CHARACTERIZATION OF THE HUMORAL IMMUNE RESPONSE TO THE BETA-CELL ANTIGENS INSULIN AND GLUTAMIC ACID DECARBOXYLASE IN PRECLINICAL AND CLINICAL TYPE I DIABETES

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OUlu 2005
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2005
Oulu, Finland

Abstract

The characteristics of humoral immunity have been proposed to reflect the bias between two T helper (Th) lymphocyte subsets: Th1 cells, which activate cell-mediated immunity, and Th2 cells, which mediate humoral immunity. The present study aimed to characterize the humoral immunity to beta-cell autoantigens insulin and glutamic acid decarboxylase (GAD65) in preclinical and clinical type 1 diabetes.

Insulin antibodies were analyzed in pregnant women with or without type 1 diabetes and their newborn infants and in prediabetic children. Epitope or/and isotype-specific GAD65 antibodies (GAD65Abs) were analyzed in prediabetic children, in children and adolescents diagnosed with type 1 diabetes, and in patients with the autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) syndrome. Antibodies were determined by radioligand immunoassays.

The humoral immune response to insulin and GAD65 was observed to be a highly dynamic process, comprising mainly the IgG1 subclass and, less frequently, other IgG subclasses. GAD65Abs were directed primarily to the middle region and secondarily to the C-terminal region of GAD65 as a consequence of epitope spreading. Young children who progressed to overt type 1 diabetes were characterized by a broad initial isotype response to insulin and GAD65 and by a strong IgG1 and IgG3 response to insulin. Children who did not progress to clinical type 1 diabetes were characterized by an emerging IgG4 response to GAD65. Rising levels of GAD65Abs targeted to the middle region of GAD65 were associated with high titers of islet cell antibodies and a decreased requirement for exogenous insulin, probably reflecting a persistent residual beta-cell mass, in patients with manifest type 1 diabetes. Non-immunoglobulin insulin-binding activity was observed to be induced by pregnancy. APECED-associated humoral autoimmunity to GAD65 did not differ markedly from that observed in subjects with type 1 diabetes alone.

In conclusion, isotype-specific GAD65 and especially insulin antibodies are valuable markers of the risk of progression to type 1 diabetes in young children. The appearance of an initial IgG3 subclass response and a strong IgG3 response to insulin in children who progressed to overt type 1 diabetes may reflect the role of cytotoxic Th1-biased immunity in the disease process leading to clinical presentation of type 1 diabetes.

Keywords: autoantibodies, autoimmune polyendocrinopathies, epitopes, glutamate decarboxylase, immunoglobulin isotypes
To my dear wife, Sanna
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Oulu, May 2005

Matti Ronkainen
Abbreviations

aa  amino acids
APC  antigen-presenting cell
APECED  autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy
AUC  area under the curve
cDNA  complement strand deoxyribonucleic acid
cpm  counts per minute
DIPP  Diabetes Prediction and Prevention project
FPIR  first-phase insulin response
GAD65  glutamic acid decarboxylase with a molecular weight of 65 000
GAD67  glutamic acid decarboxylase with a molecular weight of 67 000
GAD65Abs  autoantibodies to GAD65
GAD67Abs  autoantibodies to GAD67
GAD65-C-Abs  autoantibodies to the C-terminal region of GAD65
GAD65-M-Abs  autoantibodies to the middle region of GAD65
GAD65-N-Abs  autoantibodies to the N-terminal region of GAD65
HbA1c  hemoglobin A1c
HLA  human leukocyte antigen
IAAbs  autoantibodies to insulin
IAbs  antibodies to insulin
IA-2  insulinoma-associated protein 2
IA-2Abs  autoantibodies to IA-2
ICAbs  islet cell antibodies
IFN  interferon
Ig  immunoglobulin
IGFBP  insulin-like growth factor binding protein
IL  interleukin
JM  juxtamembrane
MHC  major histocompatibility complex
MICA  monoclonal islet cell antibody
NOD  nonobese diabetic
PEG  polyethylene glycol
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>PTP</td>
<td>protein tyrosine phosphatase</td>
</tr>
<tr>
<td>RU</td>
<td>relative units</td>
</tr>
<tr>
<td>SDS</td>
<td>standard deviation score</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>TRIGR</td>
<td>Trial to Reduce IDDM in the Genetically at Risk</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
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</tbody>
</table>
List of original articles

The thesis is based on the following articles, which are referred to in the text by their Roman numerals:


V Ronkainen MS, Härkönen T, Perheentupa J & Knip M. Characterization of the humoral immune response to glutamic acid decarboxylase in patients with APECED and/or Type 1 diabetes. Submitted for publication.
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1 Introduction

Diabetes mellitus comprises a heterogeneous group of diseases. The factor common to these diseases is hyperglycemia resulting from defects in insulin secretion, insulin action, or both. The current classification of diabetes mellitus is etiological (1). Four main categories are listed: type 1 diabetes, type 2 diabetes, other specific types of diabetes, and gestational diabetes mellitus. Type 1 diabetes, known earlier as juvenile-onset diabetes or insulin-dependent diabetes mellitus, is characterized by beta-cell destruction that usually leads to absolute insulin deficiency and a need for exogenous insulin treatment. Type 2 diabetes, previously known as non-insulin-dependent insulin or adult-onset diabetes mellitus, is characterized by insulin resistance and relative insulin deficiency. Individuals rarely need insulin treatment to survive. The third category comprises a heterogeneous group of diseases of variable origin, such as genetic defects and syndromes. Gestational diabetes is defined as glucose intolerance observed in pregnant women.

Type 1 diabetes is one of the most common chronic diseases among children and adolescents. Clinical type 1 diabetes is a severe disease: daily subcutaneous injections of insulin are required for survival, and severe complications potentially threaten patients with long-term disease. The incidence of type 1 diabetes is constantly increasing in most industrialized countries, including Finland, where the incidence of type 1 diabetes is the highest in the world. In spite of extensive research, the factors and pathological mechanisms leading to clinical type 1 diabetes are poorly known. Actual beta-cell destruction is considered to be mediated by cell-mediated immunity. However, autoantibodies to beta-cell antigens are commonly observed in the preclinical period and at clinical diagnosis, and they are useful markers to identify individuals at risk for type 1 diabetes. The present study aimed to explore the characteristics of the humoral immune response to two central autoantigens in type 1 diabetes, insulin and glutamate decarboxylase.
2 Review of the literature

2.1 Type 1 diabetes

Type 1 diabetes is an autoimmune disease resulting from selective destruction of insulin-producing beta-cells in the islets of Langerhans in the pancreas. Clinical symptoms of type 1 diabetes, i.e. polyuria, polydipsia, weight loss and fatigue, are manifested when most beta-cells are destroyed. It is widely accepted that a genetic predisposition is required for the development of type 1 diabetes. Since not all individuals with genetic susceptibility develop diabetes, some environmental factors, such as viral infections and nutrition, are assumed to have an impact on the process resulting in beta-cell destruction and insulin deficiency. The destruction of beta-cells is considered to be mediated by autoreactive T-cells.

The nonobese diabetic (NOD) mouse is commonly used as an animal model of human type 1 diabetes. NOD mice develop spontaneous diabetes resembling human type 1 diabetes. NOD mice have been widely used for e.g. T-cell transfer studies and intervention trials, which may potentially be applicable to man later. Although there are some common features in the murine model and human type 1 diabetes, such as insulitis with infiltrated mononuclear cells in the pancreatic islet cells, the presence of circulating autoantibodies, genetic complexity, and the contribution of MHC molecules, there are also remarkable differences, such as the higher incidence of diabetes among female mice, the predominant expression of GAD67 in murine beta-cells in contrast to GAD65 in human beta-cells (2), the high incidence of other autoimmune lesions, and the presence of multiple immune abnormalities in mice (3).

2.2 Epidemiology of type 1 diabetes

There is conspicuous geographical and ethnic variation in the incidence of type 1 diabetes. According to a recent worldwide study organized by the World Health Organization, there was more than 350-fold variation in the incidence over the 5-year
period 1990-1994. The highest incidence rates were observed in Sardinia and Finland (37/100 000 children under the age of 15 years) and the lowest in China and Venezuela (0.1/100 000) (4). Incidence is very high in most European countries, North America, and Oceania (New Zealand, Australia), lower in Central and South America and lowest in Africa and Asia. In other words, high incidence is characteristic of Caucasoid populations. However, tenfold variation was observed in the incidence rates on the European continent in 1989-1998, the highest rates being recorded in Finland (44/100 000) and the lowest in Macedonia (4/100 000) (5). In general, incidence rates are high in Northern and North-western Europe and low in Central, Southern, and Eastern Europe. The incidence rate does not follow a north-south gradient as closely as previously assumed, but the variation rather reflects the ethnic and racial distribution (6). The incidence rate has increased greatly over the last few decades, starting in the highly developed countries probably after the middle of the 20th century (7). Recently, the incidence rate has also increased in countries with a low initial incidence (5, 6). The relative increase has been most conspicuous among children aged under 5 years (5, 6, 8) which might result in an overestimation of the overall increase in incidence, since the increase in the youngest age group might reflect more rapid progression to clinical disease (7). In Europe, the increase seems to be leveling off in the countries with the highest incidence rates, such as Denmark, Norway, Sweden, and Sardinia but, unfortunately, not in Finland (5).

