is however predominantly localized in the cell bodies of neurons (Kaufman et al., 1991; Henry and Tappaz, 1991). It is conceivable that the localization of the pyridoxal phosphate inducible form, GAD_{65}, to the membrane of the vesicles that store and secrete its product GABA has a regulatory purpose and serves to accommodate sudden increases in GABA demand. Interestingly there are examples of other enzymes involved in the synthesis of transmitters that are anchored to the membrane of granules that store and secrete them. Thus tyrosine hydroxylase, the initial and rate-limiting enzyme in the biosynthesis of the catecholamines, and dopamine β-hydroxylase, the final enzyme in the synthesis of norepinephrine, exist in both soluble and membrane-bound forms and the latter have been localized to the membrane of chromaffin granules in the adrenal medulla (Kuhn et al., 1990; Bon et al., 1991).

Relevance for Autoimmunity

The correlation in autoimmunity toward pancreatic β-cells and brain neurons is remarkable, especially as it is revealed in the development of autoantibodies which recognize the same antigen, the 64-kD form of glutamic acid decarboxylase. A second nonallelic form of this enzyme encoded by a distinct gene, GAD_{67}, is apparently only of secondary importance. GAD_{67} lacks the primary epitopes which are antigenic in SMS (Baekkeskov et al., 1990), and evidences a lower incidence of specific autoantibodies in IDDM (our unpublished results). It is therefore provocative that these two proteins are clearly distinguished by an NH_2-terminal region which determines hydrophobic modification, subsequent palmitoylation, and consequent membrane anchoring and subcellular localization. Only GAD_{65} has these two posttranslational modifications that confer hydrophobicity, membrane attachment, and association with microvesicles. It is of note that palmitoylated influenza peptides induce potent cytotoxic T-cell responses in vivo whereas their non-palmitoylated counterparts do not (Deres et al., 1989). Perhaps one of the posttranslational modifications, or the selective localization of GAD_{65} into a membrane compartment that is secretory in nature is instrumental for its role as an autoantigen in these two diverse but coinciding autoimmune diseases.

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Chapter 3

AUTOANTIBODIES TO GAD IN TYPE-1 DIABETES MELLITUS.

3.1

Autoantibodies to Glutamic Acid Decarboxylase (GAD) in type 1 Diabetes Mellitus: Improved Analysis in Newly Diagnosed Patients Using Recombinant GAD_{65} and a Comparison with Analysis of 64kD/GAD Antibodies Using Rat Islets.

Submitted

Autoantibodies to Glutamic Acid Decarboxylase (GAD) in type 1 Diabetes Mellitus: Improved Analysis Using Recombinant GAD_{65} in Newly Diagnosed Patients and a Comparison with Analysis of Islet 64kD/GAD Antibodies .

Henk-Jan Aanstoot#*, John Kim#, Marc Jaffe#, Stephan Christgau#, Yuguang Shi#, Joan Qan#, Jacques Molenaar^, G.Jan Bruining*, and Steinunn Baekkeskov#.

From the # Depts. of Medicine and Microbiology/Immunology and Hormone Research Institute, University of California San Francisco, San Francisco, CA, * the Dept. of Pediatrics, Erasmus University Medical Center / Sophia Children's Hospital, Rotterdam, the Netherlands and ^ the Dept. of Immunology, Stichting Samenwerkende Delftse Ziekenhuizen, Delft, the Netherlands

Summary

Antibodies to the 64kD autoantigen in type 1 diabetes, now identified as the 65kD form of the GABA synthesizing enzyme Glutamic Acid Decarboxylase (GAD), are markers of ongoing ß-cell destruction. Analysis of these autoantibodies is usually performed by immunoprecipitation of islet protein fractions with patient sera. The identification of the 64kD autoantigen as Glutamic Acid Decarboxylase (GAD) allowed the development of recombinant expression systems for GAD. It has been suggested that not all 64kD immuno-activity precipitated from islets is accounted for by GAD_{65}. We analyzed if recombinant GAD_{65} is suitable for immunoprecipitation in 48 newly diagnosed diabetes patients and 135 controls. The same sera were tested using the original 64kD immunoprecipitation method with rat islets. Two eukaryotic expression systems, one in the Baculovirus infected SF9 insect cells and one in transfected COS-7 cells, were used. The SF9 expressed GAD_{65} showed aggregation of GAD_{65}, resulting in false positive samples. The COS expressed GAD_{65} was suitable for immunoprecipitations and the assay was improved using pretreatment of the protein A Sepharose®. The sera analysis showed that from 48 newly diagnosed patients, 38 were ICA positive (79%). Using COS expressed GAD_{65}, 39 patients were positive for GAD_{65} autoantibodies (81%). The combination of ICA and COS-GAD_{65} was detected 90% (43 of 48) of the patients. One control of 135 tested samples was positive (0.7%). Using the islet 64kD method 35 of the 48 patient sera were positive (73%) and 1 of the 135 controls (0.7%). The 4 disparate patients which were negative in the rat islet assay were weakly positive in the COS-7 cell assay. We conclude that 1) GAD expressed in Baculovirus infected SF9 cells is unsuitable for immunoprecipitations; 2) Analysis of GAD antibodies using COS expressed GAD_{65} can be more sensitive than immunoprecipitation of 64kD/GAD_{65} expressed in rat islets; 3) The GAD_{65} antibody positive sera include all 64kD antibody positive sera. Further simplification of the GAD-autoantibody assay will make these antibodies important markers in the prediction of type 1 diabetes.

Introduction:

Glutamic Acid Decarboxylase (GAD/ 64kD protein), the key enzyme in the synthesis of the neurotransmitter GABA, is a major target for humoral autoimmunity in type 1 diabetes mellitus (1). Two forms of GAD exist, for which two non-allelic genes code (2). GAD_{67} is a hydrophilic molecule and is present in human GABA-

ergic neurons and in ß-cells of rats (3, 4). The GAD_{65} isoform can be membrane bound (5), and is the only form expressed in human islets (6, 7). GAD antibodies are the first to appear in the preclinical stage of type 1 diabetes and can be found in up to 80% of the prediabetic individuals (8, 9). At clinical onset of the disease the antibodies are present in at least 80% of newly diagnosed patients (10). The high frequency and early appearance of the antibodies make them important parameters of beta cell destruction. Moreover, the study of GAD and GAD-antibodies provides a model that can help to elucidate the pathogenesis of autoimmune ß-cell destruction. Dissection of this process will enable to design strategies that aim at primary prevention of the disease. However, prevention will only be feasible and cost effective if adequate prediction is available. This is emphasized by the fact that in most countries only 5-10% of new cases of diabetes have a family history of the disease (11), implying that most cases occur in the general population. Thus, screening of populations will be needed to achieve prevention that is cost-effective. This will require extremely sensitive, specific and simple prediction tests. Autoantibodies to GAD are serious candidates for such a test.

The identification of the 64kD autoimmune target in type 1 diabetes as GAD_{65} (1) allowed to produce both forms of GAD by eukaryotic expression systems (3,5). In earlier studies, isolated rat or human islet were used as a source for 64kD/GAD. Expression systems for GAD have, however, not yet resulted in fast ELISA- or RIA-type tests with comparable performance as the more laborious and expensive immunoprecipitation assays with islet material. Moreover, sera from diabetes patients mainly recognize conformational epitopes of the GAD_{65} molecule, primarily in the C-terminal and middle domains (12). Thus, the recombinant material must have the correct post-translational modifications and folding. Moreover, using recombinant GAD could result in a lower incidence in autoantibodies, since it has been suggested that not all 64kD immunoreactivity can be accounted for by GAD_{65} (13).

In this study, we analyzed if recombinant GAD, produced by two different eukaryotic expression systems, is suitable for GAD autoantibody analysis. While GAD_{65}, produced in insect cells (SF9) showed to be unsuitable, the use of COS-7 expressed GAD_{65} resulted in a sensitive and specific immunoprecipitation assay. The frequencies of GAD_{65} autoantibodies at onset and in the preclinical phase were determined and compared to Islet cell autoantibodies (ICA) and Insulin Autoantibodies (IAA). We compared the incidences of GAD_{65} autoantibodies in this assay with the original 64kD autoantibody analysis using rat islet material. All the 64kD activity could be contributed to GAD_{65} immunoreactivity. Using COS-7

expressed GAD_{65}, more GAD antibody positive sera were identified than with the islet cell method. This study showed that COS-7 expressed GAD_{65} in immunoprecipitations results in a sensitive and specific tests for GAD autoantibodies.

Methods:

Patients, Prediabetic Individuals and Controls.

Sera were obtained after signed informed consent and with approval from the Hospital ethical committee from all individuals and, if needed from their parents. The sera were drawn between 1987 and 1992 and were stored at -80°C. Forty-eight Dutch newly diagnosed diabetes patients were evaluated and 135 controls. Characteristics of the patients tested are given in table 1.

Islet cell antibody (ICA) determinations:

ICA's were measured as described before (14). Briefly, coded slides of unfixed frozen thins of human donor pancreas (blood group O) were incubated with 25 µl of pure or diluted serum and evaluated after conjugation with a secondary FITC labeled antibody by two independent observers. Control sera included JDF standard serum and negative samples. End-point titers were defined as the highest detectable ICA staining. The levels were expressed in JDF units.

Insulin Autoantibody Assay:

Insulin autoantibodies (IAA) were tested using a radio binding assay as described (14). Cold insulin displacement was measured according to the international workshop on IAA standardization. The cut-off level between positive and negative values was defined as 3 x SD from the mean of normal control sera .

Analysis of 64kD/GAD autoantibodies using rat islets.

Soluble amphiphilic proteins were prepared from ^{35}S-methionine labeled rat islets as described (8,15). Isolated immune complexes were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% uniform slab gels and a modified Laemmli buffer system to achieve resolution of the GAD_{65} α- and β-chains (15). The gels were processed for fluorography as described (8). Autoradiograms were analyzed and scored positive or

negative by three independent observers without knowledge of sample identity. Positive and negative controls were used in all gels.

COS-7 Expressed Rat GAD_{65}.

a. Transient expression of GAD_{65} in COS-7 cells:

The expression vector MT-2 was used to construct a vector containing the rat GAD_{65} cDNA as described before (3). COS-7 cells were transfected by lipofection (DOTAP, Boehringer Mannheim, Indianapolis, IN) according to the manufacturers instructions. DNA, mixed with DOTAP in HEPES buffered saline (10 mM HEPES, 150 mM NaCl, pH=7.4), was added to 60-70% confluent plates of COS after refreshing of the medium (Dulbecco modified Eagles medium (DMEM) with 10% Fetal calf serum (Hyclone, Logan, UT). After 12 hours of incubation with the DNA/lipofection agent, the medium was refreshed again and the cells cultured for an additional 48 hours. The cells were washed twice with 37°C Phosphate buffered Saline (PBS) followed by a 30 min. starvation period in DMEM without methionine and with 2% dialyzed fetal calf serum. After refreshing this medium, 400 μCi of ^{35}S-methionine (Amersham SJ-1515) was added per 10 cm plate for 4hrs. The labeled cells were washed 3 times in an ice-cold harvest buffer (150 mM NaCl, 10 mM HEPES, 10 mM Benzamidine, 0.1 mM p-Chloromercuriphenyl sulfonic acid (PCMPS) and 0.1 mM Phenylmethylsulfonyl fluoride (PMSF), pH=7.4, all chemicals from Sigma, St. Louis, unless stated otherwise). All other procedures were performed at 4°C. The cells were collected with a cell scraper in 1 ml of a hypotonic lysis buffer (HEMAP: 10 mM HEPES, 1 mM EGTA, 1 mM Magnesium Chloride, 1mM aminoethylisothiouronium bromide hydrobromide (AET), 0.2 mM Pyridoxal 5'-Phosphate (PLP), 0.1 mM PCMPS, 0,1 mM PMSF and 10 mM Benzamidine, pH=7.4) and 1% Triton X-114 (TX-114, Calbiochem, La Jolla, CA), incubated for 10 minutes, followed by repeated pipetting through a bended tip for 10 minutes. The solution was cleared by ultracentrifugation at 400.000g for 1 hr in a Beckman TLA-100 ultracentrifuge with a TLA 100.3 rotor (Beckman, Fullerton, CA) resulting in an S-400 fraction. This supernatant was subjected to temperature induced phase separation (16). The detergent phase, containing the soluble amphiphilic GAD_{65}, was collected and diluted in HEMAP. Aliquots, equivalent to 30 immunoprecipitations were frozen at -80°C.

b. Analysis of 64k /GAD autoantibodies using recombinant COS-7 expressed GAD_{65}.

Autoantibodies GAD_{65} were analyzed using immunoprecipitation of GAD_{65} transiently expressed in COS-7 cells. For immunoprecipitations (IMP's), the semi purified GAD_{65} material was thawed on ice and re-spun for 30 minutes at 400.000

g to clear aggregates. An equivalent of 200.000 cpm of the semi-purified material was used for each IMP, diluted to 200 µl in HEMAP, and precleared in one batch, using a normal serum. An equivalent of 20 µl serum per 200.000 cpm was incubated for 2 hrs. Protein A sepharose (PAS, Pharmacia, Piscataway NJ) was preswollen in a large volume of an IMP washing buffer (150 mM NaCl, 10 m M HEPES, 10 mM Benzamidine, 0.5 mM Methionine, 5 mg% BSA and 5 mM EDTA, pH=7.4) and washed 3 times in the same buffer. We pretreated the PAS with an S-400 of GAD transfected COS-7 cells which were not labeled, mixed 1:1 with non-transfected COS-7-cell S400, based on the rationale that any non-specific binding of labeled GAD to PAS would be blocked by this non-radioactive material. No background precipitation in control sera was seen, including 3 month exposures (approx. one half-life of ^{35}S) of the gels. Precleared labeled COS-7-GAD_{65} was incubated with test sera for 16 hours in a total volume of 220 µl. Immune complexes were isolated using PAS, that was pretreated with cold material identically, in a 1 hr incubation. PAS with immune complexes was washed 5 times with IMP-washing buffer and 1 time with water. Laemmli buffer was added and the samples were applied on 10% SDS-PAGE slab gels after boiling. Gels were processed for fluorography and analyzed and scored positive or negative by three independent observers without knowledge of sample identity.

Baculovirus Infected SF-9 Cell Expressed GAD65.

a. Transient expression of human GAD_{65} in SF-9 cells.

Recombinant human GAD_{65} Baculovirus vectors were constructed and analyzed as described before (5). Spodoptera frugiperda (SF9) cells were infected with recombinant -GAD_{65}- containing viruses. After 48 hrs, the cells were labeled for 4 hrs with ^{35}S-methionine at 80 µCi/ml as described for COS-7 cells, except that labeling was in methionine free Grace medium (Gibco-BRL).

b. Analysis of GAD autoantibodies using recombinant SF9 expressed GAD_{65}.

Infected and labeled SF9 cells were harvested identical as described for COS-7 cells. An S-400 was obtained as described for COS-7 cells and stored in frozen aliquots after temperature induced phase separation. Immunoprecipitations were performed as described for COS-7 GAD_{65}.

Results.

ICA and IAA analysis in the newly diagnosed patients and controls.

Results of the ICA and IAA analysis are summarized in table 1. 79 percent (38/48) of the newly diagnosed patients were ICA positive as defined as > 20 JDF units. Only 5% (2/41) showed Insulin autoantibodies. In the control group, 3% showed ICA's and 1% IAA's.

TABLE 1

Data and results on 48 newly diagnosed diabetes patients and 139 control individuals.

	n =	age ± SD	M:F ratio	ICA-posi-tive	IAA-posi-tive	Islet 64kD positive	Cos-GAD65 posi-tive
Newly Diagnosed patients	48	12.1+ 9.7	0.82	79% (38/48)	5% (2/41)	73% (35/48)	81% (39/48)
Controls	139	14.0 ± 3.8	0.75	3% (4/139)	0.7% (1/139)	0.7% (1 / 139)	0.7% (1/139)

Analysis of 64kD/GAD autoantibodies using rat islets

Using the islet cell method for 64kD/GAD autoantibodies, 35 patients and one control individual were identified with 64kD / GAD autoantibodies. The results are accumulated in table 1. An example of the rat islet analysis is given in figure 1.

Analysis of GAD Autoantibodies using COS-7 Expressed GAD_{65}

Cos-7 expressed GAD_{65} was produced as described and immunoprecipitated with sera from the patient and control groups. Analysis by SDS-PAGE and fluorography showed the typical α- and β-band of GAD_{65} in positive controls (figure 2). All gels of the 48 newly diagnosed diabetes patients and controls were exposed on film for a maximum period of 3 months to be able to identify weak GAD-autoantibody positive samples. An example of these sera can be seen in figure 2.

FIGURE 1

Immunoprecipitation of soluble amphiphilic proteins from 35S methionine labelled rat islets with sera from patients with newly diagnosed type 1 diabetes and controls. Fluorogram of an SDS-PAGE showing precipitation of the 64kD / GAD doublet in most of the diabetic patients and not in control individuals. Notice the differences in GAD titers between the patients.

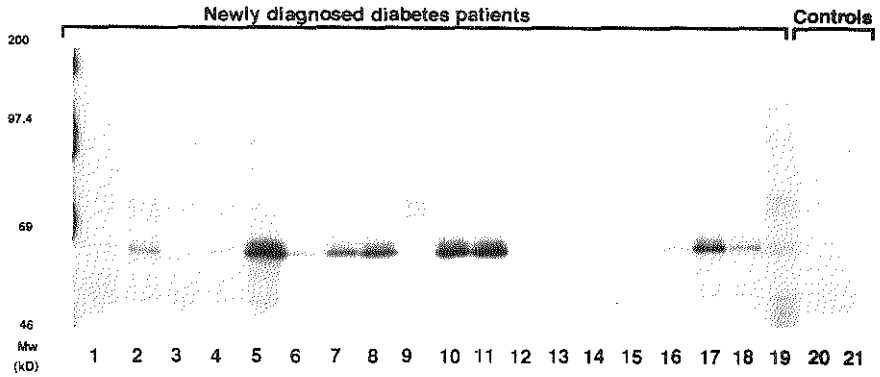
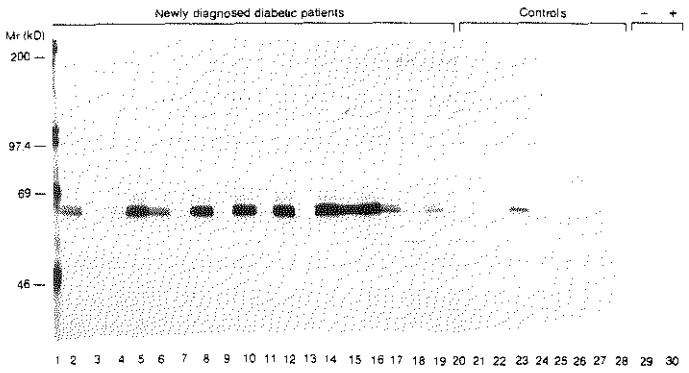


FIGURE 2

Immunoprecipitation of soluble amphiphilic proteins from GAD65 transfected COS-7 cells labelled with 35S methionine with sera fro patients with newly diagnosed type 1 diabetes and controls. Fluorogram of an SDS-PAGE showing precipitation of the GAD doublet in most diabetic patients and not in controls. One control shows a single band, which did not disappear after preincubation of the serum with unlabeled GAD, indicating that this precipitate is not GAD. The band is also positioned slightly lower.



The results of the GAD analysis in the Dutch newly diagnosed individuals and controls are collected in table 1. No background signal was observed in the control individuals (figure 2). Forty eight newly diagnosed individuals were analyzed, together with 135 controls. Thirty nine (81%) of the newly diagnosed patients showed antibodies to GAD by using the COS-GAD_{65} material, four more than with the rat islet method. One control was positive for GAD_{65} autoantibodies, identical to the positive control in the rat islet experiment. This individual has not developed diabetes in the follow-up period.

Analysis of GAD Autoantibodies using GAD_{65} Expressed in Baculovirus infected SF9 cells

Thawing SF9 expressed semi-purified GAD_{65}, showed a significant pellet after centrifugation. Analysis of this pellet by SDS-PAGE and western blot showed that this was GAD_{65} (results not shown). Cleared material was immunoprecipitated and processed as described above. SDS-PAGE showed background GAD bands in all controls. These sera were known to be negative by both islet and COS-7 GAD_{65} analysis. In order to reduce this aggregation problem, higher dilutions of the semi purified GAD were used without solving this problem. Pretreatment of the PAS, intended to eliminate non-specific binding of labeled GAD to the beads, did not solve the problem. The material was therefore not used for large scale serum analysis.

Comparison of the ICA, IAA and GAD-antibody analysis.

Table 1 shows the combined analyses for all autoantibodies tested. Thirty-eight of 48 (79%) patients were ICA positive. Four of these did not have GAD antibodies by either of the two GAD antibody methods used. Moreover, 5 GAD antibody positive patients were negative for ICA, and 5 patients were negative for both ICA and GAD autoantibodies. All IAA positive samples were also positive for ICA and GAD antibodies. When combining both GAD-autoantibodies and ICA, 43 of 48 (90%) of the patients showed signs of humoral autoimmunity directed towards ß-cell antigens.