2.3 Etiology of type 1 diabetes

The overall concept of the etiology of type 1 diabetes still remains to be resolved. There is consensus on the finding that progression to type 1 diabetes requires genetic disease susceptibility. Several genetic elements have been shown to be associated with type 1 diabetes. Mere genetic predisposition is not sufficient for the development of type 1 disease. The concordance rate for type 1 diabetes has been observed to be maximally 50% in identical twins (9-11), which supports strongly the view that environmental factors play an important role in the pathogenesis of type 1 diabetes. There is a series of environmental factors that have been implicated in the development of type 1 diabetes, e.g. dietary factors and virus infections.

2.3.1 Genetics

It has been known for a long time that there is a hereditary component in the development of type 1 diabetes. Disease concordance is relatively high among identical twins, and the risk to progress type 1 diabetes is higher in the first-degree relatives of affected individuals than in the general population (9-11). Genetic susceptibility to type 1 diabetes seems to be complex and affected by many genes. A number of putative susceptibility genes have been identified (12). However, most of these genes may confer only a weak effect. The strongest disease susceptibility is associated with alleles of the human leukocyte antigen (HLA) complex on the short arm of chromosome 6. It has been
estimated that about half of the genetic susceptibility is conferred by HLA genes (12). Another confirmed region conferring diabetes susceptibility is located in the insulin gene region on the short arm of chromosome 11.

The HLA region is one of the most polymorphic regions in the human genome. HLA genes are divided into three classes, of which the first two play a pivotal role in the activation of the T-cell response. Both the HLA class I and II regions contain several loci, and the loci of the HLA class I genes are designated as A, B, and C, and the loci of the HLA class II genes as DR, DP, and DQ. Alleles are coded by a string of four numbers that are written after the gene locus. HLA class I molecules are expressed on the surface of all nuclear cells and present antigenic peptides derived from endogenous antigens to CD8+ T lymphocytes on the surface of the host cell. HLA class II molecules are expressed normally on the surface of antigen-presenting cells (APC) and B-cells. HLA class II molecules bind antigenic peptides derived from degraded exogenous proteins and present them to CD4+ T lymphocytes. HLA loci are in linkage disequilibrium with each other and are inherited as combinations (haplotypes) (13).

Certain HLA class II alleles or haplotypes are strongly associated with genetic susceptibility to type 1 diabetes. Some haplotypes confer weak disease predisposition and some are protective. The influence of HLA class II gene products on the risk of type 1 diabetes is not known exactly, but it is probably related to their peptide-binding characteristics in antigen presentation and the activation of T-cells (13). The strongest susceptibility has been linked to HLA-DQ loci. However, the risk is strongly modified by HLA-DR alleles that are in linkage disequilibrium with the HLA-DQ alleles. In addition, some DQB1 alleles may be linked to more than one DQA1 allele, resulting in variable impacts on diabetes susceptibility. The ultimate risk of type 1 diabetes is determined by the HLA genotype defined by the combination of susceptible and protective alleles inherited from both parents, and assessment of the susceptibility to type 1 diabetes by means of HLA determinants is thus relatively complex. To sum up, the strongest susceptibility is conferred by the DQB1*0302 allele, which is linked to DRB1*04, and by DQB1*02, which is frequently linked to DRB1*03, whereas disease protection is associated with the DQB1*0301, DQB1*0602, and 0603 alleles (14). A strongly protective allele, such as DQB1*0602, dominates over a high-risk allele when they are combined. A strong genetic disease susceptibility is associated with earlier appearance of humoral markers of autoimmunity to islet cell antigens and earlier manifestation of type 1 diabetes (15-19). Table 1 presents the most frequent haplotypes with their prevalence and odds ratios in the Finnish population based on 622 families with a child affected by type 1 diabetes (20). In terms of genotypes, the strongest disease risk is conferred by the combination of DQB1*0302 and DQB1*02, while moderate or weak susceptibility is conferred by homozygosity for any of the two susceptibility alleles and by various combinations of susceptibility alleles with some neutral or weakly protective alleles (20). Recently, it has been reported that the proportion of patients carrying protective haplotypes (including DQB1*0602 and *0603) is significantly higher among patients diagnosed during recent years than among those diagnosed 50 years ago (21). This may reflect an increasing environmental load allowing the presentation of clinical disease with a weaker genetic predisposition than previously.

The insulin gene and particularly a polymorphic region upstream of the insulin gene have been observed to confer an increased risk for type 1 diabetes (22). Susceptibility has
been localized to the region comprising variable numbers of tandem repeats (VNTR) upstream of the insulin gene. Alleles with short, i.e. 23-63, repeats predispose to type 1 diabetes, whereas alleles with long repeats, i.e. more than 140, are protective (22, 23). More repeats increase the transcription of insulin in thymus, which may facilitate the induction of insulin tolerance, resulting in disease protection (24, 25).

Ueda et al. (26) recently reported that the gene region encoding the cytotoxic T-lymphocyte antigen 4 (CTLA4) on the long arm of chromosome 2 comprises polymorphisms associated with increased risk of common autoimmune disorders, such as Graves’ disease, autoimmune thyroiditis, and type 1 diabetes.

Table 1. The most frequent HLA-DR-DQ haplotypes associated with susceptibility for or protection against type 1 diabetes in the Finnish population.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Allele frequency (%)</th>
<th>Odds ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>Susceptible</td>
<td>DRB1<em>0401-DQB1</em>0302</td>
<td>28.2</td>
</tr>
<tr>
<td></td>
<td>DRB1<em>03-DQA1</em>05-DQB1*02</td>
<td>21.9</td>
</tr>
<tr>
<td></td>
<td>DRB1<em>0404-DQB1</em>0302</td>
<td>9.5</td>
</tr>
<tr>
<td>Protective</td>
<td>DRB1<em>15-DQA1</em>0102-DQB1*0602</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>DRB1<em>11/<em>12</em>1303-DQA1</em>05-DQB1*0301</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>DRB1<em>1301-DQB1</em>0603</td>
<td>1.8</td>
</tr>
</tbody>
</table>

2.3.2 Environmental factors

A series of considerations indicate that the pathogenesis of type 1 diabetes is affected by environmental factors. Firstly, the concordance rate of the disease is less than 50% among identical twins (9-11). Secondly, prevalence varies considerably between different countries and occasionally even within the country (4). Migration from an area with a low disease incidence to a high-incidence area has been reported to increase the risk to be affected (27). Thirdly, the worldwide increase in the incidence of type 1 diabetes after World War II argues in favour of the impact of environmental factors (6). However, no causative environmental factor has so far been decisively identified. It is generally presumed that environmental factors act as initial triggers of the pathogenic disease process in genetically susceptible individuals (28). A more complex model has also been implicated, in which the penetrance and expression of heritable immune aberrations are part of the life-long influences of multiple environmental factors (29). It has been similarly stated that early adaptation to the environment is an interactive process, with genetic factors modulating the gene expression and the priming of the immune system in each individual in a unique and long-lasting manner that persists throughout the remaining life span (7). Thus, the effects of multiple environmental factors may depend on the timing and quantity of exposures. It is also possible that environmental factors
might only promote and modify the disease process. Viruses and dietary factors have been perceived as the most plausible risk factors for type 1 diabetes.

2.3.2.1 Viruses

Accumulating evidence argues in favour of the role of viruses as pathogenic agents in the development of type 1 diabetes. The seasonal variation seen in the presentation of clinical type 1 diabetes (30) and in the appearance of diabetes-associated autoantibodies (18) has been interpreted as reflecting the seasonality of viral infections. A temporal relationship has been reported between certain viral infections, e.g. mumps and enterovirus infections, and the clinical presentation of diabetes. It has been reported that patients with congenital rubella syndrome have a high risk of type 1 diabetes, since 10-20% of them develop the disease at the age of 5-20 years. Some viruses have been isolated from the pancreas of individual patients with recent-onset type 1 diabetes. Serological markers of viral infections have been documented in patients with type 1 diabetes (31).

Several studies have supported the role of enteroviruses as a diabetogenic agent. It has been shown that enterovirus RNA or antibodies are more abundant in patients with newly diagnosed type 1 diabetic than in healthy subjects (32, 33). Enterovirus infections have been found to be more frequent in siblings progressing to type 1 diabetes than in siblings who remain non-diabetic (34). Enterovirus infections may induce humoral autoimmune responses to beta-cell antigens since prospective studies have indicated that enterovirus infections are associated with seroconversion to positivity for diabetes-associated autoantibodies (35-37) and precede the first appearance of autoantibodies (38). In addition, one study showed enterovirus infections to be associated with a concomitant rise in the levels of islet cell antibodies (ICAbs) and autoantibodies to glutamic acid decarboxylase 65 (GAD65Abs) but not in the titers of insulin autoantibodies (IAAbs) or antibodies to the IA-2 protein (IA-2Abs) in prediabetic subjects (39), whereas another study reported enterovirus infections also to be associated with elevated IAAb levels (34). Enhanced T-cell proliferation has been observed in response to enterovirus-infected cell lysates in children positive for diabetes-associated autoantibodies (40). Maternal enterovirus infections during late pregnancy have been implicated to increase the risk of future type 1 diabetes in the offspring (34, 41). However, there is limited direct evidence in favor of an association between enterovirus infections and beta-cell damage. According to some case reports, coxsackie viruses have been isolated from children with acute-onset type 1 diabetes (30). According to one case report, coxsackie B4 virus was isolated from the pancreas of a child who died at the manifestation of type 1 diabetes (42). Later, inoculation of mice with the isolated virus produced diabetic symptoms in the mice. However, there is no convincing evidence for a direct pathogenic role of a virus in human type 1 diabetes, and there are also studies that have failed to establish a correlation between the development of type 1 diabetes and enterovirus infections (31, 43).