Discussion

Autoantibodies to the 64kD autoantigen are important markers of autoimmune destruction . The identification of this protein as GAD_{65} (1) allowed to design recombinant expression systems. Until then, islets of Langerhans were needed for the analysis of 64kD/GAd autoantibodies. Islet isolation and labeling is time consuming and expensive. Reports describing the incidence of 64kD/ GAD autoantibodies at onset as well as in the prediabetic period using islet cell methods (8,9) result in higher percentages of positive samples than the more recently described methods using enzymatic assays (18) and ELISA's (19) do. Possible discrepancies could be due to a better sensitivity and specificity for the 64kD immunoprecipitation methods, the use of GAD from other sources such as pig brain in some new assays, the use of only peptides of GAD instead of the whole molecule (19) or the fact that not all 64kD immunoreactivity is precipitated by GAD_{65} reactivity (13). To analyze this, we compared immunoprecipitation of recombinant GAD_{65} with immunoprecipitations using the 64kD method with rat islets in a group of newly diagnosed patients and controls.

COS-7 Expressed GAD_{65} is Suitable for the Detection of GAD-Autoantibodies in Type 1 Diabetes Mellitus.

We analyzed if GAD_{65} expressed in COS-7 cells could be used for the analysis of autoantibodies in newly diagnosed type 1 diabetes patients. The transfection yielded sufficient amounts of GAD_{65} to be visible in Coomassie brilliant blue staining of gels. To avoid non-specific binding of labeled GAD_{65} to Protein A Sepharose, we pretreated the PAS with non-labeled material. Gels were exposed on film up to 3 months. No background GAD_{65} was seen in the immunoprecipitations of control sera. Both the absence of overexpression in COS-7 as well as the pretreatment of PAS with cold proteins seem to be important factors in obtaining a high resolution and low background. No evidence for aggregation or misfolding was seen. The COS-7 system results in GAD that can be recognized by the diabetic autoantibodies and is thus likely to have the correct three dimensional structure. We found four more patients positive by this assay compared to the original rat islet method. No individuals were found positive by the rat-islet method and negative on immunoprecipitation with COS-GAD_{65}, thus, all precipitated 64kD could be accounted for by GAD_{65}. The low background levels seen with the COS-GAD_{65} method seem to result in a higher sensitivity. However, this method only contains the amphiphilic proteins and treatment of the

preparation with Triton X-114 (in both methods) could have disturbed binding of GAD to an associated molecule. In our hands, no evidence for an other component of the 64kD complex was found. In summary, GAD_{65} expressed by transiently transfected COS cells is suitable for GAD antibody immunoprecipitations.

Incidence of 64kD and GAD_{65} Autoantibodies in Newly Diagnosed Type 1 Diabetes Patients and Controls Compared to other Autoantibodies.

We analyzed the frequencies of GAD autoantibodies in newly diagnosed patients with diabetes and controls and compared it with other autoantibodies in type 1 diabetes mellitus. Using the islet cell 64kD method, autoantibodies were found in 73% of newly diagnosed patients. The COS-GAD_{65} method found more (81%) of the patients positive (Table 1). One control individual was found positive in both methods. This person did not develop diabetes in the subsequent 8 years. No approval for further analysis was given.

ICA's were found in 79% of the patients and in 3% of the controls. These levels compare to other studies (20-24). The combination of GAD and ICA yielded the strongest evidence for ß-cell directed autoimmunity with 90% of the patients having either or both autoantibodies present. Four of the ICA positive newly diagnosed patients were negative for GAD autoantibodies while 5 GAD positive patients were negative for ICA. Among these 5, three had very strong titers of GAD antibodies. Strong titers to GAD were associated to non-progression of ß-cell destruction and combined with a restricted ICA staining pattern (25). The islet staining pattern was not evaluated in our study. From our study, strongly positive GAD sera are not necessarily protective.

It will be necessary to perform similar studies in prediabetic individuals. Although the percentages of positive individuals for ICA and GAD differ only slightly (79 vs. 81%) the GAD assays will be easier to standardize since they use a defined antigen and are not dependent on the quality of a biological variables such as the donor pancreas in ICA assays.

Inclusion of the IAA status did not change the prediction of ß-cell autoimmunity since all IAA individuals were ICA and GAD antibody positive.

In summary, the combination of GAD antibody with ICA analysis provides a strong marker for ongoing ß-cell destruction. Combined with other predictive

markers such as the HLA haplotype, they are candidates for family- and population based studies on the prediction of type 1 diabetes.

GAD_{65} Autoantibodies Can be Measured Using COS Expressed GAD_{65}, While SF9 Expressed GAD_{65} Shows Aggregation.

In the baculovirus expression system, Spodoptera Frugiperda (SF9) insect cells are infected with a virus-construct in which the polyhedrin gene, coding for a structural protein of the virus, is replaced by human GAD_{65} cDNA. The polyhedrin promoter drives the expression. The infected SF9 cells produce large quantities of the recombinant protein. This expression system resulted however in aggregation of GAD_{65}. Aggregates formed easily in the GAD_{65} preparations at high concentrations. This might be a result of the overexpression of GAD_{65} in the SF9 cells. For correct folding into the three dimensional conformation and to prevent aggregation of GAD_{65}, other cellular (chaperone) proteins are needed. Overload of the peptide transport- and post-translational modification mechanisms may induce faulty or absent tertiary structures. GAD_{65} becomes membrane bound in SF9 cells, identical as it does in islets (5). However, the presence of this post-translational process in the SF9 cells does not imply that the three-dimensional structure of the molecule will be correct. The correct three-dimensional structure is necessary for stability of the molecule and of paramount importance for the recognition by autoantibodies in type 1 diabetes patients, which mainly recognize conformational epitopes. Immunoprecipitations of SF9 produced GAD_{65} with diabetic and control sera, using re-centrifuged material, showed false positive signals, most likely due to aggregation of GAD_{65} and probably non-specific binding to the protein A Sepharose. Attempts to clear the preparations from aggregates and to block non-specific binding places on PAS were not successful. Dilution of the material had no effect. Although it could be argued that natural autoantibodies could be due to the immunoprecipitates in controls, we never encountered similar signals in analyses using islet materials or using the COS-GAD_{65} assay. Although the Baculovirus expression system is able to produce large amounts of GAD, we choose the COS-7 expressed GAD_{65} for our analyses.

This study provides evidence for the high incidence of GAD autoantibodies at onset of type 1 diabetes. No evidence for an additional compound of the 64kD precipitate was found. The data presented here show that the requirements for a specific and sensitive GAD antibody assay are high. GAD antibody analysis by immunoprecipitation is still not easily applicable in large scale studies. The

analysis by SDS-PAGE and fluorography requires time, although the use of phosphor-imagers can shorten this. The immunoprecipitation method can still be regarded as the most sensitive method for the analysis for GAD autoantibodies, but simpler and cheaper methods need to be developed.

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Value of Antibodies to GAD_{65} Combined with Islet Cell Cytoplasmic Antibodies for Predicting Type-1 (Insulin Dependent) Diabetes Mellitus in a Childhood Population.

Submitted

VALUE OF ANTIBODIES TO GAD_{65} COMBINED WITH ISLET CELL CYTOPLASMIC ANTIBODIES FOR PREDICTING TYPE 1 (INSULIN-DEPENDENT) DIABETES MELLITUS IN A CHILDHOOD POPULATION

Henk-Jan Aanstoot[1], Engilbert Sigurdsson[2], Marc Jaffe [1], Yuguang Shi [1],Stephan Christgau[1,2], Derek Grobbee[3], G. Jan Bruining[4], Jaques L. Molenaar[5], Albert Hofman[3] and Steinunn Baekkeskov[1,2].

1. Hormone Research Institute, Depts. of Microbiology / Immunology and Medicine, University of California San Francisco, CA 94143-0534, USA.

2. Hagedorn Research Laboratory, Gentofte, Denmark.

3. Dept. of Epidemiology, Erasmus University Medical School, Rotterdam, the Netherlands.

4. Depts. of Pediatrics, Division of Congenital / Hereditary diseases, Erasmus University Medical School and Sophia Children's Hospital, Rotterdam, the Netherlands.

5. Dept. of Immunology, Stichting Samenwerkende Delftse Ziekenhuizen, Delft, the Netherlands.

Dr. H.-J. Aanstoot's current address is: Depts. of Pediatrics, Division of Congenital / Hereditary Diseases, Erasmus University Medical School and Sophia Children's Hospital, Rotterdam, the Netherlands.

SUMMARY

The value of a test for islet cell cytoplasmic antibodies together with a test for GAD_{65} antibodies to predict the subsequent development of diabetes over a period of 11.5 years was assessed in a childhood population comprising 2,805 individuals. A single serum sample was obtained from each individual between 1975 and 1977 and screened for complement fixing islet cell cytoplasmic antibodies. Eight individuals were positive (0.29%). During the average follow-up period of 11.5 years, 4/8 islet cell antibody positive and 3 islet cell antibody negative individuals developed clinical diabetes. Sera from all individuals, who were islet cell antibody positive and/or developed diabetes (total of eleven) and from 100 randomly selected controls were analyzed for GAD_{65} antibodies. Six of eight islet cell antibody positive individuals were GAD_{65} antibody positive including all 4 who subsequently developed Type 1 diabetes. Furthermore one of the three islet cell antibody negative individuals who developed Type 1 diabetes was GAD_{65} antibody positive both in 1976 and in 1989. Thus a positive test for GAD_{65} antibodies alone correctly predicted diabetes in 5 of 7 children, who developed the disease. Only one of the children, who developed diabetes was positive for insulin autoantibodies and this individual was also positive for islet cell cytoplasmic antibodies and GAD_{65} antibodies. One of the 100 control individuals was positive for GAD_{65} antibodies (1%). The results suggest that a single GAD_{65} antibody test may have a higher sensitivity for predicting Type 1 diabetes than a test for islet cell cytoplasmic antibodies, but that a combined positive test for both antibodies increases the specificity for predicting Type 1 diabetes over a period of 11.5 years.

INTRODUCTION

The destruction of pancreatic ß-cells, which precedes the clinical onset of Type 1 (insulin-dependent) diabetes mellitus, is mediated by autoimmune mechanisms (1). The presence of islet specific autoantibodies in the prediabetic period (2-4) is likely to reflect the ongoing autoimmune process, one that eventually leads to critical ß-cell depletion and insulin dependency. A major goal of diabetes research is to develop immune interventions that block or otherwise interfere with the destruction of ß-cells and the development of clinical diabetes. Concomitant with this goal is the necessity of methods for early accurate identification of susceptible individuals (5). Antibody assays that detect early signs of humoral autoimmunity associated with ß-cell destruction are obvious candidates for this

purpose. Furthermore susceptibility to develop diabetes is associated with certain MHC-class II haplotypes (6) and analysis of both HLA haplotypes and autoantibodies provide a test of high predictive value in family members of Type 1 diabetic patients (3, 7). Since most new cases of Type 1 diabetes involve individuals without a first degree relative with the disease (8,9), such methods are not easily applicable for prediction in the general population.

The classical method to analyze islet cell autoantibodies is by immunohistology using frozen pancreatic sections. The ICA assay is however difficult to standardize (10). A better method for a standardized analyses of early humoral responses in Type 1 diabetes would involve a rapid and quantitative assay using a target antigen of humoral autoimmunity in type 1 diabetes like insulin (11), or the GAD_{65} autoantigen (12).

Insulin autoantibodies (IAA's) are only detected in up to 50% of newly diagnosed patients and are most frequent in very young patients (< 5 yrs of age) (13). IAA's alone have a limited predictive value and are not suitable as a marker to replace ICA's (5).

Incidence of 64kD or GAD_{65} autoantibodies in the prediabetic period and at clinical onset of type 1 diabetes measured by immunoprecipitation of 35-S methionine labeled human islet cell protein is 75-90% (4,15). Following identification of the 64kD protein as the smaller form of the enzyme glutamic acid decarboxylase (12,16), now called GAD_{65}, several assays have been developed and standardization is just beginning (1st GAD antibody workshop was held under the auspices of the 12th International Immunology of diabetes Workshop, Orlando, April 15-18, 1993). GAD_{65} antibodies precede Type 1 diabetes by several years and are detected concomitantly with, and sometimes before ICA and IAA (4,15,17).

GAD_{65} is a cytoplasmic protein and is amongst the target antigens of ICA's (19). Purified GAD_{65} however does not block all ICA reactivity in some sera (19) and the ICA reactivity is likely to involve other targets molecules. Furthermore sera that immunoprecipitate GAD_{65} do not always react with the protein by immunohistology and are therefore negative in the ICA assay (15,20). In most Type 1 diabetic patients, ICAs only stain frozen and not fixed sections of human pancreas. Thus ICA epitopes are dependent on conformation of the relevant antigen (21). Similarly diabetes associated epitopes in the GAD_{65} molecule are predominantly conformational and only very few patients recognize a linear epitope in the protein (12,18).

In addition to GAD_{65}, GAD is expressed as a second non-allelic form, GAD_{67} in neurons (22) and in rat and mouse ß-cells (23). Human islets only express the GAD_{65} protein (23, 24) and the incidence of GAD_{67} antibodies in Type 1 diabetes is low (25, 26). GAD_{67} does not seem to have an independent role as an autoantigen in Type 1 diabetes. Rather this protein only seems to be recognized in cases where the immune response to GAD_{65} involves recognition of epitopes that crossreact in the two proteins (26).

Previous studies have suggested that islet cell cytoplasmic antibodies (ICA) measured by immunohistology are of insufficient specificity to serve as a single marker of susceptibility (5, 27, 28). One study of the predictive value of ICA in a Dutch childhood population without emphasis on families of diabetes patients, found an incidence of 0.29%, which is close to the incidence of Type 1 diabetes in the Netherlands. In this study, all the ICA positive individuals had complement fixing ICA (CF-ICA) and the antibodies preceded the clinical onset non-familial diabetes in some individuals by several years (29). The predictive value of a positive CF-ICA test on a single occasion to predict the development of Type 1 diabetes in a subsequent 11.5 year follow-up period was 50%. The sensitivity of CF-ICA to predict Type 1 diabetes was 57%.

The present study analyzed GAD_{65} autoantibodies in all individuals in this population which were either CF-ICA positive or developed Type 1 diabetes during the 11.5-year follow-up period, as well as in 100 randomly selected individuals that remained healthy during this period. Although it was not possible to analyze GAD_{65} autoantibodies in the whole population with the methodology currently available, we have calculated the potential of a combined test for CF-ICA followed by GAD_{65} antibody analysis to predict diabetes in a childhood population at large, based on a single serum drawing per child with a minimum follow-up of 10 years. The study shows that the combination of a positive test for CF-ICA and for GAD_{65} antibodies yields a strong predictive value for subsequent development of Type 1 diabetes in individuals who are not relatives of Type 1 diabetic patients and is more specific than a single positive test for either antibody.

METHODS

Description of the population

The population studied has been described in detail (29). Approval was obtained from the local ethical committees. Briefly, 4860 schoolchildren aged 5-19 years of age from one township in the Netherlands participated in a longitudinal

study on risk factors for cardiovascular and chronic diseases. The data collected included a history on Type 1 diabetes. Serum was collected from these children between 1975 and 1977. Serum samples of 3383 individuals were available for ICA analysis. In 1988, a questionnaire was sent to all subjects for who family data on diabetes were available. 2805 children fulfilled the three criteria of having a complete first-degree family history on Type 1 diabetes, a stored serum sample and an adequate response to the questionnaire by the end of an average follow-up time of 11.5 years. CF-ICA's and IAA's were measured in this population and their predictive value analyzed (29). A second serum sample was obtained in 1989 from 10 of the 11 individuals, who were ICA positive in 1975-1977 and/or who developed Type 1 diabetes in the interim period.

Measurement of GAD_{65} antibodies using rat islets.

Autoantibodies to GAD_{65} expressed in rat islets were measured by immunoprecipitation of Triton X-114 detergent phase purified cytosolic protein fraction (12). Immune complexes were analyzed by SDS-PAGE using 10% slab gels and processed for fluorography (30). Fluorograms were analyzed visually by three independent observers, who did not know the sample identity and scored positive if the GAD_{65} a/b doublet was visible. A quantitative estimate of GAD_{65} in positive immunoprecipitates was obtained by densitometric scanning of autoradiograms using a BioRad model 620 Video Densitometer with 1D Analyst II and version 3.10 software (BioRad, Richmond, CA) and a control serum which is used as a positive standard for quantitative analyses of GAD_{65} autoantibodies and has an arbitrary value set at 10 (20). GAD_{65} antibody values in other sera were calculated from integrated peak areas by the formula: 10 x (value for unknown serum - value for negative control serum) / (value for positive control serum - value for value for negative control serum)

Measurement of GAD_{65} antibodies using recombinant GAD_{65} expressed in COS-cells

The eucaryotic expression vector pMT2 (kindly donated by Dr. R. Kaufman, Genetics Institute, Boston, MA) was used for transient expression of rat GAD_{65} in COS-7 cells. Oligonucleotide directed mutagenesis was used to change the sequence surrounding the translation initiation site of GAD_{65} into the Kozak consensus sequence (31) for optimum expression in mammalian cells before subcloning into the pMT2 vector. A 2.4 kb cDNA fragment containing the coding region of rat GAD_{65} was released from a cDNA clone (kindly donated by Dr. A. Tobin, University of California Los Angeles, CA) by *EcoR I* restriction enzyme digestion and inserted into the *EcoR I* site of pMT2. The resulting plasmid was

named pMT2-rGAD$_{65}$-10. This clone was transfected into COS-7 cells (American Tissue Culture Collection, ATCC) using a lipofection kit (DOTAP, Boehringer Mannheim, Indianapolis, IN) according to the manufacturers instructions. Cells were labeled 48hrs after transfection using 400 mCi of ^{35}S methionine (Amersham SJ-1515) per 10 cm plate for 4hrs. After labeling cells were washed 3 times in an ice-cold harvest buffer (150 mM NaCl, 10 mM Hepes, 10 mM benzamidine/HCl, 0.1 mM p-chloromercuriphenyl sulfonic acid and 0.1 mM phenylmethylsulfonyl fluoride, pH=7.4). All following procedures were performed at 4^{o}C. The cells were collected by scraping into 1 ml of HEMAP buffer (10 mmol/l Hepes, pH 7.4, 1 mmol/l $MgCl_2$, 1 mmol/l EGTA, 0.2 mmol/l pyridoxal-5-phosphate, 1 mmol/l aminoethylisothiouronium bromide hydrobromide) containing 1% Triton X-114, followed by incubation for 10 min. and repeated pipetting through a bent pipette tip for 10 minutes. The solution was cleared by ultra centrifugation at 400.000 x g for 1 hr in a Beckman TLA-100 ultracentrifuge with a TLA 100.3 rotor (Beckman, Fullerton, CA) resulting in an S-400 fraction. This supernatant was subjected to temperature induced phase separation (32). The detergent phase, containing the soluble amphiphilic GAD$_{65}$, was collected and diluted in HEMAP buffer. Aliquots containing 200.000 cpm per immunoprecipitate were precleared using normal human serum. Precleared aliquots were incubated with test sera for 16 hours in a total volume of 220 ml. Immunecomplexes were isolated by incubation for 1 hr with protein-A Sepharose (Pharmacia, Linköping, Sweden) which had been coated with cold COS-7-cell lysate to avoid non-specific absorption. Immunocomplexes bound to Protein-A Sepharose were washed 5 times in IMP-washing buffer (10mmol/l Hepes, pH 7.4, 150mmol/l NaCl, 10mmol/l benzamidine/HCl, 0.5 mmol/l methionine, 5mmol/l EDTA, 0.1% BSA) and once in autoclaved H_2O. Immunocomplexes were eluted from Protein-A Sepharose, analyzed by SDS-PAGE and processed for fluorography. Fluorograms were analysed as described above. In addition quantitative analyses of GAD$_{65}$ on gels was performed using a model 425 Phospor-Imager (Molecular Dynamics, Sunnyvale, CA) and the same positive control serum for quantitative GAD$_{65}$ analyses. The results of the Phosphor-Imager analyses and the densitometric analyses were similar except that the former measurement is linear over a longer range.

Sera analyzed for GAD$_{65}$ antibodies included all 7 individuals who developed Type 1 diabetes, the 4 individuals who were ICA positive, but did not develop diabetes, and 100 age matched controls.

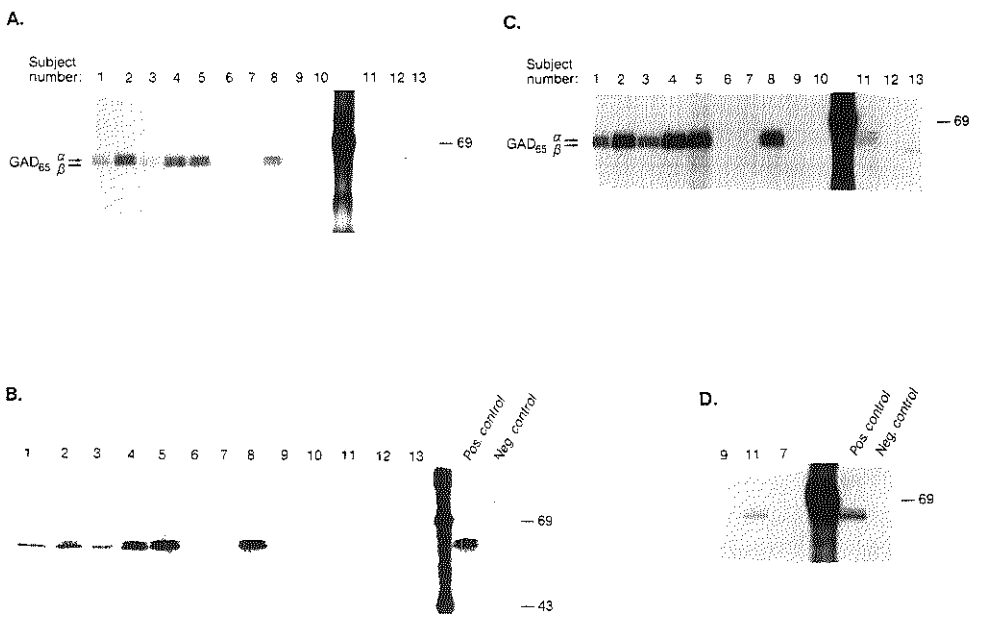
Figure 1. Analysis of GAD_{65} autoantibodies by immunoprecipitation. GAD_{65} autoantibodies were measured in a single serum sample drawn from subjects at baseline (1975-1977) (panels A,B,C) or in 1989 (panel D). Subjects 1-8 were ICA positive, subjects 9-14 were ICA negative. Subjects 1-4 and 9-11 developed Type 1 diabetes 1.8-7.1 years following the serum sampling. Subjects 12 and 13 were healthy ICA negative children.