Viruses may be involved in the destruction of beta-cells in several ways (31, 44). The direct cytolytic effect of viruses may cause beta-cell destruction, resulting in the release of intracellular autoantigens and the activation of autoreactive T-cells. The infection of beta-cells during early development may result in beta-cell-specific expression of viral
antigens that will be recognized later by the immune system as, for instance, a consequence of later infections. The infection may have an impact on the modification of antigens, MHC expression, and cytokine milieu in beta-cells. At the systemic level, a virus infection may affect the immunological balance by, for example, modulating cytokine expression and by activating autoreactive T-cells, and virus peptides mimicking host cell antigens may break down self-tolerance to such antigens (31). Much attention has been paid to molecular mimicry. The concept has been supported by the fact that there are sequence similarities between several autoantigens and viral peptides (44). Special interest has been focused on the sequence similarity between a portion of glutamic acid decarboxylase (GAD) and the P-2C sequence of the coxsackie B4 virus (45). Although the sequence has been found to be recognized by human T-cells and antibodies, no specific T-cell clones (46, 47) or monoclonal GAD65 antibodies derived from patients with type 1 diabetes (48, 49) have been shown to cross-react between the two molecules so far. Recently, the role of enterovirus in the development of type 1 diabetes was hypothesized to be similar to the impact of polio virus in poliomyelitis (33). According to this hypothesis the low frequency of enterovirus infections in the background population and, consequently, in pregnant mothers results in low concentrations of protective anti-enterovirus antibodies in newborn infants. As a consequence, early enterovirus infection in infants may spread to the internal organs, e.g. the pancreas, in the absence of neutralizing maternal antibodies. The decrease seen in the frequency of enterovirus infections in industrialized countries with a coincident increase of type 1 diabetes is considered to support the polio hypothesis.

2.3.2.2 Nutritional factors

Many nutritional factors have been implicated to play a role in the development of type 1 diabetes. Breastfeeding, nicotinamide, zinc, and the vitamins C, D, and E have been reported to protect from the disease, whereas N-nitroso compounds, cow's milk, accelerated linear growth, obesity, and psychological stress have been claimed to increase the risk (the issue reviewed by Virtanen and Knip (50)). Recently, two papers reported early exposure to cereal proteins in infancy to be a risk factor for subsequent signs of beta-cell autoimmunity (51, 52). One of these studies reported delayed exposure also to increase the risk for beta-cell autoimmunity (52).

The role of breastfeeding and cow's milk as risk factors for type 1 diabetes is highly controversial but intriguing. The diabetogenicity of cow's milk has been postulated to result from the molecular mimicry observed between several cow's milk proteins and beta-cell antigens or to derive from regulatory defects of the gut immune system hampering the induction of tolerance to cow's milk proteins (53). It has been proposed that early exposure to the bovine insulin present in cow's milk in children with regulatory defects in the gut immune system could first lead to the development of immune response to insulin, which would spread to other beta-cell autoantigens and, finally, to the development of destructive beta-cell autoimmunity (54). The observations on the increased cellular and humoral immune responses to cow's milk proteins, the expression of gut-specific homing receptors on GAD-reactive lymphocytes, and intestinal
inflammation in patients with newly diagnosed type 1 diabetes support the role of cow’s milk in disease development (53, 54).

Half of the retrospective studies that have explored the possible association between early feeding and type 1 diabetes have reported that a short duration of breastfeeding or, alternatively, early exposure to cow’s milk-based formula is associated with an increased risk of type 1 diabetes, while the other half have failed to do so (50, 53). Two meta-analyses based on a series of case-control studies indicated that short breastfeeding and cow’s milk exposure before 3 months of age are linked to an increased risk for type 1 diabetes (odds ratios 1.43-1.61) (55, 56). These retrospective observations have, however, been questioned, since the results may be affected by maternal recall bias of infants’ diets and a lower response rate among controls (55). Birth cohort studies (57-59) have failed to confirm the association between short-term breastfeeding or early exposure to cow’s milk and the emergence of markers of humoral beta-cell autoimmunity, with the exception of one Finnish study, in which short exclusive breastfeeding increased the risk for seroconversion to IA-2Ab positivity or for developing all the four autoantibody markers, i.e. ICAbs, IAAbs, GAD65Abs, and IA-2Abs (60). Trial to Reduce IDDM in the Genetically at Risk (TRIGR) is an intervention study determining whether weaning to highly hydrolyzed formula over the first 6-8 months of life decreases the cumulative incidence of diabetes-associated autoantibodies and/or type 1 diabetes in children with at least one affected first-degree relative and HLA-conferred diabetes susceptibility (61). The second TRIGR pilot performed mainly in Finland suggests that such a dietary intervention in infancy does reduce the frequency of diabetes-associated autoantibodies to about 50% of that seen in the control children (62). The studies on the association between later cow’s milk consumption and the development of type 1 diabetes are also conflicting (50). Verge et al. reported cow’s milk intake to have been more abundant in prediabetic children than in control children in New South Wales, Australia (63). The Finnish nationwide Childhood Diabetes in Finland (DiMe) study showed that abundant consumption of cow’s milk in childhood was associated with a higher frequency of diabetes-associated autoantibodies in initially unaffected siblings of children with T1D (64). There was also an almost significant association between a high level of cow’s milk consumption (≥ 3 glasses) and progression to clinical T1D. In contrast, a Swedish retrospective survey indicated that the frequency of milk intake to have been lower among children who presented with T1D than among unaffected children (65).

2.4 Pathogenesis of type 1 diabetes

2.4.1 Autoimmunity

Autoimmunity refers to a situation where the body’s immune system elicits immune responses against its own structures. Self-tolerance implies that the immune system is capable of discriminating between self and non-self-structures. Natural self-tolerance results from clonal deletion or functional inactivation of self-reactive T-cells in the thymus. This includes both positive and negative selection of T-cells: T-cells that can
recognize peptides in association with self major histocompatibility complex (MHC) molecules are selected for survival (positive selection), whereas T-cells that are strongly activated by self-MHC plus self-peptides are eliminated (negative selection). The rationale here is that T-cells that bind strongly to the self-MHC/foreign antigen complex will be activated. If autoreactive T-cells escape from negative selection, they may subsequently react against self-antigens, and cause autoimmune disease. However, it seems that the presence of potential autoreactive T-cells is a characteristic of a healthy human immune system, probably resulting either from insufficient expression of autoantigens in the thymus (e.g. the insulin expression in thymus depends on the variable number repeat (VNTR) polymorphism) or from the recognition of epitopes that are not normally exposed to the immune system (43). In most individuals, autoreactive T-cells are, however, not activated after the binding of the antigen in the periphery. This is called peripheral tolerance. Various autoregulatory T-cells (for example T helper CD4+ cells) together with cytokines may play a major role in whether peripheral tolerance to distinct autoantigens remains or breaks down. Altogether, autoimmune disease develops when the self-tolerance of the relevant T-cells is broken at either the central (thymus) or the peripheral level (lymph nodes) (66).

Several findings support the view that human type 1 diabetes is an autoimmune disease. HLA-conferred susceptibility to type 1 diabetes has been confirmed (28). Humoral and cell-mediated immune responses against pancreatic beta-cell antigens have been repeatedly observed, and the infiltration of active T-cells into the inflamed pancreatic islets has been established. Type 1 diabetes has been observed to reappear after pancreas transplantation from a non-diabetic identical twin (67). Remission after the diagnosis of type 1 diabetes has been induced and sustained by immunosuppressive therapy (68).

It is not yet clear how self-tolerance breaks down during the pathogenesis of human type 1 diabetes. It might be due to insufficient thymic negative selection, bypass of peripheral tolerance or defective suppression. The delayed expression of self-protein in beta-cells may lead to insufficient thymic selection and development of diabetes, as seen in a transgenic mice model (69). It may be possible that self-peptides normally protected from the immune system inside beta-cells are released during beta-cell damage resulting from either viral infection or other factors. Possibly, autoimmunity is elicited when T-cells react with released self-peptides in a favorable cytokine milieu, or self-peptides have been structurally altered during the destructive process or hyperexpressed in a manner that results in the activation of T-cells. Alternatively, subdominant and cryptic beta-cell antigens escape negative selection and are later encountered by autoreactive T-cells (70). Peripheral tolerance may be bypassed by molecular mimicry. Foreign peptides mimicking self-peptides may confuse T-cells to react against similar immunogenic determinants located in the self-antigen (44). Molecular mimicry has been implicated to have an impact in immune-mediated diseases, such as myasthenia gravis, multiple sclerosis, and rheumatic fever (44). The role of molecular mimicry in the pathogenesis of type diabetes is supported by the fact that there is sequence homology between diabetes-related antigens and viral and dietary proteins, which have been reported to be linked to type 1 diabetes (53). Transgenic mice expressing viral antigens on their beta-cells did not develop diabetes spontaneously but presented with diabetes when infected by the same virus strain (71). Virus infections in early life before the development of immunological
tolerance may, in parallel, induce low-level expression of viral antigens in human beta-cells. Later, the same viral infection might elicit T-cell responses that result in beta-cell destruction. Infectious agents may confuse immune regulation by up-regulating T helper 1 cell cytokines, which may assist in the activation of autoreactive T-cells as a consequence of defective suppression (44).

It has been proposed that the optimal moment for the presentation of beta-cell autoantigens would occur during the perinatal wave of beta-cell death (72). In juvenile rodents, physiological beta-cell death via apoptosis takes place about 2 weeks after birth (73). A similar event may occur in man in the perinatal period and even as late as 2 months after birth (74). Apoptosis itself has been demonstrated to be linked to the initiation of beta-cell autoimmunity in NOD mice (73). In addition, it has been shown that, if the capacity of macrophages to clear a high number of apoptotic cells is exceeded, immature dendritic cells are recruited to scavenge apoptotic cells, which may result in the maturation of dendritic cells in the favorable milieu of proinflammatory cytokines (75). In general, antigen-presenting cells (APC) may play a central role in the initiation of anti-islet T-cell responses (76). In fact, the maturation and function of dendritic cells derived from peripheral blood have been reported to be defective in patients with type 1 diabetes and in close relatives of patients (77, 78). This has been postulated to reduce the negative selection of autoreactive T-cells in the thymus or to impair the induction of regulatory T-cell in the periphery.

Autoimmunity has been proposed to be triggered by a superantigen. Superantigens are microbial products that have specificity to virtually all T-cells with a defined type of T-cell receptor. When the superantigen binds to the MHC molecule, massive T-cell activation is elicited. These T-cell clones may also include autoreactive ones, and the activated T-cell immunity will thus be directed against self-antigens as well. This has been supported by the finding that islet cell membrane preparations from patients with newly diagnosed type 1 diabetes but not from MHC-matched healthy controls were selectively able to expand T-cells with T-cell receptors characteristically recognized by superantigens (79).

2.4.2 Th1/Th2 paradigm

Cytokines are small polypeptide mediators produced by cells of the immune system. Cytokines are involved in the regulation and coordination of immune responses. CD4+ T helper cells can be divided into three subtypes according to the cytokines they produce. T helper (Th1) 1 cells secrete preferentially interferon (IFN) γ, tumor necrosis factors (TNF) β, interleukin (IL) 2, and IL-12, which promote cell-mediated immunity to destroy intracellular pathogens. Th2 cells produce IL-4, IL-5, IL-6, IL-9, IL-10, and IL-13, which activate humoral immunity, mucosal immunity, and allergy to protect against extracellular pathogens. T-cells producing both Th1 and Th2 cytokines are called Th0 cells. Cytokines produced by Th1 and Th2 cells are mutually inhibitory for the differentiation and effector functions of the reciprocal phenotype, resulting in polarization toward either a Th1 or a Th2 response (80).
Cytokines are also produced by cells other than CD4+ T helper cells. Natural killer T-cells are capable of producing large amounts of cytokines, especially IL-4, in response to primary antigenic stimulation. It has been reported that natural killer T-cells, which lack the normal capacity to produce IL-4, are associated with the development of type 1 diabetes in humans (81). In addition, there are macrophage-derived cytokines, such as IL-1, TNF-α, and IFN-α, which are referred to as proinflammatory cytokines (80). Cytokines also have an effect on the antigen-presenting and cytokine-secreting functions of APC (e.g. macrophages and dendritic cells) (82). It has been shown that there are two subclasses of dendritic cells in mice. One cell population induced Th1-biased and the other Th2-biased responses (83). IFN-γ has been reported to enhance the expression of MHC I molecules in the pancreatic islets, which has been considered an early feature of islet pathology in type 1 diabetes (84, 85).

Cytokines regulate the switching of various immunoglobulin (Ig) isotypes and subclasses. However, the pattern is complex and poorly defined. In mouse, IL-4 stimulates the production of IgG1, IgE, and probably IgA, IL-5 stimulates IgE, and IL-10 stimulates IgG3 and inhibits IgA synthesis, whereas IFN-γ induces IgG2a and IgG3 and inhibits IL-4 and IgA production (86). This pattern is not directly applicable to humans. There is evidence that IL-4 induces the synthesis of IgE and IgG4 in human B-cells (87). IL-4 is essential in the induction of hypersensitivity responses, as seen in parasite infections and in allergy. IFN-γ plays a central role in the development of cell-mediated immunity, including cellular cytotoxicity against intracellular pathogens and viruses (88), and may be associated with the formation of the subclasses IgG1 and IgG3 in human (89). In theory, the distribution of isotype-specific antibodies would reflect, at least to some extent, the polarization of the antigen-specific immune response to Th1 or Th2-like reactivity. In addition, various IgG subclasses react differently with complement, the strongest reactivity being seen for IgG3 and nearly non-existing reactivity for IgG4 in man (90).

Cytokines and particularly the polarization into Th1 or Th2-mediated immune responses have been considered to have an impact on organ-specific autoimmune diseases, including type 1 diabetes (91). A shift of the cytokine profile toward Th1-polarized immune responses has been suggested to reflect cytotoxic pathogenicity and beta-cell destruction, whereas the dominance of Th2-based responses has been associated with less aggressive immunity. Elevated concentrations of Th1 cytokines have been observed in the peripheral blood of patients with recent-onset type 1 diabetes (92). Decreased IL-4 or increased IFN-γ production by peripheral blood mononuclear cells (PBMC) has been reported in patients with newly diagnosed type 1 diabetes (93) and in high-risk relatives of affected patients, respectively (94). The inverse relationship between cellular and humoral immunity to GAD65 in subjects at risk for type 1 diabetes may reflect the polarization of GAD-specific response (95). Reduced production of IL-4-associated IgG2 and IgG4 antibodies to tetanus toxoid vaccine has been observed in young children testing positive for diabetes-related autoantibodies (96). The prevalence of Th2-polarized diseases, such as atopic disorders, asthma in particular, has been reported to be decreased among children affected by type 1 diabetes (97).

More data supporting the role of Th1/Th2 polarization has been obtained from studies in NOD mice. Transferred T-cell clones of the Th1 phenotype provoked rapid progression to diabetes in young NOD mice, whereas Th2 cells lacked the ability to trigger clinical
2.4.3 Destruction of pancreatic beta-cells

2.4.3.1 Morphology of the pancreas

The pancreas is composed of both endocrine and exocrine tissue. Endocrine tissue only accounts for about 2% of the total mass of the pancreas. Endocrine cells are located in islets that are dispersed throughout the exocrine tissue. Three main cell types have been identified within the islets: alpha, beta, and delta cells that produce glucagon, insulin, and somatostatin, respectively. In addition, there are islets comprising PP cells, which synthesize pancreatic polypeptide. Type 1 diabetes-associated autoimmunity selectively targets beta-cells (113).

2.4.3.2 Initiation of beta-cell destruction

It is generally accepted that the destruction of beta-cells is mediated by cellular immune response. This is supported by the following facts: T-cells are present in insulitis, disease progression is delayed by immunosuppressive drugs directed specifically against T-cells, and circulating autoreactive T-cells can be detected in patients at the clinical
manifestation of type 1 diabetes (3). It has remained open where the potentially islet-autoreactive T-lymphocytes are initially activated. The activation of T-cells requires the presentation of autoantigenic determinants to self-reactive T-cells by MHC II molecules. However, MHC II molecules may not be expressed normally on beta-cells in vivo. It has been shown by in vitro experiments that the expression of MHC II molecules can be induced on the surface of beta-cells by the combined effect of IFN-γ and TNF-α (114), making the activation of autoreactive T-cells possible locally in the islets. Alternatively, and even more likely, autoreactive T-cells by APC, which primarily express MHC II molecules. It has been postulated that the initial encounter of APC and naïve self-reactive T-cells takes places in the pancreatic lymph nodes (72). The activated T-cells are capable of invading the islets, where they become re-activated by encountering cognate beta-cell autoantigens and thereby initiate insulitis. It seems that the autoimmune response is antigen-driven in type 1 diabetes. This is supported by the fact that the strongest genetic susceptibility is associated with MHC class II alleles. Supporting data have been obtained from studies with NOD mice: pathogenic T-cells did not survive after the removal of beta-cells (70).

2.4.3.3 Insulitis and beta-cell death

There are only limited data on the morphology of pancreatic islets in type 1 diabetes in man because of the rare availability of fresh pancreas from patients with type 1 diabetes. It has, however, been shown that insulitis is characterized by the infiltration of mononuclear cells into the islets. The majority of infiltrating cells have been shown to be CD8+ T-cells, which strongly supports the view that beta-cell destruction is a cell-mediated disease, followed by macrophages, CD4+ T-cells, and B-lymphocytes (115-118). Hyperexpression of MHC class I molecules has been observed on beta-cells, whereas hyperexpression of MHC II molecules has been seen on endothelial cells but not consistently on beta-cells (115, 117, 118). The increased expression of intercellular adhesion molecule 1 has been detected in the vascular endothelium of the islets, in accordance with the increased infiltration of mononuclear cells from the circulation to the pancreatic islets (117, 118). It also seems that the degree of insulitis varies in different parts of the pancreas, and that not all islets are affected (117, 118). It has been estimated that 80-90% of beta-cells have been destroyed at the presentation of clinical type 1 diabetes (28, 113).

More detailed data have been obtained from NOD mouse studies. Peri-insulitis has been shown to precede invasive insulitis in mice (119). Insulitis seems definitely to be T-cell-mediated in NOD mice: Autoimmune diabetes does not develop in NOD mice that are genetically athymic or T-lymphopenic or have been thymectomized at birth (72). The precise roles of CD4+ and CD8+ T-cells in the pathogenesis of beta-cell destruction are controversial (43). CD4+ T-cells have been shown to predominate in the early phase of insulitis (120). The role of T-cell subsets has been examined by transferring various T-cell populations from diabetic NOD mice to irradiated or neonatal mice. Some studies have reported that CD4+ T-cells alone are able to cause or accelerate the onset of the disease in young NOD mice (121, 122), while others indicate that CD8+ T-cells have to be included
in efficient transfer (123, 124). It is clear that CD8+ T-cells alone are unable to invade the islets and to transfer disease (122, 125). However, autoimmune diabetes has not been observed to develop in NOD mice that lack the expression of MHC 1 molecules (126) or are treated with antibodies to CD8+ cells in early life (127), emphasizing the critical role of CD8+ cells in beta-cell destruction. It is obvious that the most effective transfer includes both CD4+ and CD8+ cells (122, 125). Most likely, both T-cell subsets are required, and the major contribution of CD4+ cells is to provide proper homing of CD8+ effector cells into the islets (125). Macrophages and dendritic cells have been shown to be the first cell types that infiltrate the islets in NOD mice (128). Macrophages play a crucial role, since they are essential for the differentiation of T-cells into beta-cell cytotoxic T-cells (129), and the inactivation of macrophages has been consistently reported to prevent the development of disease in NOD mice (130).