A: Short exposure of a fluorogram of immunoprecipitates of rat islet cell GAD_{65} on an X-ray film.

B: Short exposure of a fluorogram of immunoprecipitates of COS-7 cell GAD_{65} on a Phospor-Imager.

C: Long exposure of the fluorogram in panel A

D: Short exposure of a fluorogram of immunoprecipitates of COS-7 cell GAD_{65} on an X-ray film.



HLA haplotyping, CF-ICA analyses, and IAA analyses

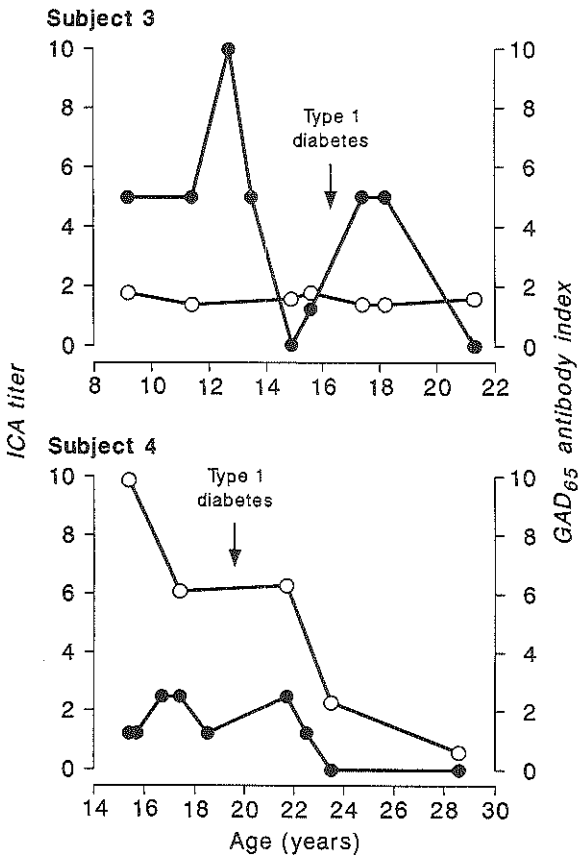
HLA haplotyping was performed as described by Giphart and co-workers (33). The ICA assay was described earlier (29). The same pancreas, which gave an unusually low background and high specificity in the analyses of the 1975-1977 samples (29), were used for analyses of the 1989 samples. ICA levels in samples were expressed in reciprocal titers. The Juvenile Diabetes Foundation world standard serum for ICA analyses and containing 80 JDF units, was positive on this pancreas up to a dilution of 1:256. Dilution of this standard serum showed a strict linear titration curve, 0.625 JDF units corresponding to a titer of 1:2. This assay participates in the International Diabetes Workshops on ICA proficiency. IAA were measured in each serum using both an ELISA assay, and a radioimmunoprecipitation assays (11). Both assays participate in the International Diabetes Workshops on IAA proficiency.

RESULTS

The GAD_{65} antibody assay

The analyses of GAD_{65} autoantibodies in this study included i) immunoprecipitation of the soluble hydrophobic form of GAD_{65} partially purified from rat islets (12,16) (Figure 1A and C), and ii) immunoprecipitation of partially purified membrane and soluble forms of recombinant GAD_{65} expressed in COS-7 cells (Figure 1B and D). The first assay is a derivative of the standard immunoprecipitation assay using GAD_{65} expressed in islet cells (4, 20) but uses only the hydrophobic soluble fraction of islets. Since very few cytosolic proteins in rat islets, except GAD_{65}, are hydrophobic, the use of this fraction results in very low background. Both methods are equally sensitive in detecting GAD_{65} autoantibodies in low-high titer sera. However the first method is more sensitive for analyses of very low titer sera, because it has a higher signal to noise ratio and allows specific detection of very low levels of the characteristic α and β doublet of GAD_{65} in immunoprecipitates (30) by long exposures of autoradiograms. This enhanced sensitivity was necessary for detection of GAD_{65} antibodies in the baseline serum from subject #11 (Figure 1C) and the 1989 serum from subject #4 in long exposure of fluorograms. By comparison the COS-7 cell assay did not detect a GAD_{65} signal above background in immunoprecipitates with those two samples after long exposures. Both assays however detected GAD_{65} antibodies in the same individuals in either earlier (subject # 4) or later (subject # 11) samples (Figure 1, Table 1).

Figure 2
Longitudinal analyses of 64kD/GAD autoantibodies and ICA in subjects 3 and 4.



Prevalence of Type 1 diabetes in the Follow-up Period

Seven children developed Type 1 diabetes, which gave a cumulative incidence of Type 1 diabetes during an average follow-up period of 11.5 years of 0.25% (7/2805). None of those 7 children had a first degree family member with Type 1 diabetes or non-insulin dependent diabetes mellitus (Type 2 diabetes).

Prevalence of CF-ICA, GAD_{65} antibodies, and IAA in 1975-1977

Eight of 2,805 (0.29%) children (#1-8 table 1) were positive for CF-ICA at the baseline timepoint between 1975-1977. Four of these (#1-4 Table 1) were among the 7 who developed Type 1 diabetes during the follow-up period. Sera from the 7 individuals who developed diabetes as well as from the other 4 ICA positive individuals were assayed for GAD_{65} autoantibodies (Figure 1A,B,C, Table 1) and IAA. Six of the 8 ICA positive individuals (#1-4, 5 and 8, Figure 1A,B,C, Table 1) were positive, including all 4 who subsequently developed Type 1 diabetes. One of those individuals (#3) was the only IAA positive individual by the ELISA assay whereas none were positive by the radio immunoprecipitation assay.

Among the three ICA negative children who developed Type 1 diabetes during the follow-up period, two (#9 and 10, Figure 1A,B,C, Table 1) were also negative for GAD_{65} antibodies in the serum sample collected between 1975-1977. One subject (#11 Figure 1, Table 1) was scored negative for GAD_{65} antibodies based on a short exposures of fluorograms of rat islet immunoprecipitates and COS-7 cell immunoprecipitates (Figure 1A). However, prolonged exposure of rat islet immunoprecipitates clearly revealed that this serum immunoprecipitated the characteristic a and b doublet of the GAD_{65} autoantigen (Figure 1C). Amongst 100 healthy ICA negative subjects included in the GAD_{65} antibody analyses, one individual, a 16 year old female, was GAD_{65} antibody positive.

GAD_{65} Antibody status 12-13 Years After the Initial Sampling

In 1989 sera were collected from all the 8 subjects who were ICA positive at baseline (#1-8) and from 2 of the ICA negative subjects (#10-11) who had developed Type 1 diabetes. Subject #9 was not available for testing in 1989. Sera were assayed for GAD_{65} autoantibodies and ICA (Figure 1D, Table 1). The ICA and GAD_{65} antibody positive individuals who had developed Type 1 diabetes had either become antibody negative or decreased significantly in titer. The ICA negative, GAD_{65} antibody positive individual (#11) had a stronger GAD_{65} immunoreactivity in 1989 than in 1977 (Figure 1D, Table 1). The two individuals who were ICA/GAD_{65} antibody positive at the baseline timepoint (#5 and 8) but who had not developed Type 1 diabetes during the observation period, were still

Table 1: Summary of autoantibody and HLA-analyses in individuals which were either positive for islet cell cytoplasmic antibodies (ICA) at baseline and/or developed diabetes during an average of 11.5 year observation period.

Subject Number/Sex	Age at Baseline	Age at Diagnosis	Interval (Years)	HLA DR	HLA DQw	ICA 1975-77	ICA 1989	GAD_{65} autoantibodies[a] 1975-77	GAD_{65} autoantibodies[a] 1989
ICCA + => diabetes									
1M	6.1	12.4	6.3	2,3	2,6	2	Neg	2.9	Neg
2M	7.5	12.4	4.9	3	2	32	Neg	4.0	Neg
3 F	9.2	16.3	7.1	3,4	2,8	16	8	1.8	1.6
4 F	15.4	19.6	4.2	3,4	2,8	4	Neg	9.9	0.6[b]
ICCA + => healthy									
5 M	7.4			3,4	2,8	32	16	14.9	13.9
6 F	7.6			2	6	4	Neg	Neg	Neg
7 M	14.7			1,w6	5,6	32	64	Neg	Neg
8 M	18.2			1,w6	5,6	16	32	14.2	12.0
ICCA - => diabetes									
9 M	5.1	12.3	7.5	NA	NA	Neg	NA	Neg	NA
10 M	12.4	14.2	1.8	4,w6	6,8	Neg	Neg	Neg	Neg
11 M	12.7	16.2	3.5	1,5	5,7	Neg	Neg	0.5[b]	3.2

[a] The quantitative values are based on Phospor-Imager analyses except for those marked by [b], which are densitometric values.

ICA and GAD_{65} positive in 1989, which was 12 and 13 years later respectively. Neither of those individuals has a first degree family member with Type 1 or Type 2 diabetes. However, a niece to subject #8 had a clinical onset of Type 1 diabetes in 1989. Subject #6 was still GAD_{65} antibody negative and had also become ICA negative in 1989. Subject #7 was still GAD_{65} antibody negative and ICA positive in 1989. Subject #10 was ICA and GAD_{65} antibody negative both at baseline and in 1989 which was 1.8 years before and 11.9 years after clinical onset of Type 1 diabetes, respectively. The positive control individual had no family history of Type 1 diabetes. She did not consent to an additional serum sampling to test whether the GAD_{65} antibodies had persisted over several years.

Longitudinal Analyses of ICA and GAD Antibodies in Two Subjects

Two girls (subjects #3, 4) also participated in a cohort study for general health risk factors. Each developed Type 1 diabetes during the period of this study. Both were re-examined every 12 months. ICA and GAD_{65} antibody titers were retrospectively analyzed and compared in the sequential sera (Figure 2). Subject #3 remained weakly positive for GAD_{65} antibodies throughout the 11 year observation period, which included 5 years following the clinical onset of Type 1 diabetes. Subject 4 showed a continual decrease from a high GAD_{65} antibody immunoreactivity during the 13 year observation period. She was weakly positive in the last sample, which was obtained 9 years following clinical onset of Type 1 diabetes. A similar pattern was observed for ICA.

Sensitivity of Individual and Combined Assays to Predict Type 1 diabetes

The sensitivity of a combined antibody assay positive for both ICA and GAD_{65} antibodies in a single sample to predict Type 1 diabetes in the 7 children who had a clinical onset during the follow-up period was 57% (4/7) or the same as for ICA alone. The sensitivity of a GAD_{65} antibody assay alone to predict Type 1 diabetes in the 7 children was 71% (5/7).

The sensitivity of a combined assay positive for both ICA and IAA in a single sample to predict Type 1 diabetes in the 7 children, who had a clinical onset during the follow-up period was 14% (1/7) or the same as the sensitivity of IAA antibodies alone and several fold lower than the sensitivity of the ICA and GAD_{65} antibody assays alone.

Predictive Value of ICA Combined with GAD_{65} Autoantibodies

The lack of GAD_{65} antibody data for all but 111 of the 2.805 children constituting this study base excludes the calculation of a positive and a negative

predictive value and a specificity for GAD_{65} antibodies alone (35,36). However, these parameters can be calculated for a combined ICA and GAD_{65} antibody assay. Thus the probability of a child acquiring Type 1 diabetes within 10 years if both autoantibodies were positive at baseline (positive predictive value) was 67% (4/6) as compared to 50% (4/8) for ICA alone. The probability of absence of clinical Type 1 diabetes among subjects not positive for both ICA and GAD_{65} antibodies (negative predictive value) was 99.89% (2796/2799) or the same as for ICA alone (2794/2797 = 99.89%). The specificity of a test positive for both ICCA and GAD_{65} antibodies to predict Type 1 diabetes within 10 years was 99.93% (2796/2798) as compared to 99.86% (2794/2798) for ICA alone.

Predictive Value of ICA Combined with IAA

The positive predictive value for a combined ICA and IAA assay was 100% (1/1), which was higher than for a combined assay for ICA and GAD_{65} antibodies. Similarly the specificity of the combined assay was 100% (2798/2798). The probability of absence of clinical Type 1 diabetes among subjects not positive for both ICA and IAA was 99.79% (2798/2804) compared to the negative predictive value for ICA alone and ICA combined with GAD_{65} antibodies of 99.89%.

HLA-Tissue Typing

HLA DR and DQ haplotypes are shown in Table 1. Subjects 1-5 and 10 were HLA DR3 and/or 4 positive. All the HLA DR4 positive subjects were DQw8 (DQB-0302) which is present in 95% of DR4 positive Type 1 diabetic patients versus 50% of DR4 positive controls (6,37). Subjects 7, 8 and 11 had the DR1 DQw5 (DQB 0501)haplotype, which is associated with a weakly increased risk of Type 1 diabetes (37). Subject 6 was HLA-DR2, DQw6 (DQB0602), which is negatively associated with Type 1 diabetes (38). Subject #9 was not available for HLA-typing. The positive control individual did not consent to HLA-typing.

DISCUSSION

The high incidence of 64kDa GAD_{65} antibodies in prediabetic individuals and their early appearance (4,15) suggests that these antibodies are an early sensitive marker of an ongoing autoimmune aggression to the insulin producing beta cells. The identification of GAD_{65} as a major humoral autoantigen in Type 1 diabetes and the availability of this protein both from brain and from cellular expression systems has greatly facilitated the analyses of those antibodies. A rapid assay of a sensitivity and specificity smilar to that of the immunoprecipitation assay is needed for an accurate assessment of the predictive value of GAD_{65} antibodies in large populations. Such an assay is however still not available.

In the meantime, we have attempted to assess the predictive value of a combined assay for CF-ICA and GAD_{65} antibodies in a single serum sample and an average follow-up of 11.5 years in a low risk childhood population. With regard to specificity, the results demonstrate that compared to CF-ICA antibody measurements alone, ICA analyses supplemented with GAD_{65} autoantibody analysis are likely to have increased specificity in predicting Type 1 diabetes. Of a normal low risk population of 2805 children who represented the study base for these analyses, 4 children would be incorrectly predicted to become diabetic in a 10-year follow-up by a single positive test for CF-ICA alone. This number was decreased to 2 subjects by requiring both CF-ICA positivity and GAD_{65} autoantibody positivity. Although analysis of GAD_{65} antibodies in 2805 sera was not possible by the current methodology, the presence of GAD_{65} antibodies in 1/100 individuals randomly selected among the ICA negative subjects, who did not develop Type 1 diabetes, confirms earlier data on the incidence of these antibodies in healthy individuals (39 for review). An incidence of 1% is however 3-4 fold higher than the cumulative incidence of Type 1 diabetes in the Netherlands suggesting that a positive test for GAD_{65} antibodies alone, measured by current methods, is not sufficient to accurately predict the clinical onset of disease within a period of 11.5 years.

This study has confirmed the early appearance of GAD_{65} autoantibodies in a non-familial study. The antibodies were detected in 5 individuals in a single serum sample obtained 31/2-7 years before clinical onset of Type 1 diabetes. None of these subjects had a first-degree family member with Type 1 or 2 diabetes. The sensitivity of GAD_{65} antibodies to detect individuals experiencing ß-cell autoimmunity may be higher than for ICA. Thus amongst the 3 CF-ICA negative individuals who developed Type 1 diabetes, one was positive for GAD_{65} antibodies at baseline and in 1989, which was 3.5 years before and 9.5 years after clinical onset respectively. The increase in sensitivity of the single GAD_{65} antibody test compared to the double antibody test was however counterbalanced by a decrease in sensitivity. It is possible however that the predictive value of a test for GAD_{65} autoantibodies alone can be increased by introducing different GAD_{65} antibody specificities. Recent studies have identified conformational epitopes associated with Type 1 (18), linear SMS specific epitopes (12), and a linear epitope associated with protection against disease (40) in the GAD_{65} molecule. Assays that distinguish GAD_{65} antibodies of different epitope specificity may therefore be the next candidate for predictive tests. Alternatively, a combination of a test for GAD_{65} antibodies for high sensitivity and early detection, and a test for antibodies that appear late in ß-cell destruction, which be indicative of massive loss

of ß-cells, may provide a better predictive value. A candidate for the latter test are the 37kD antibodies described by Christie et al. (41). Finally since ß-cell destruction in humans is likely to be T-cell mediated as in the animal models, (1) and antibodies may not have a direct pathogenic role, the predictive value of antibody tests may always have the limitations of an indirect parameter. It is certainly conceivable that an autoimmune response induced to an antigenic epitope in a particular HLA-background is dominated by cytotoxic T-cells and massive ß-cell destruction whereas the response to other epitopes in the same molecule in the same or different HLA-haplotype is restricted to antibodies and compatible with an intact or only minor loss of ß-cell mass.

In the absence of a direct test for ß-cell destruction, a combination of a positive test for CF-ICA and GAD_{65} autoantibodies together with HLA-typing may provide the most accurate predictive test currently available. In this regard it is notable however that subject #5, who has been positive for CF-ICA and GAD_{65} antibodies for more than 12 years, has the HLA-DR3, DQw2 and DR4,DQw8 haplotype, which confers the highest known relative risk of Type 1 diabetes (7). Thus even this high risk MHC allele in conjunction with both CF-ICA and GAD_{65} autoantibodies is still not sufficient to rapidly induce the onset of Type 1 diabetes.

Both CF-ICA, IAA, and GAD_{65} antibodies were absent at baseline in 2 individuals who later developed Type 1 diabetes. It is not known whether antibodies never developed in these individuals or whether antibody positive periods were missed by the single sampling at baseline. Population studies employing regular sampling of sera over 1 or 2 decades are clearly needed to assess the full value of antibody testing in predicting Type 1 diabetes, and in particular to assess whether some individuals mount an autoimmune response which is restricted to T-cells, or alternatively, experience a beta cell destruction which is not of autoimmune etiology.

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Chapter 4

THE 38kD AUTOANTIGEN IN TYPE 1 DIABETES MELLITUS.

4.1

Identification and Characterization of a 38kD Pancreatic ß-cell Antigen Which Together With Glutamic Acid DecarboxylaseMarks the Early Phases of Autoimmune Responses in Type 1 Diabetes.

Submitted

Identification and characterization of a 38kD pancreatic ß-cell antigen which together with glutamic acid decarboxylase marks the early phases of autoimmune response in type 1 diabetes.

Henk-Jan Aanstoot, John Kim, Mikael Knip* Mark Atkinson‡, Peter Mose-Larsen§, Stephen Fey§, Qin Fu, Johnny Ludvigsson¶*, Mona Landin, Jan Bruining‡‡, Noel Maclaren‡ , Hans K. Åkerblom¶¶, and Steinunn Bækkeskov**

Depts. of Medicine and Microbiology/Immunology and Hormone Research Institute, University
of California San Francisco, San Francisco, CA
*Department of Pediatrics, University of Oulu, Oulu, Finland
‡Dept. of Pathology and Laboratory Medicine, University of Florida, Gainesville, FL
§Department of Medical Microbiology, Århus University, Århus, Denmark
¶Dept. of Pediatrics, University Hospital, Linköping, Sweden
**Dept. of Internal Medicine, University Hospital, Lund, Sweden
‡‡Erasmus University Medical Center, Sophia Childrens Hospital, Rotterdam, The Netherlands
¶¶The Childrens Hospital, Second Dept. of Pediatrics, University of Helsinki, Helsinki, Finland, and the Childhood of Diabetes in Finland Study Group **(1)**

Dr. Aanstoots current address is: Erasmus University Medical Center, Depts of Pediatrics and Clinical Genetics, Sophia Childrens Hospital, Rotterdam, The Netherlands

SUMMARY

Antibodies to the enzyme glutamic acid decarboxylase (GAD_{65}) are present in 70-80% of individuals experiencing both early and later phases of pancreatic ß-cell destruction and development of type 1 diabetes. A few individuals, who are positive for islet cell antibodies detected by immunofluorescence of frozen sections of human pancreas (ICA) are GAD_{65} antibody negative. Here we identify a second ß-cell antigen, an amphiphilic membrane protein of Mr 38kD and pI 5.6-6.1, which is specifically immunoprecipitated under native conditions by IgG antibodies present at clinical onset of diabetes in a subset of patients (12/49=24% compared to 37/49=76% GAD_{65} antibody positive) some of who (6/49=12%) are negative for GAD_{65} antibodies. To address the question whether the 38kD protein is a target of early B-cell responses in this disease, we analysed sera from 44 prediabetic individuals and from 37 children who developed type 1 diabetes at a very young age. Six (14%) prediabetic individuals were positive for 38kD antibodies in the very first sample available which was 3, 9, 25, 33, 53, and 74 months before clinical onset of disease respectively. Four young children (11%) were positive for 38kD antibodies at clinical onset of disease at age 1.3, 1.6, 1.8, and 4.8 yrs. 33 (75%) and 28 (76%) patients were GAD_{65} antibody positive in the two groups. Taken together anti-38kD and/or anti-GAD_{65} antibody positive individuals accounted for all ICA positive sera in the prediabetic group but not in the newly diagnosed groups consistent with the appearence of antibodies to additional antigens in later stages of ß-cell destruction in some individuals. The diabetes associated epitopes in the 38kD protein are conformational and do not crossreact with GAD_{65} autoantibodies. Thus GAD_{65} and the 38kD autoantigen represent two distinct alternative and sometimes coinciding targets of early humoral autoimmunity associated with ß-cell destruction and type 1 diabetes.