Mathis et al. (72) proposed two different cellular mechanisms leading to beta-cell death. In the recognition-linked mechanism, cytotoxic T-cells recognize autoantigens presented by MHC molecules on the surface of beta-cells. This requires direct CD8+ T-cell/beta-cell contact. This would imply the recognition of MHC I-restricted antigens by CD8+ cells. In the activation-linked mechanism, the T-cell (either CD4+ or CD8+) recognizes the beta-cell antigens presented indirectly by APC in the proximity of islets. This is supported by the fact that MHC II molecules are not expressed on beta-cells. The activation model results in the direct killing of bystander beta-cells through cytokines and soluble death mediators produced by T-cells and in the activation of the cytocidal functions of macrophages. Final beta-cell death would occur via apoptosis in the first model, whereas soluble mediators, such as IFN-γ, IL-1, TNF-α, IL-6, and free radicals would destroy beta-cells in the latter model.

2.4.4 Role of autoantibodies

Autoantibodies to beta-cell antigens are seen in almost all prediabetic individuals and patients with newly diagnosed type 1 diabetes before or at the diagnosis of clinical disease (131, 132). The duration of the preclinical period with detectable autoantibodies can vary markedly, from only a few months to more than 16 years (133). Autoantibodies may appear in infancy or at any age (134, 135). The first detectable autoantibodies have been observed to be usually, but not exclusively, IAAbs in very young children (18, 136). A more diverse pattern has been observed in older individuals (134). At least in young children autoantibodies have been shown to appear sequentially within a short time window rather than simultaneously (19, 134, 136-138). Intramolecular epitope spreading has been documented to occur within GAD65 (139) and the IA-2 molecule (140). Single autoantibody positivity and a low expression level may represent a perfectly harmless phenomenon, whereas multiple autoantibodies reflect aggressive beta-cell damage and rapid progression to overt clinical disease (132, 141-143). Humoral autoimmunity to a single autoantigen seems to be an antigen-driven process, as demonstrated by an analysis of the variable gene regions of monoclonal GAD65Abs (144).

Autoantibodies to beta-cell antigens have not been considered to actively take part in beta-cell destruction. Rather, they are perceived as reflecting the activity of the beta-cell
destructive process. It is possible that neither antibodies nor B-cells are required for the development of type 1 diabetes in man, since there is a case report of a patient who, despite hereditary B-cell deficiency, progressed to type 1 diabetes (145). B-lymphocytes have not been shown to be definitely required for the induction of autoimmune diabetes in NOD mice, since pure T-cells from diabetic NOD mice are sufficient to induce diabetes in neonatal recipients depleted of B-cells (146). However, B-cells have been shown to be necessary for the initiation of the autoimmune process, as NOD mice with B-cell deficiency or B-cell depletion do not develop diabetes (147, 148). It has been demonstrated that B-cells act efficiently as APC through their surface Ig and, consequently, activate autoreactive T-cell responses to self-antigens in NOD mice (149). It has been shown in NOD mice that elimination of maternally transmitted autoantibodies prevents diabetes in progeny, supporting the view that maternal autoantibodies play an important role in the development of diabetes in NOD mice (150). However, maternally transmitted antibodies in children born to mothers with type 1 diabetes have not been observed to be associated with progression to type 1 diabetes (151). Rather, offspring who were GAD65Ab or IA-2Ab-positive at birth had a significantly lower risk for developing type 1 diabetes.

### 2.4.5 Autoantigens

Antibodies to antigens of pancreatic islet cells were first reported in 1974, when antibodies in sera obtained from patients with polyendocrine disorders, including type 1 diabetes, were observed to bind selectively to islets on microscopic sections of human pancreas (152). Later, islet cell autoantibodies (ICAbs) have been observed in up to 70-90% of patients with newly diagnosed type 1 diabetes (153-155), which makes them one of the most sensitive humoral markers of clinical type 1 diabetes (156). It has become evident that ICAbs represent a heterogeneous set of antibodies targeting various autoantigens expressed in beta-cells, two major autoantigens being GAD65 (157) and tyrosine phosphatase–like protein IA-2 (155). In addition to GAD65 and IA-2, insulin is also a crucial autoantigen in the pathogenesis of type 1 diabetes. Tens of other potential candidate autoantigens (e.g. islet cell antigen 69, 38 kDa jun-B, carboxypeptidase H, heat shock protein, glycosylated islet cell membrane antigen 38, Imogen 38) have been identified (158), while their role as markers of the disease process has remained modestly or poorly defined, and they are therefore not presented in detail here.

Proliferative responses of PBMC to certain autoantigens have been used to measure autoreactive T-cell responses in preclinical and clinical type 1 diabetes. However, the first international workshop for the evaluation of T-cell assays recently showed that T-cell proliferation assays were unable to discriminate between patients and controls, and there was substantial variation between the laboratories in disease sensitivity and the strength of the responses measured (159). More advanced technologies and better quality of antigen preparations will be required to detect disease-specific T-cell responses.
Properties of insulin. Mature insulin is a globular protein with a molecular weight of 5800. Insulin is composed of two separate chains, the A chain with 21 amino acids and the B chain comprising 30 amino acids, which are joined by two disulfide bridges. Insulin is processed from a single-chain precursor, proinsulin, by cleaving a signal peptide and later C-peptide from the proinsulin molecule.

Humoral and cellular immune responses to insulin. A humoral immune response to exogenous insulin is induced after the initiation of subcutaneous insulin administration (160). Autoantibodies to insulin were observed initially in 1983, when antibodies to insulin were measured before the initiation of exogenous insulin treatment in patients with newly diagnosed type 1 diabetes (161). The frequency of IAAbs has later been shown to be about 50% in patients with newly diagnosed type 1 diabetes (153, 162-166). Several facts support the view that insulin may be an important and crucial antigen in the initiation of beta-cell autoimmunity. Firstly, IAAbs have been observed to often appear as the first autoantibody reactivity in the preclinical period of type 1 diabetes (18, 136) and to be associated with early development of type 1 diabetes in humans and NOD mice (167). Secondly, an inverse correlation has been reported between IAA titers and age at diagnosis (153, 164, 167, 168), and IAA have been reported to be infrequent among patients diagnosed at adult age (>20 years) (15). Thirdly, insulin is the only beta-cell-specific autoantigen identified so far. T-cell responses to human insulin and proinsulin have been reported in prediabetic individuals and in patients with recently diagnosed type 1 diabetes (169).

Isotype distribution of IAAbs. Insulin autoantibodies have been observed to be predominantly IgG1 (83%) and, to a lesser extent, IgG4 (42%) and IgG2 (17%) at the diagnosis of type 1 diabetes (170). In very young children at risk for type 1 diabetes, IAAbs have been reported to comprise predominantly IgG1 subclass antibodies, followed by IgG4, IgG3 and IgG2 (137). A similar pattern has been observed in older IAAb-positive first-degree relatives of diabetic patients: IgG1 subclass antibodies were seen in almost all cases, IgG3 in 32%, IgG4 in 26%, and IgG2 in 24% (171). In young children, none of the individual subclasses were associated with progression to overt diabetes, whereas in older relatives, the risk was higher in those with IgG2, IgG3, or IgG4-IAA than in those without these subclasses. In one report, IgG3 have been observed to dominate over the IgG1 subclasses in IAAb-positive adults without a specific risk for type 1 diabetes (172). It has been reported that high-affinity IAA are associated with a broad IgG isotype response to insulin, the HLA DRB1*04/DQB1*0302 haplotype, young age at the initial appearance of IAAbs, and subsequent progression to multiple islet autoantibodies and/or type 1 diabetes (173). Exogenous insulin treatment has been reported to promote the IgG1 and IgG4 responses to insulin (170). According to one paper, insulin antibodies (IAbs) were predominantly composed of IgG1, followed by IgG3 more frequently than by IgG4, in insulin-treated subjects (174).

Autoantibodies to proinsulin. Specific antibodies to proinsulin have been observed in patients with type 1 diabetes prior to insulin treatment (175). However, most of the proinsulin antibodies have been shown to recognize preferentially epitopes on the insulin molecule (176, 177). Proinsulin may have a central role in the early immunizing event,
since all high-affinity IAAbs have been shown to be reactive with proinsulin, whereas low-affinity IAAbs bound insulin alone (173). Proinsulin autoantibodies have been reported initially to be more closely associated with type 1 diabetes than IAAbs (178), but this association was not confirmed by three subsequent papers (176, 177, 179).

**NOD mice studies.** Both cellular and humoral autoimmune responses to insulin have been described in NOD mice (169, 180). Interestingly, the administration of insulin or insulin peptides via a nasal, subcutaneous, or oral route has been reported to prevent or reduce the incidence of autoimmune diabetes in young NOD mice (reviewed by Gottlieb et al. (169)). The preventive effect may be mediated by the induction of Th2-type cytokine (IL-4, IL-10) responses.