INTRODUCTION

Pancreatic ß-cells in islets of Langerhans can be destroyed by autoimmune processes resulting in insulin dependent or type 1 diabetes (2). The destruction often proceeds over a long period of time before the clinical symptoms develop (3, 4). The gradual loss of ß-cells is accompanied by circulating islet cell antibodies demonstrated by indirect immunofluorescence staining of frozen sections of human pancreas. Such antibodies are referred to as ICA (3, 4). Although ß-cell destruction is believed to be mediated by T-cells (5), islet cell antibodies detected in the early phases of ß-cell destruction are likely to be directed to the same antigen as pathogenic T-cells. Furthermore antigen specific B-cells may play an important role in presentation of rare ß-cell autoantigens to maintain a chronic autoimmune

response that gradually depletes the ß-cell pool. ICA epitopes are usually only detected on frozen but not on fixed pancreatic tissue consistent with their conformational nature. Immunoprecipitation under native conditions has identified the smaller form of the neuroendocrine enzyme glutamic acid decarboxylase, GAD_{65}, as a target of islet cell antibodies associated with early as well as late stages of ß-cell destruction in 70-80% of patients (6-8). GAD_{65} is the synthesizing enzyme for the major inhibitory neurotransmitter GABA and is expressed in significant amounts in ß-cells and in GABA-ergic neurons (9). The diabetes associated epitopes in GAD_{65} are predominantly conformational and only include a linear epitope in very rare cases (6, 10, 11). The non-allelic form of the enzyme, GAD_{67}, which is expressed in human brain but not in human ß-cells (12,13), is 78% identical to GAD_{65} in the epitope areas (10,14) and yet is only rarely recognized by autoantibodies in type 1 diabetes (15,16).

A small fraction of type 1 (insulin dependent) diabetic patients have islet cell antibodies detected by immunofluorescence on frozen sections of human pancreas (ICA), but do not recognize GAD_{65} (17-19). Furthermore GAD does not always block ICA reactivity in diabetic sera suggesting that autoantibodies can be directed to additional antigens in this disease (20,21). Reactivity with denatured carboxypeptidase H (22), a 52kD protein (23) and a 69kD protein with homology to bovine serum albumin (24) on western blots has been reported in some diabetic sera. Those proteins are however unlikely to be targets of ICA in sera which only react on frozen but not fixed sections of human pancreas. We have sporadically detected an antigen of Mr 38kD in immunoprecipitates of rare preparations of human (17) and rat islets cell proteins (unpublished results) using human type 1 diabetic sera and native conditions. Furthermore antibodies to a 38kD rat islet antigen have been reported in the BB-rat, an animal model of type 1 diabetes (25). T-cell reactivity to a non-characterized insulinoma protein (26,27) of a similar relative molecular weight has also been detected in newly diagnosed diabetic patients. Recently the 38kD nuclear transcription factor jun-B was suggested to be an autoantigen in type 1 diabetes (28). Finally a 37kD tryptic fragment has been detected in immunoprecipitates of trypsinized but not intact islet cell membranes with diabetic sera (29,30). We now report that a vigorous extraction of rat islet cell proteins in detergent results in a consistent detection of a 38kD ß-cell antigen in immunoprecipitates with a subgroup of type 1 diabetic sera. The difficulty in extracting the 38kD protein indicates that it is relatively insoluble and thus has escaped detection in many previous immunoprecipitation analyses with type 1 diabetic sera. Using an improved extraction method for solubilizing islet cell proteins, we have analyzed the incidence of 38kD autoantibodies in newly

diagnosed and prediabetic individuals and determined different characteristics of the 38kD antigen.

METHODS

Preparation of islet cell extracts and immunoprecipitation analysis

Neonatal rat islets were isolated and labeled with ^{35}S methionine as described (31). Islets were swollen on ice for 10 min in HEMAP buffer (10mM Hepes pH 7.4, 1mM $MgCl_2$, 1mM EGTA, 1mM aminoethyl-isothiouronium bromide hydrobromide, and 0.2 mM pyridoxal phosphate), followed by homogenization by 20 strokes in a glass homogenizer. The homogenate was centrifuged at 100,000g for 1 h to obtain a cytosol and a particulate membrane fraction. Preliminary experiments using different detergents. The particulate membrane fraction was extracted in HEMAP-buffer with 2% Triton X-114 for 2h by repeated dispersion through a bended pipette tip, followed by centrifugation at 100,000g to remove debris. Amphiphilic proteins in both the cytosol fraction and the membrane extract were purified by temperature induced TX-114 phase separation (6). The detergent phase of either membrane or cytosol fractions were precleared with a normal human serum before immunoprecipitation with the indicated sera (31). Extracts of 250-500 rat islets was used per immunoprecipitate. Immunoprecipitates were analyzed by SDS-PAGE using 15% gels and processed for fluorography (31). A quantitative estimate of the GAD_{65} and 38kD protein immunoreactivity of sera (antibody index) was obtained by densitometric scanning of bands corresponding to the proteins on autoradiograms using a Model 620 Video Densitometer with 1D Analyst II and version 3.10 software (BioRad, Richmond, CA). Serum I_{15} was used as an internal 38kD antibody positive control in all analyses and its value arbitrarily set at 10. 38kD antibody indexes in other sera were calculated from integrated peak areas by the formula: index = 10 x (value for unknown serum - value for negative control serum)/(value for serum I_{15} - value for negative control serum). Similarly GAD_{65} antibody indexes are expressed in relation to a standard positive control serum which is a Juvenile Diabetes Foundation (JDF) world standard for ICA analyses and is also used as a standard for quantitative analyses of GAD_{65} autoantibodies (18).

The βTC3 cell line was derived from a transgenic insulinoma (32). The αTC cell line was similarly derived from a transgenic glucagonoma (33) and was a gift from Dr. D. Hanahan (University of California San Francisco, CA). The GT1 cell line was derived from a gonadotropin releasing hormone secreting tumor from a transgenic mouse (34) and was a gift from Dr. R. Weiner (University of California San Francisco, CA). The rat hepatoma cell line HTC (35) was a gift from Dr. A. Reuser,

University of Rotterdam. All other cell lines (table 1) were obtained from American Tissue Culture Collection (Rockville, MD). Cell lines were cultured according to established methods and labeled with ^{35}S methionine (31). Membrane extracts were prepared, extracted and immunoprecipitated as described for neonatal rat islets using serum I_{15} and serum C_1. The immunoprecipitates were analysed by SDS-PAGE and fluorography (31).

Two-dimensional gelectrophoresis and determination of isoelectric points

Two dimensional gel-electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension was carried out as described (31,36). The isoelectric points were determined by coelectrophoresis with carbamylated creatin phosphokinase charge chain markers (BDH, Poole, Dorset, U.K.) and with total HeLa cellular proteins containing several hundred marker proteins of known Mr, pI and location in the two dimensional pattern. The radioactive spots were analysed by image scanning using the Bio-Image software version 4.6 (Millipore, Bedford, MA, U.S.A.).

Description of patient groups and sera

Sera were collected following informed consent from three groups of patients, i) 49 newly diagnosed Dutch and Swedish type 1 diabetic patients who developed diabetes at > 5yrs of age and 25 control individuals in the same age group; ii) 37 Finnish and Dutch newly diagnosed type 1 diabetic patients who developed diabetes at ≤ 5yrs of age, and 38 control individuals in the same age group ; and iii) 44 North-American and Dutch prediabetic individuals (age 2.6-49.9). The first (and sometimes only) serum available from the individuals in this group was sampled 3-85 months before clinical onset of type 1 diabetes.

An antiserum to jun-B (37) was from Dr. R. Bravo (Bristol-Myers Squibb Institute for Pharmaceutical Research, NJ). Two patient sera which recognize a 37kD fragment generated by trypsin digestion of rat islet cell membranes (29,30), were a gift from Dr. M. Christie (Oxford University, U.K.). A GAD_{65} antibody positive serum from a stiff-man syndrome patient was from Dr. R. Layzer (University of California San Francisco, CA).

ICA analysis

Islet cell cytoplasmic antibodies were analyzed by indirect immunofluorescence of frozen sections of human pancreas from cadaveric kidney donors of bloodgroup 0 (38-40) Samples were titrated and end point titers were defined as the highest titer of detectable ICA-staining. Positive samples were expressed in Juvenile Diabetes

Foundation-units by comparing their end point dilution to a standard calibration curve using the international Juvenile Diabetes Foundation reference serum provided by the Immunology of Diabetes Workshops (41). The test laboratories are participiants of the ICA proficiency program conducted under the auspices of the Workshops (42).

RESULTS AND DISCUSSION

The 38kD protein is an amphiphilic ß-cell membrane protein of pI 5.6-6.1

Extraction of the GAD_{65} antigen from islets of Langerhans is complete after 30 min in 1% non-ionic detergent. In contrast the 38kD protein is only sporadically detected in such extracts by immunoprecipitation with a serum from a newly diagnosed type 1 diabetic patient, I_{15}. In preliminary experiments several detergents (CHAPS, ß-octyl-glucoside, sodium deoxycholate, Triton X-114) were tested for their ability to extract the 38kD protein. A 2% concentration of each detergent effectively solubilized the 38kD during a vigorous 2 hour extraction of islet cell membranes. Triton X-114 for was selected for all further experiments to facilitate a partial purification of the 38kD protein by a temperature induced phase transition and separation of amphiphilic membrane proteins into the Triton X-114 detergent phase. The relative insolubility of the 38kD protein suggested that it was membrane bound. Cytosolic and membrane proteins were subjected to a Triton X-114 phase separation to assess the amphiphilicity of the 38kD protein (Figure 1A). In contrast to the GAD_{65} autoantigen which is found both as a soluble hydrophilic, a soluble amphiphilic, and a membrane bound amphiphilic form (43,44) (Figure 1A compare lanes 16 and 18), the 38kD protein was only detected in the particulate fraction, where it partitioned into the detergent phase (Figure 1A, lanes 15 and 16). Thus the 38kD protein is an amphiphilic membrane protein. The relative insolubility of the 38kD protein suggests that it is an integral membrane protein in contrast to GAD_{65}, which is anchored to membranes via lipid residues (44).

The 38kD protein was detected as a broad band on fluorograms of SDS-gels suggesting heterogeneity in size and/or charge (Figures 1 and 2). Two dimensional gel electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (31,36) revealed 7 spots of similar relative molecular weight and isoelectric points of 5.6-6.1 (results not shown).

In an analysis of neuroendocrine and non-endocrine cell lines, the 38kD antigen was only detected in immunoprecipitates of ßTC3 cells derived from a transgenic mouse ß-cell tumor (32), whereas glucagon producing αTC cells (33) as well as 13

other cell lines of endocrine and non-endocrine origin were negative (Table 1). Thus expression of the 38kD protein seems to be restricted to pancreatic ß-cells.

TABLE 1: Analysis of expression of the 38kD protein in different cell lines:

Cell Line	Origin	Expression
βTC3 (ref. 32)	Mouse pancreatic insulinoma	pos
αTC-2 (ref. 33)	Mouse pancreatic glugagonoma	neg
GT1 (ref. 34)	GnRH neuronal tumor	neg
PC12*	Rat adrenal pheochromocytoma	neg
CHO*	Chinese hamster ovary	neg
HeLa*	Human ovarian adenocarcinoma	neg
$T_{47}D$*	Human ductal breast carcinoma	neg
Sk-NEP-1*	Human nephroblastoma	neg
Cos-1*	Monkey kidney tumor	neg
CV-1*	Precursor of Cos-1	neg
HepG2*	Human hepatocellular carcinoma	neg
BHK-21*	Baby hamster kidney	neg
HTC (ref. 35)	Rat hepatoma	neg
TERA-2*	Human teratocarcinoma	neg
CCD-118Sk*	Human fibroblast	neg

*American Type Culture Collection, Bethesda MD; GnRH: gonatotropin-releasing hormone

The 38kD protein is not a fragment of GAD_{65}

Christie et al., have described antibodies in sera from type 1 diabetic patients which recognize a 37kD trypsin fragment of an islet cell protein of similar molecular mass as GAD_{65} (29,30). Such sera do not always recognize the full length native GAD_{65} molecule, suggesting either that a sequestered epitope may become exposed upon trypsinization or that the 37kD fragment is derived from a different protein from GAD_{65}. In light of this report we have addressed the question whether the 38kD protein is a fragment of GAD_{65} or otherwise related to GAD_{65}. Islet cell membrane extracts were sequentially immunoprecipitated with sera that contained GAD_{65} or 38kD antibodies. Supernatants depleted for immunoreactive GAD_{65} were still positive for the 38kD protein (Figure 2). Similarly supernatants depleted for immunoreactive 38kD protein still contained the GAD_{65} molecule. Thus the native 38kD protein and the native GAD_{65} molecule do not display immunological

Figure 1A. Immunoprecipitation of the 38kD protein from Triton-X-114 detergent phase purified particulate and cytosol fractions of islet cells by diabetic sera.

Fluorogram of an SDS-PAGE showing immunoprecipitation of membrane and cytosol fractions of ^{35}S methionine labeled islet cell proteins with sera from newly diagnosed diabetic patients I_1-I_{16} (lanes 1-18), a stiff-man syndrome serum (lane 19) and sera from healthy controls C1-C3 (lanes 20-22). GAD_{65} which splits into two bands a and ß, can be seen in immunoprecipitates from both membrane and cytosol fractions with serum from patient I_{16} (lanes 16 and 18) whereas the 38kD protein is only detected in immunoprecipitates from the membrane fraction with serum from patient I_{15} and I_{16} (compare lanes 15-16 with lanes 17-18).

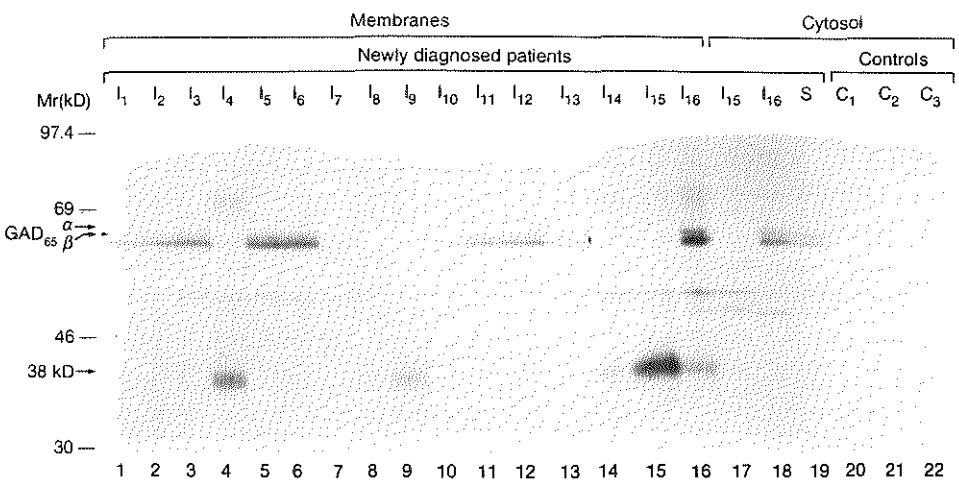
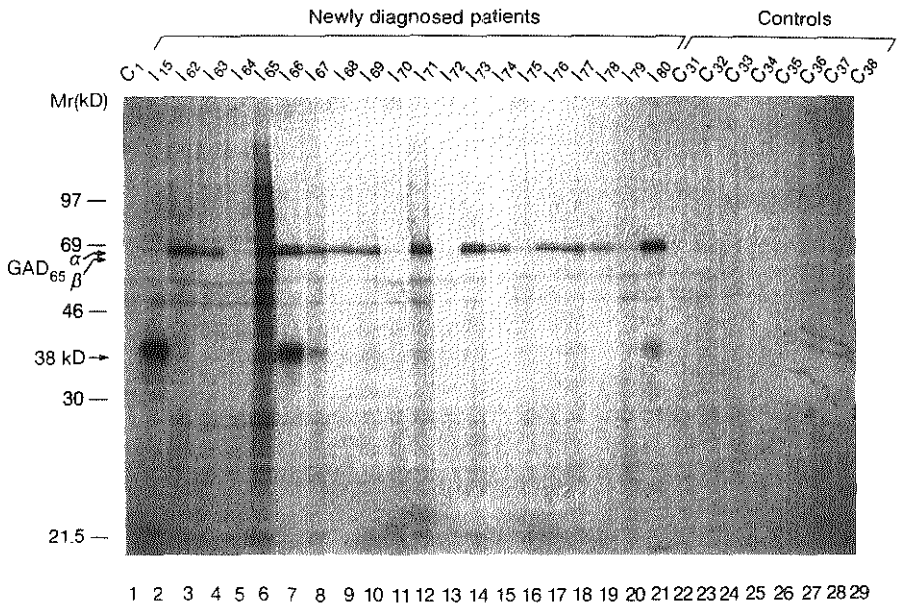


Figure 1B. Immunoprecipitation of the 38kD protein from Triton-X-114 detergent phase purified particulate and cytosol fractions of islet cells by diabetic sera.

Immunoprecipitation of membrane fractions of ^{35}S methionine labeled islet cell proteins with sera from newly diagnosed diabetic patiens I_{15} and I_{62}-I_{80} (lanes 3-21) and healthy controls C_1 and C_{31}-C_{38}. The diabetic sera recognize either the 38kD protein alone, the GAD_{65} protein alone, both proteins or no specific protein.



crossreactivity. Next we analysed whether 38kD sera recognize a 37/40kD fragment of GAD_{65}, which was generated by a mild trypsin digestion of either protein. Although several GAD_{65} antibody positive sera recognized the 37/40kD GAD_{65} trypsin fragment, the sera which were only positive for 38kD antibodies did not recognize this fragment (results not shown). Finally two 37kD antibody positive sera (29,30) (kindly provided by Dr. M. Christie, Oxford University) did not immunoprecipitate the 38kD antigen (results not shown). In sum those results show that the 38kD protein is distinct from GAD_{65} and distinct from the 37kD fragment described by Christie et al. (29,30).

The 38kD ß-cell antigen in type 1 diabetes is not jun-B

Honeyman et al. (28) reported recently the isolation of a cDNA clone encoding jun-B by antibody screening of an expression library from islets and placenta using a human antiserum from a type 1 diabetic patient. They subsequently detected peripheral T-cell responses to jun-B in some type 1 diabetic patients and their relatives and concluded that jun-B is a target autoantigen in type 1 diabetes. Jun-B has a Mr of 38kD. However jun-B is a soluble nuclear protein and its isoelectric point is significantly more basic than that of the 38kD autoantigen described here. Thus rat jun-B has a pI $\geq$ 9 on our 2D-gels and the calculated pI for human jun-B based on the amino acid sequence (45) is 9.58. Furthermore antibodies to jun-B did not immunoprecipitate the 38kD protein (results not shown). We therefore conclude that the 38kD antigen described here is not jun-B.

Autoantibodies to the 38kD antigen are present in a subgroup of newly diagnosed diabetic individuals and complement GAD_{65} autoantibodies

Using the improved method for solubilization of the 38kD antigen, we analysed 38kD antibodies in 49 individuals who developed diabetes at > 5yrs and 25 controls in the same age group (Figure 1, Table 2). Twelve patients (24%) and none of the controls were positive for antibodies to the 38kD protein. In comparison 37 (76%) patients and none of the controls were positive for GAD_{65} antibodies. Six patients (12%) were positive for 38kD antibodies only. Thus the cumulative incidence of GAD_{65} and/or 38kD antibodies was 84%

Both the 38kD antigen and GAD_{65} are targets of early B-cell responses associated with ß-cell destruction

ß-cell destruction can progress for several years before the first clinical symptoms of type 1 diabetes appear. It is conceivable that the prolonged autoimmune

destruction may result in secondary autoimmune responses to some molecules which spill out of ß-cells in numbers or forms which are not tolarized during development. To assess whether the 38kD antigen is a target of early rather than late immune responses in type 1 diabetes we analysed the presence of 38kD antibodies in i) a group of 44 individuals (age 2.6-49.9 yrs at clinical onset) from whom sera were available 3-85 months prior to clinical onset of type 1 diabetes and ii) 37 children who developed diabetes ≤ 5yrs of age and 38 controls in the same age group (Table 2). Six of the 44 prediabetic individuals (14%) were positive for 38kD antibodies in the first serum sample available, which was 3, 9, 25, 33, 53, and 74 months respectively before clinical onset of type 1 diabetes. Two individuals were positive for 38kD antibodies only and 4 individuals were positive for both GAD_{65} and 38kD antibodies. GAD_{65} antibodies were detected in a total of 33 (75%) of the prediabetic patients in the first sample available 3-85 mo before clinical onset of disease, a result consistent with our earlier studies (7,8). Thus both 38kD and GAD_{65} antibodies can be detected up to several years before clinical onset. Follow-up samples were available for 34 of the prediabetic individuals. None of the individuals changed from antibody negative to antibody positive status in later samples. Thus antibody negative individuals remained negative and single antibody positive individuals did not become double antibody positive, suggesting that neither the GAD_{65} antibodies nor the 38kD antibodies are a consequence of prolonged ß-cell destruction. Rather they may be determined by genetic or environmental factors which are either present or absent at the onset of the autoimmune process.

Amongst the young newly diagnosed children, 4 of 37 patients (11%), 1.3, 1.6, 1.8 and 4.8 years of age respectively, were positive for 38kD antibodies. 28 patients, including all of the 38kD antibody positive individuals, were positive for GAD_{65} antibodies (76%). None of the healthy control individuals were positive for GAD_{65} or 38kD antibodies.

Summary of antibody data and implications for type 1 diabetes

Taken together amongst the 130 patients, who were analysed either in the prediabetic period or at the clinical onset of disease, 22 (17%) were 38kD antibody positive compared to 98 (75%), who were GAD_{65} antibody positive. Eight patients were positive for 38kD antibodies only, wheras 14 had both 38kD and GAD_{65} antibodies. Thus 106 (82%) were positive for antibodies to either or both antigens. 38kD as well as GAD_{65} antibodies were detected at clinical onset in children who developed type 1 diabetes as early as 1.3 and 0.8 years of age respectively. Since the duration of ß-cell autoimmunity in those very young

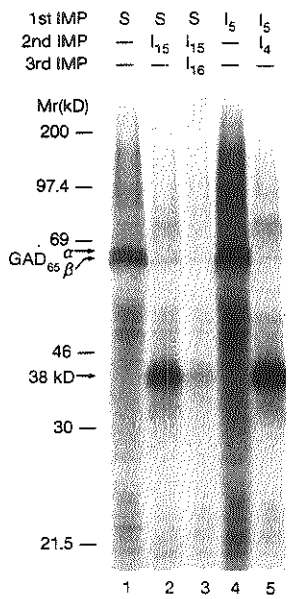
TABLE 2: Incidence of autoantibodies to a 38kD β-cell membrane protein in type 1 diabetes and comparison with GAD_{65}ab and ICA:

GROUP	n	Avg. age at diagnosis or at sampling of sera (controls) years	Range years	F/M	ICA	Incidence 38kD autoantibodies	Incidence GAD_{65} autoantibodies	Incidence 38kD and/or GAD_{65} autoantibodies
Newly diagnosed diabetic patients > 5 years of age	49	13.1 ± 9.22	5.1 - 57.0	20/29 (0.7)	38*/49 77%	12‡/49 (24%)	37/49 (76%)	41/49 (84%)
Healthy controls > 5 years of age	25	14.9 ± 12.6	5.1 - 54.2	10/15 (0.7)	0/25 (0%)	0/25 (0%)	0/25 (0%)	0/25
Newly diagnosed diabetic patients < 5 years of age	37	2.9 ± 1.4	0.8 - 5.0	15/22 (0.7)	32§/37 (86%)	4¶/37 (11%)	28/37 (76%)	28/37 (76%)
Healthy controls < 5 years of age	38	2.8 ± 1.3	0.9 - 5.0	18/20 (0.9)	0/38 (0%)	0/38 (0%)	0/38 (0%)	0/38 (0%)
Prediabetic individuals 3-85 mo. before clinical onset	44	19.2 ± 12.5	2.6 - 49.9	14/30 (0.5)	28**/44 (64%)	6‡‡/44 (14%)	33/44 (75%)	35/44 (80%)

* Six of whom were both GAD_{65} and 38kD antibody negative
‡ Six of whom also had GAD_{65} autoantibodies
§ Four of whom were both GAD_{65} and 38kD antibody negative
¶ All of whom also had GAD_{65} autoantibodies
** None of whom were both GAD_{65} and 38kD antibody negative
‡‡ Four of whom also had GAD_{65} autoantibodies

Figure 2. Lack of immunological crossreactivity between GAD_{65} and the 38kD protein.

Anti-38kD antibodies and anti-GAD_{65} antibodies recognize distinct proteins. Fluorograms of an SDS-PAGE showing sequential immunoprecipitation of ^{35}S methionine-labeled rat islets membrane proteins (material from 500 islets per lane) with anti-38kD antibodies and anti-GAD65 antibodies positive sera . Lanes 1 and 5 show immunoprecipitation with an anti-GAD65 antibody positive stiff-man syndrome serum (Lane 1), and a diabetic patient serum I_5 (lane 4). The supernatant after immunoprecipitation was then subjected to a second immunoprecipitation with anti-38kD antibody positive diabetic sera I_{15} and I_4 (lanes 2 and 5). The supernatant after immunoprecipitation in lane 2 was then subjected to a third immunoprecipitation with an anti- 38kD and anti-GAD_{65} antibody positive serum I_{16} (lane 3). GAD_{65} and the 38kD protein do not affect the immunoprecipitation of each other.



children must have been significantly shorter than is often the case in older individuals (7,8) this result suggest that both proteins may be targets of primary rather than secondary autoimmune processes directed to the ß-cell in the human disease. This notion is supported by the appearance of antibodies to both antigens several years before the clinical onset of type 1 diabetes. Thus both 38kD and GAD_{65} antibodies mark periods of early ß-cell destruction.

The incidence of ICA detected by immunofluorescence of frozen sections of human pancreas was 75% (98/130). In the three groups, the 38kD and/or GAD_{65} immunoprecipitation assays detected a total of 18 individuals which were negative for ICA by the immunofluorescence assay indicative of a lower sensitivity of the latter method to detect antibodies to those antigens. Blocking experiments have shown that the ICA response can progress from GAD-restricted to non-GAD restricted during the prediabetic period in some individuals which suggests a spreading of antigen reactivity during prolonged periods of ß-cell destruction (20). Interestingly, all ICA positive individuals in the prediabetic group were positive for either 38kD antibodies, GAD_{65} antibodies or both. In contrast 4 and 6 ICA positive individuals in the young and older newly diagnosed groups respectively were negative for both 38kD and GAD_{65} antibodies, suggesting that the humoral autoimmune response in those individuals may include other target molecules at the time of clinical onset. Immunoprecipitations did not reveal islet cell protein(s) that were specifically recognized by those sera (results not shown). It is conceivable that ICA reactivity in those 38kD and GAD_{65} antibody negative sera may be directed to non-protein molecules like gangliosides (46). Finally 8 individuals in the prediabetic group and 2 and 3 individuals in the young and older newly diagnozed groups respectively were negative for antibodies by both immunofluorescence and immunoprecipitation assays

The 38kD antibodies analysed in this study recognized their target under native but not denaturing conditions, suggesting that 38kD antibodies, much as GAD_{65} antibodies, are primarily directed toward conformational epitopes. Regarding immune recognition, it is well established that the MHC-haplotype is influential. More than 90% of all individuals who develop type 1 diabetes are HLA-DR3 and/or DR4 positive (47). HLA-data could only be obtained for 12 of the 22 38kD antibody positive individuals. The DR4 haplotype was particularly abundant in those individuals (10/12 compared with 4/12 for DR3, and 2/12 for DR1, and DR7 respectively). The dataset is, however, too small to analyse for statistical significance.

We have extensively analysed immunoprecipitates of detergent lysates of ^{35}S-methionine labelled islets with diabetic and control sera by one and two dimensional gel-electrophoresis in attempts to detect additional islet cell proteins that are specifically recognized by autoantibodies in type 1 diabetes under native conditions. The stringent conditions of immunoprecipitation require that antibodies must 1) be of the IgG isotype (for binding to Protein A-Sepharose), and 2) be of sufficient affinity and specificity to recognize their target protein in the midst of an abundance of other islet cell proteins. Using this assay, we have been unable to detect proteins other than GAD_{65} and the 38kD protein that are consistently and specifically recognized by a significant fraction of diabetic sera. In particular, carboxypeptidase H, a 52kD protein, 69kD protein with homology to bovine serum albumin, and insulin, all reported to be targets of antibodies in diabetic sera (22-24, 48) are not detected in immunoprecipitates (31 and unpublished results). Thus strong IgG responses to conformational protein epitopes may be limited to the GAD_{65} and 38kD molecules in the human disease. Since high titer GAD65 and 38kD IgG antibodies are detected in the early phases of ß-cell autoimmunity, we predict the presence of activated $CD4^+$ T-helper cells recognizing each of those molecules. In fact, reactivity to both GAD (49-51) and to a 38kD ß-cell protein (26,27) has been demonstrated using T-cell lines from newly diagnozed diabetic patients. The relation of the 38kD antigen described in this report with that 38kD T-cell stimulatory protein (26,27) remains to be clarified. An outstanding question now is, whether GAD and the 38kD antigens are targets of pathogenic $CD8^+$ T-cells which are believed to mediate ß-cell destruction in type 1 diabetes (5,52), a result which would suggest these proteins have a potential for antigen specific immunotherapy aimed to prevent the disease.

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Chapter 5

GENERAL DISCUSSION

5. General Discussion

The work described in this thesis has resulted in the identification of the 64kD autoantigen in type 1 diabetes mellitus as the neurotransmitter γ-amino butyric acid (GABA) synthesizing enzyme Glutamic Acid Decarboxylase (GAD) (1). Additional immunological and biochemical analyses resulted in further characterization of this antigen(2, 3) The 38kD autoantigen was studied (chapter 4) and, although not yet fully identified and characterized, the value of the autoantibodies against this target evaluated. Analysis of autoantibodies to both peptides opens new ways for disease prediction in type 1 diabetes mellitus and these molecules now be evaluated for studies on their role in the pathogenesis.

The identification of the 64kD antigen has triggered further research efforts on this molecule. Several laboratories started to develop antibody assays that would allow fast and reliable testing for antibodies to GAD. A first workshop on the standardization of such GAD assays was held in Orlando, Florida in April 1993. This meeting was held under auspices of the Juvenile Diabetes Federation (JDF), which also orchestrated the standardization of ICA and IAA assays. Although different assays were tested and the organizers had supplied the different groups with standard sera, the immunoprecipitation techniques proved to be the best, thus setting a sort of 'golden standard'. In addition to establish the predictive value of GAD autoantibodies, several projects were initiated to determine the role of GAD as a model-autoantigen in type 1 diabetes. Such studies will also help to determine how tolerance or absence of tolerance to such peptides is established. Several papers have addressed the GAD molecule since 1990. Despite of all the research, it is at present not known if GAD, or any other characterized antigen is a primary target of autoimmunity in type 1 diabetes. The next paragraphs discuss the work presented in this thesis and discuss directions for further research.

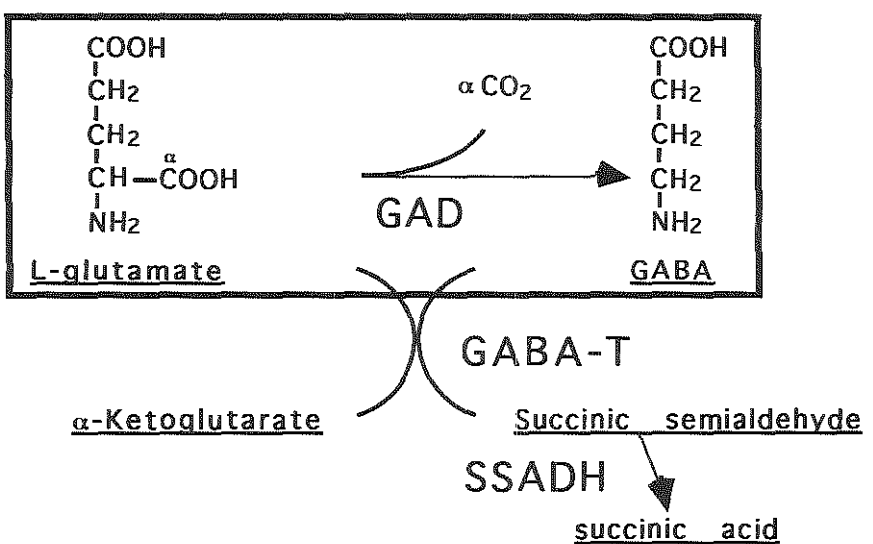
5.1 The 64kD antigen is GAD: Biochemical Function and Characteristics in Brain.

As outlined in the different papers presented in this thesis, the discovery that the 64kD autoantigen was a neuronal enzyme was somewhat surprising. One isoform, GAD_{67}, was actually cloned years before. As indicated in the introduction, ß-cells do share several features in common with neurons and GAD is a typical

example. Since this enzyme has been studied for years by researchers in neurobiology and neuroscience, it is obvious that researchers studying in ß-cells and in type 1 diabetes can learn important lessons especially on the chemical, functional and cell biological aspects of the protein. The next section discusses some of the features on GAD. The interesting links between GAD, neurons and diabetes are important avenues to explore in the future.

The enzyme Glutamic Acid Decarboxylase (GAD, E.C. 4.1.1.15) catalyses the conversion of L-glutamate into γ-amino butyric acid (GABA). GABA is the major inhibitory neurotransmitter in the brain and it has been estimated that up to 30% of the neurons are GABA-ergic ((4) for review). The decarboxylation of the α-COOH group from L-glutamate is the rate limiting step in GAD formation. In neurons, GAD is the major GABA synthesizing enzyme, but other mechanisms of GABA synthesis are present. GABA can also be formed from glutamate by an aerobic pathway via succinic semialdehyde.

Figure 5.1 : GAD catalyzes the alpha decarboxylation of L-glutamic acid to form γ-amino butyric acid (GABA). GABA transaminase (GABA-T), catalyzes the transfer of the amino group from GABA to α-ketoglutarate. This reversible reaction results in the formation of L-glutamate and succinic semi-aldehyde. Succinic semi-aldehyde is oxidized to succinic-acid by succinic semi-aldehyde dehydrogenase (SSADH).



Additional mechanisms for GABA synthesis include production from putrescine via MAO (monamine oxidase), contributing about 0.5% of the GABA pool in brain., and

GABA synthesized from ornithine (5). Neurons do express enzymes involved in GABA breakdown. These are GABA transaminase (GABA-T) and Succinic semi-aldehyde dehydrogenase (SSADH) (4) The three enzymes GAD, GABA-T and SSADH form together the so-called GABA-shunt in which GAD is the rate limiting step. The GABA shunt acts as a closed loop system, that conserves the supply of GABA: GABA is re absorbed from the synaptic cleft by a specific GABA uptake and transport mechanism (GABA transporter) and is either restored in vesicles or transported to the mitochondria for breakdown. GABA breakdown produces glutamate (via the intermediate α-ketoglutarate). Glutamate is the precursor of GABA, thus closing the loop in the GABA shunt

In the nervous system GABA is released from small synaptic vesicles and enters the synapse ((3, 6, 7) for review on synaptic mechanisms see (8). The inhibitory effect of GABA is mediated through GABA receptors of which two types exist. $GABA_B$ receptor activation results in cell inhibition through closure of Ca^{2+} channels or opening of K^+ channels (9, 10) $GABA_A$ receptors are made of (at least) 6 subunits, which together form a chloride channel, that opens upon binding of GABA to one of the subunits. The receptor has additional binding sites for benzodiazepines and steroids and can also be influenced by other chemicals (10-14). Although GABA is a general neuron inhibitor, some other specific effects of GABA in the nervous system are known. GABA has been colocalized in cells that secrete other neurotransmitters or peptidergic neurotransmitters (15, 16) It may possess a regulatory role, particular in hypothalamic and pituitary cells (9, 17). Such a regulatory role in secretion was found in the pineal gland (18) Of great importance is the role of GABA in development of the neuronal networks (19-25). Finally, GABA serves as an energy source in several cell types.

Conflicting results existed in neurochemical literature about the molecular weight. Erlander and Tobin, collected and summarized these data: GAD is composed of two polypeptides with molecular weights between 59-67kD (4). A gene for GAD was cloned in 1986 (26). At that stage, it was believed that this gene would encode for both GAD proteins and that the different forms would be due to alternative splicing or post-translational modifications. In 1991 Erlander and coworkers showed however (27) that 2 genes existed, coding for 2 different proteins, now referred to as GAD_{67} and GAD_{65}. GAD belongs to the family of pyridoxal-5-phosphate (PLP) dependent decarboxylases. These enzymes have in general been highly conserved over evolution (28) The active form of GAD, called holoGAD, has PLP covalently bound to an amino group of a lysine residue in the

PLP binding site of the enzyme. The inactive form, apoGAD, lacks PLP. In whole brain homogenates, only 50% of total GAD is in the active holo form, even though the concentration of PLP in the brain is adequate to saturate GAD (29, 30). This indicates that GAD and PLP may be confined to different compartments in vivo. Under depolarizing conditions, PLP associates with apoGAD to form holoGAD thereby increasing GABA production in nerve terminals with increased neuronal activity (4). PLP levels seem to be of major importance for regulating GAD activity. GAD_{65} makes up most of the apoGAD reservoir in GABA-ergic neurons (30) while GAD_{67} predominantly exists in the holo-form, is less regulated by PLP, and binds this coenzyme more tightly (30). Other molecules can regulate GAD activity, such as GABA, glutamate and aspartate which may induce apoGAD formation, while inorganic phosphate promotes holoGAD (31). It has been suggested, that GAD_{67} is more involved in a steady state production of GABA, and that GABA formed by this isoform functions as an energy source rather than a neurotransmitter (29). This is in line with observations showing that GAD_{67} is localized in the perikaryon, while GAD_{65} is more confined to the synaptic area.

GAD_{67} activity is regulated mainly at the transcriptional level. An increase in neuronal activity has been strongly correlated with increased GAD_{67} RNA (32). Analysis of the 5' region of the GAD_{67} gene has revealed 2 different promoter regions and several potential transcription factor binding sites (33) During development of the rat embryo, the translation of GAD_{67} is regulated by a splicing event. It was found that a 86 base pair exon contains a UGA-codon which is believed to result in a truncated form since the UGA codon represents a stop-codon in the genetic code (34). The UGA stop-codon, however, has recently been found to be overread and can result in methionine, tryptophan or selenocysteine (35) incorporation. This addition to the genetic code has been identified in several species now, but a role for this stop-codon in GAD has not been analyzed. In human fetal brain samples, some larger instead of truncated GAD-forms are present (H.J. Aanstoot, G.J. Bruining, unpublished observations). Aberrant forms of GAD could have different activities, a possibility supported by studies showing that GAD in newborns is more vulnerable to heat. This observation has been implicated in the etiology of febrile seizures (36). Other evidence for a differences in forms or activity of GAD in newborns comes from studies on the role of vitamin B6 (a precursor of PLP) in so called pyridoxine dependent convulsions (37, 38) In addition, syndrome of GAD deficiency in a newborn with convulsions has been described (39) although it is not clear from that study if one or both forms were absent. In rats, differential expression of the two forms was found during

development (40, 41). While GAD_{67} was predominantly present in the embryological period, GAD_{65} expression dramatically increased in the neonatal period, concomitant with a period of intense synapse formation. After 2 weeks, the expression normalized to levels found directly after birth. These observations indicate that our knowledge on the role of GAD and GABA in neuronal dysfunction and convulsive disorders, is incomplete and needs more attention. It also indicates that studies on brain GAD will be important to study GAD and the GABA system in ß-cells.

5.2 Tissue Distribution and GABA-ergic System in the Pancreas.

GABA-ergic neurons are widely present in the central nervous system. Their abundance has lead to the suggestion that the brain is in a constant inhibited state. GABA-ergic neurons vary widely in morphology (42). Coexpression with other neurotransmitters has been found in some GABA-ergic neurons (15). In the peripheral nervous system, GABA and GAD containing neurons are present (43, 44). Outside the nervous system, GAD has been found in several tissues (see (45) for review) including adrenal, thymus, stomach, duodenum, gall bladder, muscle and thyroid. However, GAD concentrations as found in ß-cells and brain (46) are only present in fallopian tubes (oviducts) and testis. Several of the studies performed used enzymatic assays to measure GAD activities (see (1) for method) and did not directly visualize the protein by biochemical techniques such as Western Blotting. The enzyme assay is not specific. Other enzymes with decarboxylation activity can be measured. Recently, it was shown that GAD present in oviducts is GAD_{65}, while in testis GAD_{67} was present (47). GAD_{67} in sperm probably functions as an energy source (48) while in the oviduct with GAD_{65} signaling might be the primary role. GAD activity in the fallopian tubes in rats is highly dependent on the hormonal status (49, 50) with fluctuations during the menstrual cycle, but the exact role for GAD in these tissues is not established.

The presence of GABA system components in the pancreas has been recognized since 1986 (51). However, their functional significance was not understood. Preliminary in vitro experiments involved the study of effects of GABA and GABA-inhibitors on insulin secretion, but controversial results were obtained: both increase as well as inhibition of insulin release was reported (52-55). More recently, Rorsman and co-workers suggested that inhibition of glucagon secretion by glucose is mediated by GABA, cosecreted with insulin (56). $GABA_A$ receptors on

the α-cells are present. These findings were supported by a study that showed a 20% inhibition of glucagon secretion by GABA in mouse and rat pancreas (57). $GABA_A$ receptors are also present on δ-cells (58). These data support a paracrine role of GABA within the islets of Langerhans.