### 2.4.5.2 Glutamic acid decarboxylase (GAD)

*Properties of GAD.* Glutamic acid decarboxylase is an enzyme that catalyzes α-decarboxylation of L-glutamic acid to gamma-amino butyric acid (GABA), which is an inhibitory neurotransmitter. Two GAD isoforms are expressed in humans, with molecular weights of 65 000 (181) and 67 000 (182). Each isoform is encoded by single genes, the GAD65 gene located on the short arm of human chromosome 10 and the GAD67 gene on the long arm of chromosome 2 (182). The amino acid sequences of the isoforms are highly homologous in their middle and C-terminal region, whereas there is less similarity in the N-terminal region. GAD is expressed in the central and peripheral nervous system and in many endocrine tissues, including pancreatic beta-cells (183). In the islets, GAD is synthesized in the cytoplasm as a hydrophilic soluble molecule, which undergoes several hydrophobic modifications before its N-terminal end is reversibly anchored to the membranes of microvesicles (184). The function of GAD in the islets is still open. Both GAD isoforms are expressed in human brain, while full-length GAD65 is expressed only in human islets (185). In addition, a splice variant of GAD67, with a molecular weight of 25 000, is produced in human islets (186). The three-dimensional structure of human GAD65 has been predicted by using homolog-scanning mutagenesis and detailed mapping of autoreactive epitopes recognized by a set of monoclonal islet cell antibodies (MICA) derived from patients with type 1 diabetes or Graves’ disease. (187). According to that data, the GAD65 molecule comprises three structural domains: the N-terminal one comprising the amino acid residues 1-200, the middle one covering the residues 200-460, and the C-terminal one including the residues 461-585.

*Humoral and cellular immune responses to GAD.* Autoantibodies to GAD65 were documented for the first time in 1982, when sera from children with newly diagnosed type 1 diabetes were observed to precipitate a protein with a molecular weight of 64 000 from a lysate of human islets (188). Later, the antigen was identified as GAD65 (189). GAD65Abs can be detected in about 70-80% of patients with recently diagnosed type 1 diabetes (190-195). It has been shown that GAD65Abs persists for years after the clinical diagnosis of diabetes, and the levels of GAD65Abs rise in some patients after clinical presentation (196-199). Autoantibodies to GAD67 (GAD67Abs) have been detected in 18-26% of patients with newly diagnosed type 1 diabetes (192, 200). However, the binding of GAD67 is generally blocked by unlabeled GAD65, suggesting that detectable
GAD67Abs may, in fact, represent antibodies to shared epitopes with GAD65 (45, 200). GAD67 may, in some cases, be a unique autoantigen in type 1 diabetes, as implicated by the observation of two type 1 diabetes-prone individuals still showing GAD67 binding after competitive inhibition with GAD65 (139). Autoreactive T-cell responses to both GAD65 and GAD67 have been observed in patients with newly diagnosed type 1 diabetes (201, 202).

High levels of GAD65Abs may be a sign of reduced risk for type 1 diabetes. The prevalence of GAD65Abs has not been observed to decline with age at clinical presentation of type 1 diabetes in contrast to IAAbs and ICAbs (203). Among first-degree relatives of patients with type 1 diabetes, the levels of ICAbs, IAAbs and IA-2Abs have been reported to be higher among those who progress to overt type 1 diabetes than in those who remain healthy, whereas no such association has been observed in the case of GAD65Abs (156, 171). High titers of GAD65Abs have been reported to correlate inversely with the T-cell response to GAD in patients with type 1 diabetes (95) or to spontaneous secretion of IFN-\(\gamma\) from PBMC in the high-risk relatives of patients (94).

Antigenic determinants recognized by GAD65Abs. Monoclonal islet cell antibodies (MICA) derived from GAD65Ab-positive patients (204-207) and deletion mutants of GAD65 and GAD65/67 chimeric molecules have been used to map autoreactive epitopes within the GAD65 molecule (45, 187, 200, 208, 209). Almost all epitopes recognized by patient-derived MICA identified so far are conformational and located in the middle and C-terminal domains of the molecule (Table 2). Only a few epitopes have been mapped to the N-terminal region of GAD65. The extreme portion of the N-terminal end of the molecule (aa 1-39) is not recognized by any MICA, indicating that this region may be buried in the folded molecule or masked by membrane interactions (187, 210).

GAD65Ab-positive sera from individuals at risk for or diagnosed with type 1 diabetes recognize predominantly the middle and C-terminal epitopes, whereas N-terminal epitopes are recognized rarely (139, 211, 212). It has been documented that the middle region of GAD65 is the most immunodominant one, and it is recognized by the early immune response, whereas the other regions of GAD65 are recognized mainly together with antibodies targeting the epitopes in the middle region (139, 211). Spreading of the immune response from the middle region to the other regions, primarily the C-terminal region, has been documented in single pre-diabetic individuals (139). Two papers have reported that none of the epitope-specific reactivities or their changes are associated with type 1 diabetes (139, 212), whereas one paper has shown that elevated levels of C-terminal antibodies distinguish patients with type 1 diabetes from unaffected GAD65Ab-positive individuals (211), and two other papers have reported that young GAD65Ab-positive patients with recently diagnosed type 1 diabetes show significantly less binding to rat GAD67 and the N-terminal region of human GAD65 than adult GAD65Ab-positive healthy individuals, first-degree relatives, and patients with slow-onset autoimmune diabetes (213, 214). Reduced reactivity of GAD65 and GAD67 to the N-terminal region has been reported in GAD65Ab-positive pregnant women with gestational diabetes compared to GAD65Ab-positive relatives of patients with type 1 diabetes (215).

Isotype distribution of GAD65Abs. It has been consistently documented that GAD65Abs are mainly composed of the IgG1 subclass in individuals at risk for type 1 diabetes and in diabetic patients (137, 171, 215-221). The occurrence of other IgG subclasses has been reported at variable frequencies, probably resulting from differences...
in study populations and variability in the assays used. No isotype switching or specific isotype distribution within the GAD65Ab response was observed to be associated with progression to diabetes in three surveys (137, 171, 220), whereas one paper reported increased levels of IgE and IgM-GAD65Abs to be a sign of a low risk for future diabetes in siblings of patients with type 1 diabetes (217), and another paper showed the high levels of IgG2 and IgG4-GAD65Abs to be typical of ICAb-positive first-degree relatives of diabetic patients (216) who did not progress to type 1 diabetes.

**NOD mice studies.** Both cellular and humoral immune responses to GAD67 and GAD65 have been observed in NOD mice (222). GAD has been considered to play a central role in the pathogenesis of diabetes in NOD mice, since autoreactivity to GAD has been reported to develop prior to other autoantigens coincidentally with early insulitis (222, 223), and the expression of antisense GAD transgene in beta-cells blocked the generation of diabetogenic T-cells, diminished the T-cell responses to other beta-cell autoantigens, and prevented diabetes in NOD mice (224). In addition, the administration of GAD65 or its peptides and GAD67 to young NOD mice by a variety of routes (e.g. intrathymically, intravenously, intranasally) has been observed to result in tolerization to GAD and prevention of diabetes (222, 223, 225, 226). The preventive effect of GAD administration seems to result from the induction of CD4+ Th2 cell responses. This is evidenced by findings showing that the secretion of Th2-like cytokines compared to Th1-like cytokines and the production of GAD65Abs, especially the Th2-related IgG1 subclass, were increased in treated mice (223, 226). T-cell responses have been observed to spread intramolecularly within the GAD molecule in NOD mice, being dependent on the stage of the disease (223).
Table 2. Monoclonal islet cell antibodies to GAD65

<table>
<thead>
<tr>
<th>Clone</th>
<th>Source</th>
<th>Required amino acid residues (Ref. 187)</th>
<th>Subclass</th>
<th>Ref.</th>
</tr>
</thead>
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<tr>
<td>N-terminal domain (aa 1-200)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPB</td>
<td>T1D, DR3/DR4</td>
<td>39-173</td>
<td>IgG1</td>
<td>205</td>
</tr>
<tr>
<td>DPD</td>
<td>T1D, DR3/DR4</td>
<td>96-173</td>
<td>IgG1</td>
<td>205</td>
</tr>
<tr>
<td>Middle domain (aa 201-460)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPC</td>
<td>T1D, DR3/DR4</td>
<td>231-234 and 366-413</td>
<td>IgG1</td>
<td>205</td>
</tr>
<tr>
<td>MICA6</td>
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<td>242-282</td>
<td>IgG1</td>
<td>204</td>
</tr>
<tr>
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<td>242-282</td>
<td>IgG1</td>
<td>206</td>
</tr>
<tr>
<td>MICA4</td>
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<td>308-365</td>
<td>IgG1</td>
<td>204</td>
</tr>
<tr>
<td>b96</td>
<td>Graves’ disease</td>
<td>308-365</td>
<td>IgG1</td>
<td>207</td>
</tr>
<tr>
<td>C-terminal domain (aa 461-585)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>483-499 and 556-585</td>
<td>IgG1</td>
<td>204</td>
</tr>
<tr>
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<td>T1D, DR4/DR11</td>
<td>483-499 and 556-585</td>
<td>IgG1</td>
<td>206</td>
</tr>
<tr>
<td>DPA</td>
<td>T1D, DR3/DR4</td>
<td>483-499 and 556-585</td>
<td>IgG1</td>
<td>205</td>
</tr>
<tr>
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<td>512-585</td>
<td>IgG3</td>
<td>204</td>
</tr>
<tr>
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<td>Graves’ disease</td>
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<td>IgG1</td>
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<td>IgG1</td>
<td>204</td>
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<tr>
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<td>96-173 and 451-585</td>
<td>IgG1</td>
<td>206</td>
</tr>
<tr>
<td>MICA9</td>
<td>T1D and Graves’ disease, DR3/DR3</td>
<td>96-173 and 451-585</td>
<td>IgG1</td>
<td>206</td>
</tr>
</tbody>
</table>

2.4.5.3 Insulinoma-associated protein 2 (IA-2 protein)

Properties of IA-2. Insulinoma-associated antigen 2, also known as islet cell antigen 512, is a 979 amino acid long integral membrane protein expressed in cells of neuroendocrine origin, including human brain and pancreatic islets (227, 228). It contains an extracellular domain, a transmembrane region and an intracellular protein tyrosine phosphatase (PTP) catalytic domain. The extracellular domain locates in secretory granules, while the intracellular domain resides in the cytoplasm (228, 229). No enzymatic activity has been observed for the IA-2 protein (229). The intracellular domain of the IA-2 protein is highly homologous to the catalytic domain of receptor-type tyrosine-phosphatase proteins (227, 229). The precise function of IA-2 protein in beta-cells is still open.