It can be argued that GABA has no regulatory function in ß-cells, but is used as an energy source. ß-cells, as extremely specialized cells with high peptide synthesis rates, are in the need of a high energy production to keep up with the demands. GABA production offers an alternative fuel. The production of GABA and localization of GABA and GAD_{65} however, has many parallels to neurons that energy needs are not likely to be the reason for a ß-cell to express GAD. The specific subcellular localization of GAD_{65} is an additional reason to propose that GAD is not simply an enzyme to cover energy demands. The role of GABA in the ß-cells can be tested in vitro by stimulating or inhibiting GABA synthesis and secretion. Several GABA-ergic compounds are available and have been extensively characterized in neurons and the brain (59-63) Interestingly, ßTC3 tumor cells have, despite the fact they are tumor cells, several features in common with normal ß-cells. They can be induced to develop neuronal outgrowths (64). Such cells could be studied after stable transfection with GAD_{65} (to induce overexpression) to see if GAD behaves similar in these ß-cells as it does in neurons: binding to small synaptic vesicle-like microvesicles. This model would allow to test functional aspects of GAD, but also to do additional studies on subcellular localization. This could reveal to which side of vesicles GAD_{65} is localized. Moreover, the transport mechanisms involved in GAD synthesis, posttranslational modification and membrane binding could show if these steps are involved in the visualization of GAD to the immune system .

Localization studies showed that GAD expression is limited to islets of Langerhans and in particular, the ß-cell (51, 65-68). Recently, Petersen and colleagues described the presence of GAD in α- and δ-cells (69). Human islets express only GAD_{65}. In other species, such as rat, mouse, dog and pig, both GAD_{67} and GAD_{65} are expressed in islets (1, 70). In rats GAD expression is ß-cell specific (69). In developing human islets the cells can express more than one hormone simultaneously. This is however, never described in the adult pancreas. Although GAD_{65} expression was found in a very limited number of human adult α–, δ–, and PP- cells, the pathophysiological importance of this observation is not clear. If GAD is an important antigen, autoimmunity would also include such cells, unless the presentation of GAD to the immune system is different. Also within the ß-cell population, there is a striking difference in GAD expression (71). Using

confocal microscopy of whole islets, it was shown that some ß-cells express predominantly insulin and contain low amounts of GAD, while others predominantly express GAD and contain little insulin. It is conceivable that different types ß-cells exist. These differences could fluctuate in time (i.e. the cells switch from one phenotype to another, perhaps under the influence of metabolic changes) or if they represent two more or less different populations is unknown. After the identification of the 64kD protein as GAD_{65}, several labs cloned the human GAD genes It was established that the GAD_{65} gene is localized on chromosome 10, while the GAD_{67} gene is located on chromosome 2 (72-74).

In proliferating neurons, GABA synthesis is needed for neurite outgrowth and inhibition of GABA synthesis results in underdeveloped neuronal networks (75, 76). A similar trophic role could also be present in the islets, where the endocrine cells share several characteristics with neurons. We did see expression of both GAD and GABA in the developing human endocrine pancreas. Islet formation is a process that starts with endocrine cell formation in the ducts and continues through steps where some of the cells migrate to form an islet (77). This islet formation and migration may, similar to what is proposed in neurons, be dependent on compounds such as GABA. Other, relatively simple molecules have been implicated in cellular differentiation and proliferation processes such as sodium butyrate (78). In summary, analysis of GAD at the embryo-fetal level will help in our understanding of (non-) tolerance to GAD and will contribute to our knowledge of ß-cell and islet formation. These processes are not very well studied and the observations of heterogeneous ß-cell populations plus the fact that autoimmunity has, at least partly, to do with the levels and absolute amounts of antigen, urge for a more detailed look into the role of the ß-cell itself in this (ß-cell specific) disease.

In summary, the function of GABA in islets is not clear yet, but likely paracrine. Cells expressing GABA might represent a special subgroup. Further research on GAD and GABA in the pancreas, during development and later in life, could contribute to our understanding of the pathology of the endocrine pancreas. If GABA has a clear relation to insulin, studies on the GABA metabolism in the pancreas could offer new ways to establish and monitor ß-cell function.

5.3 Type 1 Diabetes Mellitus: An Immunological Disturbance.

Type 1 diabetes mellitus is considered to be an autoimmune disease. As described in chapter 1.5, several lines of evidence support this concept. The mechanism of the disease is selective ß-cell destruction. This process is T-cell mediated and T-cell dependent. However, the reason why the immune system starts the attack is far from clear. A major question regarding the autoimmune destructive process is what causes the breakdown or lack of tolerance to a ß-cell component. This question addresses the nature of the primary autoantigen involved in the autoimmune process. It must be stressed that, although the disease seems T-cell mediated and T-cell dependent, it is not determined if this whole process is actually initiated by T- or other cells of the immune system. The selectivity of the attack by the immune system and the need for a specific ß-cell component indicate an important role of the ß-cell itself: a malfunction of ß-cells (overexpression of non-tolerated antigens, expression of different peptides than usual, virus infection, etc.) could initiate a primed immune system (presence of macrophages, dendritic cells, autoreactive T-cells, a favorable HLA haplotype). Thus, the immune system could be another susceptibility factor, just like the genetic system seems to be. Additional studies are needed to determine the role of the ß-cells in type 1 diabetes.

Studies concentrating on the role of the ß-cell in the pathogenesis must be based on immunological studies, which determine the antigens involved in the immune process. Characteristics of such antigens need to be explored in order to determine how and why such antigens are presented to the immune system and induce autoimmunity. The existence or lack of tolerance to such antigens needs to be explored. Experiments will include the study of these antigens in different conditions such as high glucose concentrations or the levels and forms expressed during viral ß-cell infections. It will be clear that optimal assay systems are required to determine if up regulation of an antigen actually results in triggering of the immune system.

If the ß-cell plays such an important role towards the immune system, and can initiate the first steps of autoimmunity, we have to assume that susceptibility for diabetes also include the presence of autoreactive T-cells. It is now clear from several experiments that autoreactive cells are actually present and can escape the mechanisms that exist to delete such cells. Autoreactive T-cells were found in control individuals in several autoimmune diseases, including multiple sclerosis (MS) and autoimmune thyroid disease. In MS, autoreactive T-cells are believed to be directed towards the autoantigen myelin basic protein (MBP). Other evidence

for the existence of autoreactive T-cells in non-disease situations comes from studies in normal rats, where the T-cell repertoire contains cells with the potential to cause type 1 diabetes (79) and from studies in NOD- and normal mice (80). If autoreactive T-cells are present, it is actually surprising how little autoimmunity is seen. Silencing of such T-cells is obviously an important mechanism. Special circumstances may activate such cells. As outlined in chapter 1.5, anergy or ignorance would allow the presence of such autoreactive T-cells without initiation of autoimmunity. Evidence for the presence of silenced autoreactive T-cells comes from studies where autoimmune disease could be initiated by partial T-cell depletion (81, 82). It has been proposed that anergy, i.e. silencing of such cells, is a delicate balance between different functional subtypes of CD4+ (helper) T-cells and that this heterogeneity in subtypes is dependent on differences in cytokine production by these cells (83, 84). Which cytokines are produced is partly determined by the nature of the antigen presenting cell and can either result in a so called Th1 or a Th2 response. Th2 cells produce for example, interleukin-4 (IL-4) which inhibits cytotoxic (Th1) responses, suggesting that Th2 cells are inhibitors of cellular immunity. Such data suggest that immune system exists as a dynamic and finely tuned balance between different immune response pathways. Autoimmune reactions are the result of imbalances of this system. Disturbances in Th1/Th2 responses can be the result of environmental factors like viral infections. Thus, with the presence of autoreactive T-cells, disturbances of the balance between stimulating and inhibiting factors could determine if autoimmunity ensues. In the case of type 1 diabetes this involves induction of an autoreactive T-cell clone by a ß-cell specific or a mimicking peptide. Alternatively, it is possible that the peptide which induces this reaction is only visible or accessible for the immune system in the ß-cells, and expression of the peptide in other cells is invisible or protected from the immune system. The absence of tolerance to this primary antigen can, upon presentation to the immune system, result in autoimmunity. Transgenic mouse models showed that in the absence of tolerance to a ß-cell antigen, autoimmunity can be absent. However, these models showed that a triggering event, such as a viral infection, containing similar or mimicking antigens, could imbalance the system and initiate autoimmune ß-cell destruction (85, 86).

These proposed mechanisms directly pose the question what triggers the immune system imbalance. The activation of T-cells takes place by an interaction of the T-cell receptor and an antigen presenting cell (APC). In addition, accessory molecules are involved, such as adhesion molecules and molecules that induce or transduce a so-called second signal (co-stimulatory signal), needed to initiate a

proliferative T-cell response and preventing the autoreactive cell from switching into an anergic mode. It is clear form immunological studies that the susceptibility for type 1 diabetes is determined by the MHC complex on chromosome 6. Until recently however, no conclusive evidence was presented, showing that the MHC molecules (i.e. the HLA molecules) are indeed involved in the immune response in type 1 diabetes. The association could merely be a phenomena of linkage between the MHC and other unidentified 'diabetogenic' genes in the same region. Recently it was shown in the NOD animal model of type 1 diabetes, that blocking of the specific NOD MHC molecule (A^{g7}) by a designed peptide resulted in a strong reduction of autoimmune diabetes (87). The designed peptide blocked the antigen presentation by the unique NOD class II molecule (A^{g7}) in vitro. It also inhibited responses in A^{g7} specific T-cells. These effects were not seen in animals treated with a control peptide, which does not bind to A^{g7}, and demonstrated that antigen presentation by the NOD specific A^{g7} has a role in the disease. A similar role of susceptibility HLA molecules in humans needs further studies.

Except for MHC-peptide complex that binds to T-cells via the T-cell receptor (TCR) and CD4 or CD8 co-receptors, a second or co-stimulatory signal is needed to result in T-cell activation (88). This signal is antigen independent and lack of this signal results in anergy. The second signal is induced by the interaction of receptors (such as CD28 or CTLA4) on T-cells with a molecule called B7, present on specialized ('professional') antigen presenting cells (APC): macrophages and dendritic cells (DC's). DC's are outposts of the immune system and are present in (non-lymphoid) several tissues including the pancreas. Although their antigen processing is not as good as some other APC's, they have a high density of MHC class II molecules and are extremely good antigen presenters, able to deliver second signals. Thus, these cells have an important role in the initiation of immune responses. Dendritic cells are present in islets (89) and may function as the cell type that is able to trigger autoreactive T-cells, although the factors needed for this trigger are yet unknown. It is established now however, that the outcome of an antigen encounter with an autoreactive T-cell is dependent on the type and abilities of the APC and the intensity of the stimulation. Such factors will also be dependent on the type of T-cell response initiated: Th1 or Th2 (reviewed in (90)). There is still controversy about the role of APC's in the islets: Treatment with silica, which disables macrophages, was found to have no effect on the incidence of diabetes in animal models (91), thus the question remains how macrophages or Dendritic cells function in the first phases of type 1 diabetes . As proposed, the ß-cell itself may have a more prominent role where differences in expression of antigens (amount,

peptide composition etc.) could result in a different processing by APC's in islets, thereby enhancing presentation of such peptides to autoreactive cells. The importance of APC's as antigen presenters is clear, the mechanism and position in the sequence of events needs to be further established.

At present the antigen involved in this initiation of type 1 diabetes is not known. For such an antigen it must be shown that tolerance is lost, or never established. Moreover, in other experiments, it should be analyzed if immunization induces tolerization. Additionally, breaking of such tolerance should induce the disease in appropriate models. It is important to realize that such experiments can be performed in animal models only. In humans we do not yet know if GAD is tolerized or if in type 1 diabetes and SMS, tolerance to GAD is lost. Non-establishing tolerance to GAD in the human situation may have different causes. GAD is a specialized molecule, only present at significant levels in few tissues. The expression may have started only after completion of the immune repertoire as outlined above. It is striking that several enzymes, expressed in specialized tissues and sometimes protected by barriers for the immune system, can become targets for autoimmunity, such as thyroid peroxidase and 17α-hydroxylase(92). Late expression can result in non-tolerance. The availability of fetal human tissue to our lab allows to address this question in more detail and with regard to pancreatic GAD. Preliminary experiments do show the expression of GAD_{65} mRNA in islets, using in situ hybridization, in at least some pancreas cells of 14-18 week old fetuses. Detailed studies on protein expression have not yet been possible, but the availability to purify endocrine (precursor) cells from the human fetal pancreas (93) will help to establish if fetal islet do express this protein. It is possible that, although the adult human pancreas solely expresses GAD_{65}, the fetal endocrine pancreatic cells temporarily express GAD_{67}.

The levels of expression will be dependent on the functional state of the ß-cell. This functional state a main topic in research in type 2 diabetes. Differential expression within the islet ß-cell population has been observed for key enzymes and proteins such as glucokinase, a ß-cell specific enzyme and GLUT-2 the ß-cell glucose transporter, all of which have important roles in the regulation of insulin synthesis and secretion (94, 95). Thus some cells are more involved in insulin synthesis and secretion, while others are likely to be primarily involved in regulation and paracrine signaling. Type 1 and type 2 diabetes are clearly different diseases , but the diseases cluster more within families than can be expected if they were completely unrelated. Some genetic studies indicate common

haplotypes in the two diseases (96, 97). The heterogeneity of the ß-cells could be a common aspect involved in both diseases. It can be hypothesized that individuals with a lack of islet regulation (role of GABA?) or with too many GABA-containing regulatory ß-cells starts, for example, overexpression of GAD.

5.4 GAD and the 38kD Molecule in Future Type 1 Diabetes Research.

Experiments directed to unravel the questions on tolerance focus on the cellular immunity. For most antigens described in the context of type 1 diabetes, we only deal with the humoral arm of the immune system and we only know that antibodies are induced. The initial mechanisms of loss of tolerance are T-cell dependent. Secondary effects are B-cell proliferation and antigen production plus extension of the T- and B-cell response to other antigens that become available upon ß-cell death. Most of the work published to date involves either B-cell responses (autoantibodies) or T-cell responses at clinical onset of the disease. The 'original' T-cell, which started the cascade of reactions, may be present at very low frequencies at the time of clinical onset. If autoantibodies are initiated, even the levels of such antibodies might have dropped by the time of diagnosis. Aggressive T-cell responses have a Th1 phenotype, with IL-2 and interferon production (83, 84). It is suggested that such T helper responses are not initiating strong B cell responses (which require Th2 response), thus antibody levels to a primary antigen may be low (83) Although these factors may make a humoral target for autoimmunity less attractive to play a key role in the onset of the disease, the following characteristics make GAD is an interesting molecule:

1. GAD antibodies are the earliest detectable marker of ß-cell destruction, suggesting them as the humoral representatives of an early autoimmune reaction towards ß-cells (98, 99).
2. Autoantibodies to GAD have an incidence of ≥ 80% in sera from both newly diagnosed patients and pre-onset individuals, higher than antibodies to any other previously studied autoantigen including the novel antigen 'ABBOS', a fragment of BSA (100).
3. Autoantibodies to GAD are present in two animal models of type 1 diabetes, the BB-rat and the NOD mouse(101, 102).
4. GAD may be a target for 'molecular mimicry', a possible mechanism by which autoimmunity is triggered by viral proteins (103).

The smaller form of GAD (GAD_{65}) is therefore still a candidate as primary

autoantigen in type 1 diabetes. This is also true for the 38kD protein, to which a lower incidence of autoantibodies is found. If present, they are also very early markers.

GAD was characterized in islet cells, where some specific biochemical characteristics were studied. The importance of such studies is that the mechanism by which GAD becomes a target for autoimmunity is a crucial aspect in the pathophysiology of tolerance to such molecules. We found that the two forms of GAD present in brain, were identical to the two forms found in islets and that they differed with regard to cellular compartment and hydrophobicity. Later the cDNA for two pancreatic and neuronal GAD isoforms were cloned. GAD_{65} is the form that can be hydrophilic and soluble, hydrophobic and soluble or membrane bound. GAD_{67} is always hydrophilic. Using eukaryotic expression systems for both GAD's, we were able to extend the observations that the differences at the DNA level have important implications for the localization and likely for the way the immune system recognizes GAD_{65}. The GAD_{65} molecule shows major differences with GAD_{67} at the N-terminus. We were able to show that this N-terminus is the site where GAD_{65} can become membrane bound. Several mechanisms exist for proteins that lack a hydrophobic stretch to become membrane bound. GAD_{65} is hydrophilic after its synthesis. It goes through a 2 step modification process of which the second step results in a firm membrane anchoring. This involves posttranslational, hydroxylamine sensitive, palmitoylation. More recently, it was documented by us and others, that GAD_{65} is the only form present in human islets (69, 104) in contrast to rat and mouse, where both forms can be found. It was proposed and shown that membrane bound GAD_{65} is linked to the small synaptic like vesicles, the endocrine cells counterpart of small synaptic vesicles. Recently, Solimena and co-workers showed that in transfected cells (CHO), GAD_{65} becomes localized in the Golgi system, the area where such vesicles are formed (105). The cells used in this experiment do not posses the complete system of small vesicles and it will be interesting to see if expression in endocrine or neuronal cells will result in the localization in vesicles and transport of these vesicles through the cell with subsequent presentation at the cell surface. Alternatively, pharmacological techniques to stimulate or inhibit GAD, the transport mechanisms or stimulate secretion of vesicles, could help to study this. The importance of subcellular localization studies is both that these studies can identify how the immune system encounters GAD, but additionally that such studies can provide clues to the function of GAD and GABA in the ß-cell. A localization inside vesicles opens the possibility of a direct presentation to the immune system. Moreover, since the

secretory activity of the ß-cell has been linked to induction of autoimmunity (106) and with observations that GABA secretion is linked to insulin secretion (54, 107) the effect of the functional activity of the ß-cell on induction of autoimmunity may be studied. Recently, GAD expression in ß-cells was shown to be related to the rate of insulin secretion (108).

Autoantibodies to GAD were found in two diseases: type 1 diabetes and Stiff-man syndrome (SMS). We hypothesized that in SMS the pathophysiology is caused by a direct effect of the antibodies to GAD_{65}. If expressed inside the vesicles of GABA-ergic neurons, it could become visible at the plasmamembrane upon the merging of the synaptic vesicle with the plasmamembrane. Antibodies could have direct access to GAD and inhibit its function. Animal models of Stiff-man syndrome could be developed to study such a mechanism.

GAD as a target for autoimmunity in two different diseases may raise questions about the fact if such an antigen is interesting as a primary target. However, the immune response towards GAD is different in the two diseases, thus allowing the possibility of two different mechanisms. In Stiff-man syndrome, at least one linear epitope is present that distinguishes the antibodies from diabetes. Further analysis is under way to determine this epitope. Determination of this epitope could help to develop animal models of SMS. Immunization with this linear SMS epitope of GAD could result in high antibody levels as are generally seen in SMS. Disturbing the blood brain barrier (i.e. by pertussis toxin) could create a similar situation as seen in SMS patients: high titer antibodies, recognizing a linear epitope of GAD in both serum and cerebrospinal fluid. Specific therapy against the antibodies could result in diminishing of symptoms, showing if antibodies are indeed involved in the disease. Moreover, the close relationships between the two diseases (at least 30% of the patients with SMS develop type 1 diabetes) make them excellent models in autoimmune research. It should be noted that our current knowledge on SMS is limited. Sera tested so far, usually include patients who were diagnosed years before. We do not have adequate knowledge on GAD titers in earlier stages of SMS. Moreover, HLA data in these patients are lacking, although a recent abstract indicated that most patients were DR3 (DQB1 *0201) positive, comparable to diabetes where DR3 is also present in many patients.

Christie and colleagues described that trypsinization of immunoprecipitated GAD resulted in tryptic fragments of GAD (109, 110). As described in chapter 4,

these 37/40 kD fragments found do not have a relation to the 38kD protein described in this thesis. The 37/40 kD fragment seems to be either a cryptic fragment of GAD or a separate, co-precipitated protein. The protein is not seen in 10% SDS-PAGE, indicating that it is probably part of a larger molecule prior to trypsinization. The importance of the finding by Christie is, that antibodies to the 37/40 kD fragment seem to be highly associated to onset of clinical diabetes, thus providing a test to distinguish individuals with latest stages of ß-cell destruction from individuals with early onset ß-cell destruction. Further studies are needed to study this in more detail. The 38kD protein described in this thesis has no relation to the 37/40kD fragment as is discussed in chapter 4.

Studies of epitope recognition of GAD are important not only for the proposed SMS studies, but especially for further studies in diabetes. After induction of humoral autoimmunity, a polyclonal response of B-lymphocytes will be evoked. However, it is possible that in the beginning, the responses will be more or less limited to the same epitope region as which triggers the T-cells. Thus, early B-cell responses, may reflect T-cell epitopes. It will be of interest to analyze which epitopes are most common in type 1 diabetes, in particular in the early phases. Prediabetic sera, or sera from very young children with diabetes (< 5 years old, since the time passed between the start of autoimmunity and the disease is supposedly still short) can be tested to see if GAD-epitopes are more restricted. A recent study on epitope recognition from the lab where this thesis was prepared, showed that the C-terminal part of the GAD_{65} molecule is the predominant site of autoantibody recognition in type 1 diabetes patients (111). This study was possible using site-directed mutagenesis of the GAD_{65} molecule together with the availability of human monoclonal antibodies (112). This result can also be used in further designs of GAD antibody assays. The technical difficulties encountered in these tests could partly be due to the fact that the non-soluble phase tests hamper the recognition of the conformational epitopes. Interference with the molecule may result in loss of such epitopes and subsequent underestimation of GAD autoantibodies in patients as is described for ELISA's (113). Since the N-terminal is not involved in type 1 diabetes, this site could be modified to improve solubility or be used as a site for immobilization of the GAD molecule without interfering with conformational epitopes.