Humoral and cellular immune responses to IA-2. Antibodies to the IA-2 protein were reported for the first time in 1990, when sera from patients with type 1 diabetes were observed to recognize previously unknown fragments with molecular weights of 37 000 and 40 000 (230, 231). Some years later, sera of patients with type 1 diabetes were independently observed to recognize the islet cell antigen 512 isolated from an islet cDNA expression library (229, 232) and the IA-2 protein was cloned from human insulinoma cells (227). Later, the 40 000 dalton fragment was shown to be the intracellular portion of the IA-2 protein (233-235), while the 37 000 fragment was
derived from the islet antigen 2β (IA-2β) protein (236). IA-2Abs have been reported to be present in 50-80% of patients with newly diagnosed type 1 diabetes (233, 237-240). T-cell autoreactivity to IA-2 has been demonstrated in patients with type 1 diabetes (241).

**Antigenic determinants of IA-2Abs.** IA-2Abs have been shown to recognize the cytoplasmic domain of IA-2 (aa 605-979), while no reactivity has been observed to transmembrane or extracellular regions (234, 242, 243). IA-2Ab-positive sera have been shown to recognize epitopes in the PTP domain somewhat more frequently than epitopes in the juxtamembrane (JM) region (242-244). It has been reported that antibodies to the IA-2-specific JM epitope tend to appear first in young IA-2Ab-positive children, followed by the appearance of antibodies to IA-2 specific PTP and finally by antibodies to the PTP epitopes shared by IA-2 and IA-2β molecules (140, 245). To note, IA-2β is a transmembrane PTP expressed in pancreatic islets, sharing a high degree of homology with the intracellular part of IA-2, and the majority of antibodies to IA-2β observed in patients with newly diagnosed type 1 diabetes cross-react with IA-2Abs, probably originally targeting the IA-2 protein (140, 236). Progression to diabetes has been reported to be associated with the presence of antibodies to the JM region (245, 246) or with reactivity to multiple IA-2 and/or IA-2β epitopes (140, 171).

**Isotype distribution of IA-2Abs.** The humoral response to IA-2 has been shown to comprise dominantly IgG1 subclass antibodies in both prediabetic and diabetic subjects (137, 220, 246, 247). It has been reported that an exclusive IgG1 response is associated with a higher risk for diabetes progression in very young children compared to those with other IgG subclass specific IA-2Abs (137), whereas a contradictory finding has been reported in older IA-2Ab-positive relatives of patients affected by type 1 diabetes (171). In addition, an IgG4-restricted response to IA-2 has been reported to be related to protection from type 1 diabetes in ICAb-positive siblings of patients with type 1 diabetes (247).

### 2.5 Prediction and prevention of type 1 diabetes

The risk of future type 1 diabetes can be assessed based on a positive family history or on genetic, immunological, and metabolic markers. Prevention of type 1 diabetes can be categorized into three groups: primary, secondary, or tertiary prevention (248).

**Family history.** Susceptibility to type 1 diabetes is inheritable. Thus, the risk of type 1 diabetes is increased among relatives of patients with type 1 diabetes. The risk to develop disease before adult age is approximately 5-6% in the offspring of an affected father, whereas the risk is lower, being 2-3%, in a child with an affected mother. If a child is affected by type 1 diabetes, the risk of any sibling is dependent on the degree of HLA identity with the index case, being 16-20% in HLA-identical siblings but only 1% in HLA-nonidentical siblings (248).

**Genetic markers.** Genes in the HLA region are considered to confer major genetic susceptibility to type 1 diabetes (14, 20). The ultimate risk is determined by a combined effect of various susceptible or protective HLA alleles. Risk values associated with the most common haplotypes in the Finnish population are presented in Table 1. The positive
predictive value (PPV) of genetic risk markers remains relatively low, since susceptible HLA alleles are frequently also present in healthy individuals.

**Humoral immune markers.** Autoantibodies detectable in the preclinical period of type 1 diabetes are useful predictive markers for future disease. All four classical antibody reactivities, i.e. ICAbs, IA-2Abs, GAD65Abs, and IAAbs, have a relatively high positive predictive value among first-degree relatives (131, 156, 249). The risk for future type 1 diabetes increases if multiple autoantibody reactivities are present (131, 143, 156, 249). Similarly, the disease risk increases as a function of rising antibody levels other than GADAbs (156). The combination of genetic and autoantibody markers improves the PPV of each autoantibody but results in conspicuously reduced sensitivity (250). In the Finnish population, the following PPVs and sensitivities of autoantibodies for progression to type 1 diabetes over a period of 8.9 years have been measured in siblings of affected children: PPV of IA-2Abs 57%, ICAbs 46%, GAD65Abs 42%, IAAbs 31%, and multiple antibodies 57%, with respective sensitivities of 64%, 79%, 64%, 24%, and 76% (250). It seems that the PPV of single antibody markers for progression to type 1 diabetes remains relatively low in the general population (132, 143, 251). Only the PPV of multiple autoantibody positivity may be applicable in the assessment of risk for type 1 diabetes in the general population.

**First phase of insulin response.** A reduced first-phase insulin response (FPIR) has been observed in autoantibody-positive individuals, and it predicts strongly future type 1 diabetes (252-255). Especially antibody-positive relatives with an abnormally low FPIR have a high risk for developing type 1 diabetes. A reduced FPIR may represent subclinical beta-cell dysfunction. Testing for FPIR is an invasive procedure and hence hardly applicable in the estimation of the risk at the population level.

**Primary prevention.** Primary prevention aims to minimize the effect of the factors that initiate the pathogenic process towards beta-cells in individuals with or without genetic disease susceptibility. The TRIGR study is an example of a primary prevention study. It is a randomized, double-blind trial to determine whether it is possible to reduce the cumulative incidence of beta-cell autoimmunity and/or clinical diabetes up to the age of 10 years by weaning infants to highly hydrolyzed formula over the first 6–8 months of life (61). The second TRIGR pilot study performed among Finnish infants showed that a dietary intervention of this kind in infancy appears to reduce the cumulative incidence of type 1 diabetes-associated autoantibodies over the first 4 years of life (62). The actual international TRIGR study, appropriately powered, was initiated in 2002 to provide a definite answer to the question of whether avoidance of complex dietary proteins over the first 6 months of life reduces the risk of progression to overt type 1 diabetes among young children with increased genetic disease susceptibility.

**Secondary prevention.** Secondary prevention aims to stop the course of the disease before any clinical disease manifestation has emerged in subjects with signs of on-going beta-cell destruction. A few secondary prevention trials have been completed recently. Nicotinamide (vitamin B3) has been shown to prevent or delay the development of experimental diabetes (256). Nicotinamide has been considered to preserve beta-cell function by inhibiting the function of noxious enzymes and toxic radicals in the pancreatic islets. The German Nicotinamide Intervention trial (DENIS) was aimed at reducing the cumulative incidence of type 1 diabetes within 3 years from 30% to 6% in high-risk first-degree relatives by using daily oral administration of nicotinamide. The
trial was terminated when the second interim analysis showed no protective effect of nicotinamide (257). Similarly, the large-scale European Nicotinamide Diabetes Intervention Trial (ENDIT) failed to show any effect of daily administration of nicotinamide on the progression to clinical type 1 diabetes among ICAb-positive first-degree relatives of affected children (258).

Insulin administration through various routes has been shown to prevent or delay the onset of diabetes in NOD mice (169). Insulin treatment has been postulated to relieve beta-cell stress by decreasing insulin secretion, by inducing immune tolerance against insulin or by modulating cytokine expression. The aim of the American Diabetes Prevention Trial 1 (DPT-1) was to reduce the incidence of type 1 diabetes in high-risk relatives of affected patients by a low daily dose of subcutaneous insulin plus annual intravenous insulin infusions or by oral insulin treatment. After follow-up for 4 years, parental insulin treatment was not found to reduce the disease incidence in treated relatives as compared to untreated subjects (259). The oral arm of the study similarly resulted in a disappointment, as there was no difference in the progression rate between the group on oral insulin and the placebo group (Skyler JS, personal communication).

The Finnish DIPP study aims to test whether daily nasal insulin administration delays the clinical manifestation of type 1 diabetes when started soon after the appearance of signs of persistent beta-cell autoimmunity (260). In this study, all infants born at three university hospitals in Finland are invited to take part in genetic screening for HLA-susceptible alleles. Children with the risk alleles (HLA DQB1*02/0302 or *0302/x, where x is other than *02, *0602 or *0301) are followed up with sequential sampling at intervals of 3-12 months up to the age of 15 years. The children that present with at least two autoantibodies in two consecutive samples are invited to take part in the intervention trial based on daily treatment with nasal insulin (1 IU/kg/day). The study is randomized, double-blind, and placebo-controlled. The study was initiated in 1994.