As described in Chapter 2, analysis of 64kD / GAD antibodies was initially hampered by the difficulties in obtaining islet material. After the identification as GAD, recombinant expression systems were developed, but new problems arose

(see chapter 3). The assay system used was immunoprecipitation as described in the papers of this thesis. Other assays are described. Some studies use the enzyme activity of GAD (114). We used a similar approach to show enzyme activity of GAD of the 64kD antigen (1). Autoantibodies precipitate this activity and allow a comparison to control sera. The disadvantage is a relatively low sensitivity and the possibility of interference of antibodies with the active site of the molecule. Other groups developed RIA-like assays which compare better to the immunoprecipitation technique used in previous studies (115). The development of faster assays with high sensitivity and specificity, is of importance since further analysis of the predictive value of GAD (and 38kD) autoantibodies will need large study groups.

Antibodies to the 67kD isoform of GAD are present in a subgroup of patients. Three groups published data on this isoform (103, 113, 116) with varying results ranging from 9-75% of the patients positive for GAD_{67}. Our preliminary data show 8% of newly diagnosed patients positive for GAD_{67}. Although it is suggested that GAD_{65} is the target for autoimmunity in type 1 diabetes and that GAD_{67} antibodies only develop secondary to GAD_{65} autoimmunity, we identified one patient who only showed GAD_{67} antibodies. However, this patient developed type 1 diabetes at a relatively old age (35 years). Antibodies to GAD_{65}, may have been present in the earlier phases of ß-cell destruction. In summary, GAD_{67} antibodies are not likely to be an important parameter of autoimmune destruction in type 1 diabetes. T-cell responses to a partial GAD_{67} molecule (aminoacids 208-404), expressed in bacteria, were found in newly diagnosed diabetes patients in one study (117)

A second antigen of Mr 38kD was identified by the same techniques as originally used for the 64kD protein in islets. Both are targets for humoral autoimmunity in type 1 diabetes mellitus. Detection of autoantibodies to these targets provides a specific way of determining ongoing ß-cell destruction. Autoantibodies to these antigens can therefore be used as markers for type 1 diabetes risks. Since the revelation as GAD, described in chapter 3, several groups have embarked on further studies, both on the molecule itself and how it becomes a target for autoimmunity, as well as on the predictive value of GAD autoantibodies for diabetes as presented in chapter 4. The 38kD antigen is a new marker. We are currently working on the identification of its molecular structure. The incidence of autoantibodies to it is lower, both in the prediabetic period as well as at onset. However, the results in chapter 4 indicate that both proteins are not necessarily simultaneous targets for autoimmunity. The assays complement each

other and increase the predictive value for type 1 diabetes. Moreover, the complete correlation between GAD plus 38kD antibodies and Islet cell antibody (ICA) reactivity in the prediabetic period, indicate that both are major targets for the immune response.

5.5 Prediction and Prevention of Type 1 Diabetes Mellitus

Methods of prediction:

No disease prevention is possible without disease prediction. This prediction method must be sensitive and specific. The best method in type 1 diabetes would be to have superb susceptibility risk factors detectable before any attack of the ß-cell takes place. This would imply that environmental factors that trigger the disease could be prevented. Unfortunately this is not the case. Prevention and prediction after an initiating event, implies that detection humoral or cellular autoimmunity is theoretically possible.

Prediction of type 1 diabetes is based on the following items: ICA's, IAA's, genetic markers and functional ß-cell testing. However, most studies performed describe family related diabetes cases. As outlined in previous chapters, most cases of diabetes occur without a first degree relative having the disease, thus the incidence studies have a 'natural' bias. Other drawbacks in those studies are the fact that the duration of follow up is relatively short, and the fact that only data on limited groups are available. It is estimated that worldwide, only 100-200 'real' prediabetes cases are documented. It can be concluded, that a lack of prediabetes samples is a severe limitation in diabetes prediction studies. This argues for the establishment of national registries of new cases of diabetes. A first improvement could be made from here by sampling first degree relatives on a large scale, which will eventually result in a collection of prediabetic samples.

The prediction assays tests used, have been described in previous chapters except for functional ß-cell tests. Although oral glucose tolerance tests are not helpful in detection of ongoing ß-cell destruction, it was found that intravenous glucose tolerance testing (IVGTT) was more precise. In particular the first phase insulin response (FPIR), defined as the sum of the 1 and 3 min. insulin concentration after a 3 min. infusion of 25% glucose (0.5 g/kg body weight), was found to be related to ß-cell destruction. However, large differences in IVGTT protocols made comparisons of FPIR difficult. Standardization of IVGTT was obtained (118). Analysis of insulin responses after partial pancreatectomies in baboons showed

however that relatively large percentages of the pancreas can be removed before ß-cell function tests were useless. This could indicate that such tests are detecting ß-cell mass aberrations within the first 50% of ß-cell destruction, but the exact detection limits are not known. Analysis of repeated IVGTT's and FPIR's showed a significant intra-person variation, especially in children. It might be questioned whether the FPIR is not an early test for diagnosing diabetes instead of determining prediabetes. An outstanding question is, if autoimmune destruction can take place without appearance of autoantibodies. Palmer and coworkers showed that first degree relatives of patients can show abnormal insulin secretion without the presence of ICA's (119, 120). Such a finding could indicate to a non-linear decrease in ß-cell function or cell mass after initiation of the destruction. However clear data on development of clinical diabetes in these individuals are not available. Others doubt the findings on the basis of a linear decrease in ß-cell function observed in twins (121)

As described in the paragraphs on ICA's and IAA's, combination of these tests alone or with HLA data can provide prediction. The presence of IAA's and ICA's increases the risk for diabetes. In one study, more than 70% of individuals with both antibodies developed diabetes, compared to 42% of ICA positive alone and 17% for IAA positive alone in an average period of 5 years follow-up. On the basis of IAA's and IVGTT's, a dual parameter model was described (122) Another group proposed a model for prediction where the first step is based on ICA determinations in at risk families and on genetic markers in populations (123). At risk individuals in the population would be screened for ICA, if positive for IAA and additional markers. In family members, ICA titer will determine further steps: If ≥20 JDF units, further screening would include HLA typing, GAD, IAA and FPIR. This approach is based on an important issue. Improvement of sensitivity of a given test will result in a decrease in specificity and vice versa(Bayes' theorem). Thus, for each situation (family vs. population) the requirements and approach of screening need to be different. The different articles describing prediction show no consensus yet. . This is most likely the result of the current insufficient specificity and sensitivity of the tests employed.

Finally, it should be stated that prediction methods without therapeutic consequences can be a psychological burden for individuals who participate in such studies. Since most studies are performed in families, the participants are in general aware of the impact a positive prediction can give. Few studies are performed that deal with this. A study from Florida found increases anxiety levels in

family members who where found positive for ICA (124). The avoidance of coping strategies and denial of their possible future diabetes was found more frequent in ICA positives than in their relatives. Other family members often believed that their ICA+ relative would develop diabetes. In general, the psychosocial impact of the prediction assay was limited, although long-term effects remain to be seen.

Attempts to Prevent Type 1 Diabetes Mellitus:

Several studies have been published that aimed at secondary prevention of type 1 diabetes. The wealth of such strategies was the reasons for the American Diabetes Association to publish a position statement on prevention of type 1 diabetes (125). Some intervention studies on the basis of current prediction methods are approved in this statement, although strong recommendations are given to limit such studies since the cause of the disease is not known, thus intervention is still a risk and could induce unwanted effects.

The earliest attempts to provide a permanent treatment for type 1 diabetes were pancreas transplantations. The severe medical problems connected with both transplantation (rejection and re initiation of autoimmunity to the donor islets) together with an organ full of proteolytic enzymes, hampered this technique in becoming a standard treatment. Current status of pancreas transplantation is given in a recent review (126). One important observation was made using pancreas tissue from the non-diabetic discordant twin half to transplant into the diabetic twin. After transplantation, the autoimmune process reactivated and also destroyed the transplanted ß-cells (127). Transplantations are still performed, in particular combined with kidney transplantations in patients with severe nephropathy with end-stage kidney disease. It has been tried, as in kidney transplants, to use part of the pancreas of a relative. The donors showed however, after hemipancreatectomy for this purpose, a deterioration of insulin secretion and glucose tolerance measured one year later. If this increases the chances for clinical diabetes later in life, is not yet established (128). A different approach is islet or islet cell transplantation. Two methods are currently employed to prevent rejection: immuno-alteration of islets (for example by physical influences such as culture at low temperatures, removal of HLA class II expressing cells) and encapsulation of islets to prevent attack by the immune system. Transplantation of islets is reviewed in (129)

An approach that has been often applied in type 1 diabetes is immunomodulation. This usually implies however the modulation of the whole immune system. As described before in the chapter on T-cells, several

immunosuppressives have been used either alone or in combinations. First attempts were limited to newly diagnosed diabetic patients, but currently trials are underway that involve ICA positive family members with aberrant FPIR's . A recent review summarized over 30 methods that have or are employed to influence either the honeymoon period or delay the (presumed) onset of the disease (130)

The first immunotherapy studies used glucocorticoids combined with immunosuppressives such as azathioprine and Cyclosporine A. In newly diagnosed patients no persistent beneficial effect was obtained (131-133). No published data are available on large scale studies of this approach in prediabetes.

Currently nicotinamide (NA) has regained attention as an anti-diabetic compound after its first effects were described in 1947 (134). It is a water soluble vitamin (B3) derived from nicotinic acid, and is also known as niacinamide or nicotinic acid amide. Nicotinamide is believed to restore ß-cell NAD pools by inhibiting poly(ADP-ribose)synthetase (PARS, an enzyme involved in repair of DNA breaks) (135) and to function as a free radical scavenger (136). In a recent conference on the feasibility of a nicotinamide trial in Europe (Copenhagen, Denmark, December 6-7, 1992) some reports were presented showing that these effects were indeed described with nicotinamide, but not in the concentrations used. Tissue levels would not reach the required levels for such an action and could actually reverse the effect on PARS or lead to carcinogenic effects (M. Smulson, R.W. Pero, personal communication). Moreover, nicotinamide was reported to influence growth in young rats. NA has been used at clinical onset of the disease, both in animals and humans. No significant restoration or sustaining of ß-cell function was observed (137, 138). In prediabetic animal models conflicting results were obtained (138-140). A preliminary report from New Zealand, where a nation wide trial of nicotinamide takes place, showed no convincing beneficial effect of NA (141). Moreover, this study enrolled all ICA positive individuals into the treatment group, so no control group is available. The taste of the drug has, although improvements were made by the industries, helped to reduce patient compliance to 60-70%. It will be difficult to conclude clear effects from such studies. Other studies in prediabetes have been initiated or preliminary reports are presented (142, 143). The NA treatment is claimed to be save. Hypervitaminosis and liver damage have been described.

Insulin therapy is the cornerstone of therapy in type 1 diabetes. Some

groups however, use insulin before the clinical diagnosis, in prevention studies. Three different mechanisms are proposed:

1) Hyperglycemia is toxic for ß-cells. Insulin therapy would therefore help to improve ß-cell function. In the phases just before clinical onset, short periods of hyperglycemia would alter the function of the ß-cells.

2) Insulin has been pinpointed as a candidate primary antigen. Insulin autoantibodies are present in some individuals before diagnosis and insulin is ß-cell specific. Tolarization with insulin would provide a way to adapt the immune system. Such adaptations could be done either by subcutaneous or intravenous ways, but alternatively oral administration of insulin could induce tolerization. Similar approaches were undertaken for other autoantigens (144, 145), but the mechanism by which oral tolerance induction would work are unknown. Currently, after succesful experiments in animals (146, 147), first degree relatives are under treatment in the Joslin Clinic, Boston (Dr. Richard Jackson) with subcutaneous insulin, in an attempt to re-instruct the immune system and prevent (presumed) diabetes.

3) It has been proposed that the activity of the ß-cell is related to the immune response. Active ß-cells are presenting more antigen and are therefore more immunogenic than resting ß-cells (106). Insulin therapy (subcutaneous) would help to rest the ß-cells, thereby reducing the antigen expression and the chances for diabetes.

Animal models have provided evidence that direct interference in the immune recognition mechanism can block autoimmune diseases. Components of the trimeric complex can be blocked with specific monoclonal antibodies. Such approaches are used for the TCR (148), MHC (149), CD4(150), CD8 (151) and cytokines (152). In addition, peptide-mediated immunotherapy would be a possibility where modified peptides bind to the MHC and TCR but do not elicit a proliferative response.

Based on the assumption of loss, breakdown or non establishment of tolerance, other approaches have been tried with interesting results. Intrathymic transplantation of islets resulted in tolerance for ß-cell components and prevention of diabetes in susceptible animals (153-155). These approaches have not yet been established for animals with overt disease, but this approach could result in the re-instruction of the immune system. If such treatment could be offered to humans in the early phases of ß-cell destruction, further deterioration might be stopped. Several technical, medical and ethical problems will however be needed

to overcome before such therapies would be useful in clinical practice. At this moment, the are not feasible and only stress the need for better prediction methods.

In general, the immuno-interventions performed at present are not specific and may have several side effects. For many researchers, including our group, such current interventions attempts are started too early and additional data on etiology and pathogenesis are needed. Moreover, prediction methods still suffer from lack on specificity and sensitivity. The scarce amounts of prediabetic individuals for both prediction and prevention studies is an additional problem. The strategy followed in the work presented here is to first obtain improved prediction methods accompanied by further studies to unravel the immune mechanisms that cause type 1 diabetes. Only than, intervention studies will not only prevent the disease in at risk relatives, but can also be applied in populations.

GAD and 38kD autoantibodies: Better prevention?

The antibodies to GAD and 38kD will have a role in prediction. Specific molecules are identified now and can be applied in prediction tests, which is a main improvement compared to the former assays such as islet cell antigen (ICA) testing. ICA's are analyzed in a 'bioassay (human donor pancreas) and no specific target molecules are known. Several markers for type 1 diabetes are presented in this thesis: MHC molecules, antibodies and T-cell analysis. A major problem is that technical, economical and ethical reasons do not allow to tests every available marker yet. It will be clear that research is needed to determine the best approach in prediction of type 1 diabetes mellitus. Two main approaches are possible. Family studies of first degree relatives provide a relatively large high risk group for the development of type 1 diabetes. However, prediction and prevention are only likely to be cost-effective if applied in the population at large, since most cases develop in families without first degree relatives with the disease. Therefore, family studies are only a primer to assess predictive values of markers. In a second phase the results obtained in such studies can be applied in population studies. We are currently involved in a family related study of 80 families (1989-1992) and want to increase this to 300-500 families in the next few years. At this moment groups around the world take two main approaches in diabetes prediction. Some groups use ICA determination as an entry criteria for further studies (123, 156). ICA positive individuals are subsequently HLA typed. The risk can be established further by adding other markers. Others use insulin autoantibodies as an entry criteria for high risk (122). An approach that has been hampered by high costs would be to test for susceptibility at the HLA level in the population and only study

for signs of ß-cell destruction (autoantibodies, T-cells) in those with a typical risk haplotype. HLA typing has been too costly, but new developments may change this rapidly. Using PCR techniques and capillary blood sampling, population size screening could allow the detection of at risk individuals. A major problem of this approach is, that not all diabetic patients will have 'a' diabetogenic haplotype. Furthermore, 'diabetes associated' haplotypes differ between races. Analysis of genetic susceptibility could be improved by the use of extended haplotypes (including the TAP-genes, Bcl-2 gene and other diabetes related genes as they have been identified in the NOD-mouse). HLA typing and extended haplotype analysis might provides a good way to identify susceptible individuals, but this method is still too expensive to apply in population based studies.

Alternatively, fast assays for GAD could allow the analysis of large study groups. We propose to use this marker in conjunction with genetic screening. Repeated analyses might be important to detect autoantibodies as marker of ß-cell destruction. The advantage of GAD analysis is that the identification of the target allows precise quantification of the assays, something that is not possible for ICA's. Already now, the immunoprecipitation method can be quantified using laser scanning of phosphoimager screens. Moreover, research on epitopes will allow to develop even more specific tests. Since large scale HLA analysis is yet expensive and ethically not approved, analysis of GAD autoantibodies, supplemented with 38kD antibodies will provide a quantitative basis for type 1 diabetes prediction (See chapter 4.2) and will be also be feasible in large studies using new assay systems.

5.6 Conclusion

Antibodies to GAD_{65} and the 38kD protein are present in the early phases of ß-cell destruction and may represent the humoral response to a primary T-cell antigen in human type 1 diabetes. Additional studies are needed to evaluate why tolerance to GAD and the 38kD protein are lost or never established. The role of the ß-cell in such events needs full attention. T-cell responses to these targets can help to analyze why proteins or peptides are no longer ignored and are the cause of a specific attack to ß-cells. Analysis of the epitopes in GAD_{65} shows that specific parts of the molecule may be involved in type 1 diabetes. Further analysis may reveal if a restricted primary epitope is present and if prolonged ß-cell destruction results in broadening of the epitopes on GAD_{65}. GAD and the 38kD protein are

not yet identified as causative ß-cell components of type 1 diabetes but provide models to explain both ß-cell and immune system related mechanisms of autoimmunity. The further identification, characterization of these molecules is likely to provide information that can help to design applications for prediction and prevention of the disease.

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Chapter 6

List of Abbreviations

Summary

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Acknowledgments

Curriculum Vitae

LIST OF ABBREVIATIONS

AET : Aminoethylisothiouronium bromide hydrobromide
APC: Antigen Presenting Cell
BB: Bio Breeding
CMV: Cytomegalo virus
CNS : Central nervous system
EAE : Experimental allergic encephalomyelitis
EDTA : Ethylenediaminetetra-acetic acid
EGTA : Ethyleneglycol-bis-(ß-aminoethyl ether) N,N,N',N'-tetra acetic acid
ELISA : Enzyme linked immunosorbent assay
GABA : γ-amino butyric acid
GABA-T : GABA transaminase
GAD : Glutamic acid decarboxylase
IAA : Insulin autoantibodies
ICA : Islet cell cytoplasmatic autoantibodies
IDDM : Insulin dependent diabetes mellitus
IL-1 : Interleukin 1
INF-γ : Interferon-γ
HLA : Human leukocyte antigen
HLB : Hypotonic lysis buffer
IL:Interleukin
LDCV: Large Dense Core Vesicle
MBP : Myelin basic protein
MHC : Major histocompatibility complex
MS: Multiple sclerosis
Mw : Molecular weight
NEM : N-ethylmaleinimide
NIDDM : Non insulin dependent diabetes mellitus
NK: Natural Killer
NOD : Non-obese diabetic mice
NSE: Neuron specific Enolase
PAS : Protein A Sepharose
PBS : Phosphate buffered saline
PBMC : Peripheral blood mononuclear cells
PCMPS : p-chloromercuriphenylsulfonic acid
PLP : Pyridoxal-5'-phosphate
PMSF : Phenyl-methyl-sulfonyl fluoride
PVDF : Polyvinylene-diflouride

RIA : Radio-immuno-assay

SDS - PAGE : Sodium-dodecylsulphate - polyacrylamide gel electrophoresis

SG: Secretory Granule

SLMV : Synaptic like microvesicle

SMS : Stiff man syndrome

SSV: Small Synaptic Vesicle

SZ : Streptozotocin

TBS : Tris buffered saline

TCR : T-cell receptor

TH: Tyrosine Hydroxylase

TNF-α : Tumor-necrosis-factor-α

Tx-100 : Triton X-100

Tx-114 : Triton X-114

Summary

Diabetes Mellitus is a chronic disease, characterized by the inability of the body to maintain control over glucose levels. The hormone insulin is needed for this control. Insulin is produced in specialized cells (ß-cells) in small clusters of endocrine cells (islets of Langerhans) in the pancreas. Two major forms of this disease exist: type 1 or insulin-dependent diabetes mellitus, and type 2 or non-insulin dependent diabetes mellitus. This thesis describes work performed on type 1 or insulin-dependent diabetes mellitus.

Chapter 1 gives an overview of this disease and discussed the current knowledge on the pathophysiology of this disease. Type 1 diabetes is the result of immune destruction of the ß-cells. This abolishes the possibility to produce insulin completely. Type 1 diabetes occurs predominantly in childhood and adolescence. Only about 25% of the cases occur later in life. Type 2 diabetes occurs mainly after the age of 35 years. The symptoms do not present because of a lack of insulin, but because the insulin cannot be secreted and function properly. Other diabetic syndromes are the result of other pancreatic diseases or problems with insulin usage. Type 1 diabetes can present with life-threatening complications in the acute phase. There is a therapy for the disease (daily injections with insulin), but no cure. Most people can live a relatively normal life with this disease, but long-term complications after 20-30 years of diabetes result in a high morbidity and mortality.

The cause(s) for the ß-cell destruction in type 1 diabetes is (are) not known. The disease has a genetic background i.e. the susceptibility can be inherited, but not the disease itself. Additional triggers are needed, and a role of environmental factors has been shown by epidemiological studies. However, which factor(s) is (are) responsible is less clear. Viral infections, certain chemicals and food components have all been suggested, but no proof has been provided yet.

The pathological hallmark of the disease is infiltration of the islets of Langerhans by cells of the immune system. These immune cells are able to destroy the ß-cells selectively. This immune reaction is directed to a self-component and is hence called an auto-immune reaction. All immune reactions are the result of an encounter of immune cells with components that they regard as non-self: bacteria and viruses. In auto-immune reactions such responses are directed to self-peptides. At present it is presumed that in those individuals, who inherit the genetic susceptibility for type 1 diabetes, this disease can ensue.

The major question in type 1 diabetes research is which component of the ß-cell is responsible for this faulty reaction. Normally, the immune system develops tolerance towards self-components. It is believed that in autoimmunity, the immune system has lost tolerance to this self-protein or was never able to develop tolerance. The identification and analysis of the lack of tolerance belongs to the aims in this thesis : if the responsible peptide can be identified, prediction of the disease becomes feasible. Further unraveling of the pathophysiology could result in prevention. A candidate for this was identified as a 64kD protein. **Chapter 2** describes how the 64kD protein was identified as the neuroendocrine enzyme Glutamic Acid Decarboxylase or GAD. The rare neurological disease Stiff-man Syndrome (SMS)is associated with type 1 diabetes. About one third of the SMS patients also have type 1 diabetes. SMS patients were found to have antibodies to GAD, which is present in special neurons (GABA-ergic neurons). GAD was known to have a molecular weight of about 64kD. Initially, some researchers thought that the 64kD protein could not be identical to GAD, since 64kD antibody containing sera did not recognize GAD as SMS patients did. The antibodies to GAD in type 1 diabetes were shown to be highly dependent on the three dimensional structure of the GAD molecule, while antibodies SMS sera recognize also the denatured molecule. Additional work showed that rat ß-cells contained two forms of GAD, GAD_{67} and GAD_{65}. Also the GABA-ergic neurons contain the same two forms. GAD_{65} is a molecule that can be bound to small vesicles in such neurons and in ß-cells. This binding is performed by a lipid modification of the molecule (palmitoylation) allowing the molecule to stay close to the place where its product (GABA) is needed. This subcellular and biochemical localization work helps to analyze how the lack of tolerance to this molecule can result in autoimmunity. In addition, Chapter 2 describes the differences between the two diseases in which GAD becomes a target for autoimmunity: SMS and type 1 diabetes. Except for the differences in immune recognition by the antibodies, the specific localization of neurons behind a so-called blood-brain barrier are believed to play a role. Moreover, it is suggested in this chapter that, while in type 1 diabetes the immune cells play a major role by destroying the ß-cells, in SMS the antibodies are the cause of neuronal dysfunction.

The antibodies to GAD in type 1 diabetes are present in 80% of the newly diagnosed patients, but also long before the clinical diagnosis of type 1 diabetes. This allows their use as an early warning system of ongoing ß-cell destruction. However, it will be clear that prediction is only useful once prevention is available. On the other hand, it is important to be able to predict diabetes, once prevention

methods become available. Most cases of this disease occur in individuals who have no first degree or second degree family member with type 1 diabetes, in other words, they 'pop-up' in the population without a 'warning'. Only 5% of the cases are family related. This implies that preventive measures, once available, are only useful if the population at large is screened and not if screening is limited to those families where type 1 diabetes is already present. This implies that sensitive and simple prediction methods must become available. The use of GAD autoantibodies in prediction of type 1 diabetes are analyzed in **Chapter 3**. Although the identification of the 64kD protein as GAD allowed the development of recombinant GAD systems, the assays were hampered by technical problems. An improved method was developed (Chapter 4.1) which allows a sensitive detection of GAD antibodies. The incidence of GAD autoantibodies in newly diagnosed diabetic children was used as a reference group. The method was compared to the original method using pancreatic islet cells and was found to be extremely sensitive. Chapter 4.2 describes the use of GAD autoantibodies in a population of schoolchildren in the Netherlands, showing this method as the best available marker of humoral autoimmunity at present. This study also clearly showed that further studies on diabetes prediction will need large sample sizes and that even a combination of several markers will not predict all cases of type 1 diabetes. A second target of autoimmunity in type 1 diabetes was further characterized in **chapter 4**. To this 38kD protein autoantibodies had been found in earlier studies but results were inconsistent. A method was developed to improve analysis of 38kD autoantibodies. These autoantibodies were found to complement the GAD analysis. Individuals negative for GAD antibodies are sometimes positive for 38kD antibodies, although most 38kD positive patients also have GAD antibodies. In prediabetic samples, defined as sera obtained from the period before clinical onset of diabetes, the 38kD antibodies were shown to be a very early marker of auto-immunity just like antibodies to GAD are. The 38kD autoantigen is a strongly membrane bound protein, likely to be glycosylated and has no cross-reactivity to GAD. Further analysis has started to identify the molecular structure of this protein. Analysis of 38kD autoantibodies adds to the predictive value of type 1 diabetes. The early appearance of 38kD antibodies makes the molecule an other candidate of a primary antigen involved in the pathogenesis of type 1 diabetes. **Chapter 5** discusses the results of the thesis and discussed how and why further research on GAD, 38kD and other antigens can be conducted. In summary, the identification and characterization of two targets of autoimmunity in type 1 diabetes has resulted in applications of this knowledge in both the prediction of diabetes and in studies on the pathogenesis of this disease.

Samenvatting

Onder Diabetes Mellitus (suikerziekte) wordt een aantal chronische ziekten begrepen die als hoofdkenmerk hebben dat het lichaam niet langer de bloedsuikerspiegels, nodig voor het goed functioneren van alle organen, kan regelen. Het hormoon dat bij deze regulering betrokken is heet insuline. Insuline wordt gemaakt in speciale cellen, zogenaamde ß-cellen, die samen met andere hormoon-makende cellen in kleine groepjes (eilandjes van Langerhans) in de alvleesklier liggen. Twee hoofdvormen van diabetes mellitus kunnen worden onderscheiden alsmede een aantal bijzondere vormen. Dit proefschrift gaat over type 1 of insuline afhankelijke diabetes, vroeger ook wel jeugd diabetes genoemd. Daarnaast bestaat type 2 of niet-insuline afhankelijke diabetes, vaak ook ouderdoms suikerziekte genoemd.

Hoofdstuk 1 geeft een literatuuroverzicht van de specifieke kenmerken van type 1 diabetes en de insuline-makende ß-cel. Type 1 wordt gekenmerkt door een geleidelijke maar uiteindelijk volledige afbraak door het eigen afweersysteem van alle insuline producerende ß-cellen. Deze vernietiging is een proces dat jarenlang kan duren. Deze periode wordt dan ook de prediabetische fase genoemd. Door het tekort aan ß-cellen, en dus aan insuline, ontstaan de symptomen. Deze vorm van suikerziekte komt vooral voor bij kinderen en jong volwassenen. Een deel van de gevallen (± 25%) ontstaat later in het leven. Type 2, of niet-insuline afhankelijke diabetes ontstaat meestal op latere leeftijd. Hierbij wordt wel insuline gemaakt, maar de insuline komt niet goed in het lichaam en kan niet goed gebruikt worden door de lichaamscellen. Naast de typen 1 en 2 zijn er bijzondere vormen van suikerziekte, bijvoorbeeld ontstaan ten gevolge van medicatie, zogenaamde insuline ongevoeligheids syndromen en andere ziekten. Type 1 diabetes kan gepaard gaan met ernstige complicaties in de acute fase. Er is wel een therapie beschikbaar in de vorm van dagelijkse insuline injecties, maar genezing is niet mogelijk. Een redelijk normaal leven is mogelijk voor mensen met diabetes, maar de ziekte kenmerkt zich door complicaties die zich na 20-30 jaar openbaren, veel medische problemen geven en ook levensbedreigend kunnen zijn. Het betreft complicaties aan de ogen (retina), de nieren (nefropathie) en sommige zenuwcellen (neuropathie).

De oorzaak van type 1 diabetes is onbekend. Bekend is wel dat de vatbaarheid voor de ziekte erfelijk is, de ziekte zelf niet. Andere factoren zijn nodig om in vatbare personen de ziekte te doen ontstaan. Epidemiologische studies tonen aan dat dergelijke factoren uit het milieu afkomstig kunnen zijn, zoals

infecties, chemische verbindingen en bepaalde componenten in de voeding. Op dit moment is echter geen enkele van deze factoren een bewezen oorzaak van type 1 diabetes. Uitsluitend zijn correlaties van deze stoffen met de ziekte beschreven en geen oorzaak.

De pathologie van type 1 diabetes kenmerkt zich door een ontstekingsreactie in de eilandjes van Langerhans. Immuuncellen vallen de ß-cellen aan en vernietigen deze. Dit kan gezien worden als een afweervergissing. Normaal zorgt het immuunsysteemsysteem ervoor dat vreemde onderdelen van het lichaam worden afgeweerd. In het geval van type 1 diabetes vergist het afweersysteem zich en valt het een 'eigen' onderdeel van de ß-cel aan. Dit proces wordt autoimmuniteit genoemd. Waarom het immuunsysteemsysteem deze vergissing maakt en tegen welke lichaamseigen stof een dergelijke reactie begint is het doel van veel onderzoek en ook van dit proefschrift. Als deze lichaamseigen stof, die het immuunsysteemsysteem 'op hol' doet slaan, kan worden gevonden wordt het voorspellen van de ziekte mogelijk en komt het voorkómen van de ziekte door middel van speciale herinstructie van het immuunsysteem binnen handbereik. Een kandidaat eiwit voor de rol als veroorzaker van type 1 diabetes was jarenlang het z.g. 64kD (kilo Dalton, een maat voor de molekuulgrootte) eiwit dat in ß-cellen werd gevonden. Patiënten met diabetes hebben al zeer lang voor het uitbreken van de ziekte antistoffen tegen dit eiwit (dat een antigeen wordt genoemd omdat het een immuunreactie teweegbrengt). Wat dit 64kD eiwit precies was, was onbekend. **Hoofdstuk 2** beschrijft hoe de identiteit van dit eiwit is uitgezocht. Hiervoor was niet alleen laboratoriumwerk nodig, maar bleken klinische gegevens van een zeldzame ziekte van groot belang. Het bleek dat in de zeer zeldzame neurologische ziekte 'Stiff-man syndrome' (SMS), die vaak (1/3e van de patiënten) met type 1 diabetes gepaard gaat, antistoffen tegen zenuwcellen aanwezig waren. Deze antistoffen waren gericht tegen een bepaald onderdeel van de zenuwcellen, het enzym (ook een eiwit) Glutaminezuur decarboxylase (GAD). Dit is een belangrijk enzym dat een boodschapper-stof voor zenuwcellen maakt. Het GAD molekuul is ongeveer 64kD groot. Gekeken werd of GAD hetzelfde was als 64kD, omdat een aantal kenmerken van het molekuul overeenkwamen en omdat diabetes relatief vaak voorkomt bij SMS. Hoewel sommige onderzoekers eerst dachten dat er geen overeenkomsten waren, toont hoofdstuk 2 aan dat het 64kD molekuul identiek is aan GAD. De antistoffen in patiënten met diabetes reageerden ook met GAD. Op verschillende manieren werd vervolgens bewezen dat het 64kD antigen in type 1 diabetes het molekuul glutaminezuur decarboxylase is. Verder werk toonde aan dat de antistoffen bij diabetes alleen het GAD

herkennen als dit molekuul in de juiste conformationele vorm bestaat, d.w.z. zoals het in de natuur voorkomt. Bij SMS kunnen de antistoffen ook door onderzoekstechnieken veranderd en niet meer in de juiste configuratie verkerend GAD herkennen. In dergelijke testen verandert het molekuul van driedimensionale structuur. Dergelijke gegevens tonen aan dat de structuur van eiwitten een belangrijke rol speelt bij immuunreacties en dat resultaten van methoden die dergelijke veranderde eiwitten opleveren, voorzichtig geinterpreteerd moeten worden. Sommige onderzoekers hadden door dergelijke veranderingen de overeenkomst tussen 64kD en GAD nooit opgemerkt.

GAD is een bijzonder molekuul. Het enzym is van groot belang voor het functioneren van ongeveer 1/3e van onze hersencellen. Hoewel het de boodschapper-stof GABA maakt, is de rol van dit GABA in de eilandjes van Langerhans onbekend. Waarschijnlijk is dat het ook hier een boodschapper-functie heeft tussen de verschillende celtypen in het eilandje en mogelijk er buiten. Er zijn twee vormen van GAD: GAD_{65} en GAD_{67} (genoemd naar de exacte molekuulmassa) en voor elke vorm bestaat een apart gen. In eilandjes van de rat komen beide vormen voor en deze zijn identiek aan de vormen die in zenuwcellen in de hersenen worden gevonden. Recentelijk is gebleken dat GAD_{65} echter de enige vorm is die bij de mens in ß-cellen voorkomt. GAD_{65} kan op een bijzondere manier aan kleine blaasjes in de zenuwcellen en ß-cellen worden gekoppeld (via een palmitoyleringsstap, dat wil zeggen, dat een vetstaart aan het molekuul wordt gekoppeld). De lokalisatie in de cel is van belang om te zien hoe GAD in de cel wordt getransporteerd en aan het immuunsysteem wordt getoond. Hoofdstuk 2 bespreekt eveneens de verschillen en overeenkomsten die gevonden werden tussen Stiff-man syndrome en type 1 diabetes. Naast verschillen in herkenning van GAD door de antistoffen zijn er verschillen in de lokalisatie van het GAD: het immuunsysteem kan bijvoorbeeld veel moeilijker bij de hersencellen komen. Het grootste verschil tussen de twee ziekten is dat in type 1 diabetes de ziekte door infiltrerende immuuncellen (T-lymfocyten en macrofagen) wordt veroorzaakt, terwijl in Stiff-man syndrome de antistoffen (gemaakt door B-lymfocyten) waarschijnlijk een rol spelen bij de symptomatologie.

Antistoffen tegen GAD worden bij zo'n 80% van de nieuw gediagnostiseerde patiënten met type 1 diabetes aangetroffen. Van groter belang is echter het feit dat lang voor de diagnose (tot 15 jaar ervoor) deze antistoffen al aangetoond kunnen worden en dus een voorbode zijn van op handen zijnde diabetes. Het gebruik van GAD antistoffen als merkers voor dit proces wordt beschreven in **Hoofdstuk 3**. Voorspelling van diabetes op een dergelijke manier heeft natuurlijk alleen nut als er een afdoende methode is om dit proces van ß-cel vernietiging te

stoppen voordat het klinische symptomen geeft. Omgekeerd is een goede voorspellingsmethode nodig omdat de meeste gevallen van diabetes zich aandienen zonder dat de ziekte in de naaste familie voorkomt. Slechts 5% van de gevallen komt voor in families waar de ziekte al bij een van de familieleden aanwezig is. Met andere woorden, een nuttig effect van een preventieve behandeling kan alleen worden bereikt als alle personen met een verhoogd risico worden opgespoord in de populatie. Dit vereist een zeer gedegen predictietest. Gekeken werd of GAD antistoffen hierbij van nut kunnen zijn. Allereerst werd een nieuwe methode voor de bepaling van deze antistoffen ontwikkeld, gebaseerd op recombinant geproduceerd GAD. Vroeger waren eilandjes van Langerhans, verkregen uit ratten, nodig voor de bepaling. Diverse technische problemen werden opgelost en een zeer gevoelige methode werd ontwikkeld. In serum van patiënten met net ontdekte diabetes werd de methode getest, vergeleken met de oorspronkelijke methode met eilandjes van ratten en beter gevonden. Verder onderzoek richtte zich op een bevolkingsstudie die eerder was uitgevoerd en waarbij een andere merker voor ontwikkelende diabetes werd getest (ICA's, islet cell antibodies). De GAD methode bleek gevoeliger voor het vaststellen van het proces van ß-cel vernietiging, maar de methode is nog arbeidsintensief en verbeteringen zijn nodig. De studies toonden aan dat voor een betrouwbare voorspelling zeer grote groepen patiënten en personen moeten worden geëvalueerd en dus een eenvoudige methode nodig is.

Naast GAD/64kD werd destijds een tweede interessant merkstof gevonden, een 38kD eiwit. Eerdere studies gaven geen duidelijkheid over de frequentie van antistoffen tegen 38kD in type 1 diabetes. **Hoofdstuk 4** beschrijft een nieuwe methode voor het aantonen van 38kD antistoffen. 38kD antistoffen verbeteren de voorspellende waarde van testen waarbij naar GAD antistoffen en ICA gekeken werd, doordat sommige GAD antistof negatieve patiënten 38kD antistof positief bleken te zijn. In een relatief grote groep van prediabetische monsters werd gevonden dat 38kD net als GAD een zeer vroege merker is van ß-cel destructie. Het eiwit is sterk membraangebonden en bezit suikerketens. Het heeft geen relatie met GAD. Verder onderzoek naar de molekulaire structuur van 38kD is gestart naar aanleiding van de gevonden resultaten. **Hoofdstuk 5** vat het onderzoek samen en beschrijft nieuwe recente ontwikkelingen rond type 1 diabetes en GAD. Hoewel veel onderzoek gericht is op de rol van het immuunsysteem, moet er in de toekomst meer gekeken worden naar de rol die de ß-cel zelf speelt bij het op gang brengen van een afweerrespons. GAD en 38kD zijn eiwitten waartegen het immuunsysteem de normaal aanwezige tolerantie heeft verloren of misschien nooit

heeft opgebouwd en bieden daardoor modellen om deze tolerantie of het gebrek hieraan te onderzoeken.

Samenvattend heeft de identificatie van het 64kD eiwit als GAD en de verdere karakterisering ervan een bijdrage geleverd aan het onderzoek dat de oorzaak van type 1 diabetes wil ontrafelen. Naast GAD is ook het 38kD antigen een interessante kandidaat voor de lichaamseigen stof die het immuunsysteem dwingt tot een afweervergissing. Beide stoffen worden toegepast in de ontwikkeling van voorspellingsmethoden om type 1 diabetes vroegtijdig te herkennen. Nader onderzoek zal de rol van GAD en 38kD in type 1 diabetes verder duidelijk maken.

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It will be clear from the lists of coauthors that this work was only possible with an extensive network of collaborators around the world. This has been the basis for many fruitful discussions, collaborations and friendships. I want to thank everyone who has participated and hope that this will continue in the future. I am very greatful for all the help and friendliness from everyone at the Hormone Research Institute / UCSF San Francisco, but my special thanks go to Stephan,

Kathi, Yuguang, Qin, Joanne, Mark and John ('Kimster'): I miss the Scandinavian Deli food (not). Na terugkomst in Nederland bleek dat het continueren en uitbreiden van het onderzoek uiteraard geen gemakkelijke zaak was. Greetje, Manou en Ronald wil ik dan ook bedanken voor hun hulp en geduld. Alle andere collega's en vrienden dank ik voor de continue steun en stimulering die ze hebben gegeven. Ik spreek mijn hartelijke dank uit aan de verschillende instanties en bedrijven die geholpen hebben om het fellowship in de V.S en de voortgang van het onderzoek in Nederland financieel mogelijk te maken. Tot slot wil ik alle kinderen en ouders bedanken die hebben meegewerkt, en nog steeds meewerken aan de diverse onderzoeken die hopelijk uiteindelijk leiden tot het verdwijnen van diabetes.

Curriculum Vitae

De schrijver van dit proefschrift werd of 8 februari 1957 geboren te Deventer. In deze plaats bezocht hij de lagere school hetgeen gevolgd werd door de Rijksscholengemeenschap. De middelbare school tijd werd vanaf 1970 voortgezet te Zierikzee en het eindexamen Atheneum werd in 1976 behaald. Na een propedeutisch jaar aan de Landbouw Hogeschool Wageningen, werd in 1977 begonnen met de studie Geneeskunde aan de Erasmus Universiteit Rotterdam. Het artsexamen werd in januari 1984 afgelegd. Gedurende de studie was hij, aansluitend aan het zogenaamde keuzepracticum (1980), werkzaam als student-assistent op de afdeling Kindergeneeskunde (Hoofd: Prof.Dr. H.K.A. Visser). Voorts werkte hij in een zg. studententeam op de Intensive Care Neurochirurgie van het Dijkzigt ziekenhuis. In 1984 en 1985 was hij als militair arts gedetacheerd op de afdeling Hematologie van het Radboud ziekenhuis (hoofd destijds: Prof.Dr. C. Haanen) te Nijmegen alwaar gewerkt werd op de therapeutische aferese afdeling en een onderzoeksproject werd uitgevoerd naar de flowkaryografische detectie van chromosomale schade door bestraling. Aansluitend op de militaire diensttijd werd dit werk voortgezet als arts-assistent tot in oktober 1984 werd aangevangen met de opleiding tot Kinderarts in het Sophia Kinderziekenhuis te Rotterdam (Hoofd: Prof.Dr. H.K.A. Visser). Registratie als kinderarts vond plaats in oktober 1989. Na de opleiding vertrok hij naar Denemarken (Hagedorn Research Laboratory) en San Francisco (Hormone Research Institute University of California San Francisco) alwaar hij tot zomer 1992 werkte in de laboratoria van Dr. S. Baekkeskov. Het in dit proefschrift beschreven onderzoek werd daar uitgevoerd. Na terugkomst in Nederland werd met steun van de Stichting Klinische Genetica Rotterdam en de Sophia Stichting voor Wetenschappelijk Onderzoek het onderzoek voortgezet in laboratorium en kliniek. De schrijver is getrouwd met Aleid Brandsma. Ze hebben twee dochters, Inge en Saskia.