Tertiary prevention. After the clinical diagnosis, tertiary prevention aims to preserve the residual beta-cell function or to prevent clinical complications of the disease.

### 2.6 Pregnancy and the immune system

A successful pregnancy requires that the maternal immune system does not reject a genetically different fetus. It is poorly understood how immunological unresponsiveness is achieved during pregnancy. It may be provided by immunomodulation of the maternal immune system, especially locally at the site of the maternal-fetal interface (261). It probably involves down-regulation of potentially dangerous T-cell-mediated immune responses, while activating the humoral and innate immune system (261, 262). This is supported by the view that T-cell-mediated diseases, such as rheumatoid arthritis (263), tend to become less severe during pregnancy, while diseases characterized by excessive autoantibody production, such as systemic lupus erythematosus, become aggravated (264). Immunosuppression has been proposed to be mediated by the activation of Th2 cytokine production and the suppression of Th1 cytokine production (265, 266). However, this issue is controversial (261, 262). The counts of B-cells and the production of IgG probably remain unchanged during pregnancy (261, 262). The decrease in IgG
concentration observed during pregnancy is the consequence of hemodilution as seen in the analyses of sequential samples from pregnant women (267, 268). It has remained an issue of controversy as to whether autoantibody production changes during pregnancy, but most likely, it remains unchanged (269, 270). Some studies have reported stable IAb levels in diabetic mothers during pregnancy (271-273), while a slight decrease in the levels of IAb was reported in one paper (274).

It is well established that maternal IgG are actively transferred to the fetus, even to the extent that the total IgG concentrations are higher in cord blood than in the maternal circulation at term (275). It is also known that IgG2 is transferred less readily than the other IgG subclasses, and IgG2 is often detected at lower concentrations in cord blood than in maternal blood (276). Consistently, the IAb observed in newborn babies appear to have been transferred transplacentally from the maternal circulation during pregnancy in mothers treated with exogenous insulin (135, 277). It has been reported that the levels of IAb are higher in cord blood of newborn infants of non-diabetic mothers than in the mothers (278). Later, it has been shown that the IAb in such newborn infants represent unknown anti-insulin activity that is non-IgG mediated (279).

### 2.7 APECED and autoimmunity to GAD

Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) is a rare autoimmune disease characterized by the breakdown of tolerance to several organ-specific self-antigens. It is also known as autoimmune polyglandular syndrome type 1 (APS1). The disease is inherited as an autosomal recessive trait (280). The AIRE gene defective in APECED has been identified. Its exact role is obscure. It seems that AIRE encodes a protein that may act as a transcriptional activator and may play an essential role in the development and maintenance of self-tolerance (281). APECED is clinically characterized by three disease components: autoimmune polyendocrinopathies, chronic mucocutaneous candidasis, and ectodermal dystrophies. In 68 Finnish patients with APECED, the largest series of APECED patients reported so far, the most common endocrinopathies were hypoparathyroidism (79%), adrenal failure (72%), and ovarian failure (60%) (282). Candidasis was observed in all patients. Among ectodermal dystrophies, enamel hypoplasia (77%) and nail dystrophy (52%) were seen most frequently. Type 1 diabetes was present in up to 23% of APECED patients (283). The prevalence of type 1 diabetes increases as a function of age, which means that approximately 50% of APECED patients will develop diabetes before the age of 50 years (282).

Organ-specific autoantibodies are characteristic of patients with APECED (284). APECED patients with type 1 diabetes have frequently tested positive for ICAbs (55%) and GADAbs (73%) and less frequently for IA-2Abs (36%) and IAAbs (36%) (285). The frequencies follow the pattern seen in patients with isolated type 1 diabetes, the prevalence of IA-2Abs being somewhat lower. As a peculiarity, GADAbs have been observed in one-third of non-diabetic patients with APECED (285-287), while IA-2Abs and IAAbs have not been detected in non-diabetic patients with APECED (285). GAD65Abs may not be related to impaired beta-cell function in APECED, since C-
peptide levels and FPIR values have not been observed to differ between non-diabetic APECED patients with or without GAD65Abs (285). GAD65Abs have been shown to be present at extremely high levels (286), to share more frequently epitopes with GAD67 (286, 288), and to display different epitope recognition (289) in APECED patients compared to GAD65Ab-positive patients with isolated type 1 diabetes. T-cell proliferation to GAD has been reported in APECED patients irrespective of whether type 1 diabetes is present or not (290). Type 1 diabetes has not been found to be associated with HLA class II risk alleles in patients with APECED (286), while the protective DQBI*0602 allele has been reported to protect non-diabetic APECED patients from clinical type 1 diabetes (285).

In addition to APECED, GADAbs have been observed in many other autoimmune and neurological diseases, e.g. autoimmune polyendocrine syndrome 2 (APS 2) (291) and stiff-man syndrome (292). Most of the patients affected by these diseases are non-diabetic, indicating that GADAbs are not a specific marker for type 1 diabetes. The levels of GADAbs are typically higher in these patients than in patients with type 1 diabetes. It also seems that epitope recognition is partly different in stiff-man syndrome and APS 2 compared to that seen in type 1 diabetes (207, 293, 294). No infiltration of lymphocytes, beta-cell destruction, or other immune abnormalities within pancreatic islets were seen in three non-diabetic, autoimmune endocrine patients with high levels of GADAbs antibodies (295). This indicates that humoral autoimmunity to GAD does not necessarily associate with islet inflammation. It has been proposed that the high levels of GADAbs seen in patients with polyendocrine disease or stiff-man syndrome may reflect the deviation of immunity toward Th2 polarization (293, 296).
3 Aims of the present study

Several observations in diabetes research in the 1990’s contributed to the initiation of the present dissertation project in the late 1990s. Firstly, humoral immune responses to specific beta-cell autoantigens had been established in many studies, but the impact of autoantibodies on the pathogenesis of type 1 diabetes was not well understood. Secondly, an interest in the role of the Th1/Th2 paradigm in the pathogenesis of type 1 diabetes had been triggered. Some preliminary observations on the isotype distribution of islet cell autoantibodies had been reported. Thirdly, specific and sensitive radio-immunoassays had been developed for the detection of autoantibodies, e.g. a novel microassay for insulin antibodies had been described in 1997. Similarly, radio-immunoassays for the measurement of epitope-specific antibodies had been established. In addition, sequential serum samples had been obtained with short sampling intervals from young children who seroconverted to antibody positivity in the DIPP study started in 1994. This made it possible to explore the dynamics and characteristics of the autoantigen-specific humoral immune response in detail.

The main aim of the present study was to characterize the humoral immune responses to insulin and GAD65, and to study whether the epitope and isotype characteristics of IAAbs or GAD65Abs reflect the Th1/Th2 polarization of the immune response in individuals with detectable antibodies. The specific aims of the sub-studies were:

1. To study the humoral immune response to insulin in pregnant mothers with or without type 1 diabetes and their newborn infants, and to test the concordance between two assays used in the detection of IAbs.
2. To investigate the isotype characteristics of IAAbs during the prediabetic period in young, genetically susceptible children observed sequentially from birth.
3. To elucidate the epitope and isotype characteristics of the humoral immune response to GAD65 during the prediabetic period of young genetically predisposed children observed sequentially from birth.
4. To study the humoral immune response to GAD65 in young children and adolescents after the clinical presentation of type 1 diabetes by analyzing epitope-specific GAD65Abs.
5. To compare the epitope and isotype characteristics of the humoral immune response to GAD between APECED patients and patients with type 1 diabetes.
4 Subjects and methods

4.1 Subjects

The subjects in study I had initially been recruited for the second TRIGR pilot study. This study aimed to evaluate whether weaning to highly hydrolyzed formula over the first year of life decreases the cumulative incidence of diabetes-associated autoantibodies (61). The first 104 mothers and their newborn infants from families with at least one family member affected by type 1 diabetes were included in study I. Blood samples were obtained from the mothers at the end of the first trimester (originally obtained for routine screening of rubella and syphilis in pregnant women) and at delivery and from the cord blood of the newborn infants. Thirty-eight of the 104 mothers (36.5%) had type 1 diabetes and the remaining 66 were unaffected.

The population included in studies II and III comprised children taking part in the DIPP study (260). In the DIPP study, newborn infants representing the general Finnish population are recruited for genetic screening of type 1 diabetes-associated HLA-DQB1 alleles. Families with an infant carrying an increased HLA-conferred risk are invited to a follow-up study with sequential monitoring of the appearance of signs of beta-cell autoimmunity at intervals of 3-6 months up to the age of 2 years and subsequently at intervals of 6-12 months. ICAbs are used for primary screening of beta-cell autoimmunity. If ICAbs are detected, antibodies to GAD65, the protein tyrosine phosphatase-related IA-2 protein, and insulin are analyzed in all subsequent and previous samples from that individual. Sequential samples are obtained from autoantibody-positive subjects at 3-month intervals. Children who have persistent autoantibodies (at least two consecutive positive samples) are invited to take part in a randomized placebo-controlled intervention trial to assess whether it is possible to postpone the manifestation of clinical diabetes by daily administration of nasal insulin.

Index cases who had seroconverted to positivity for IAAbs or GAD65Abs and subsequently had at least two positive samples were included as subjects in studies II and III, respectively. Altogether, 61 IAAb-positive children were identified by the end of May 2000 and 36 GAD65Ab-positive children by the end of spring 1999. Of the 61 children testing positive for IAAbs, 15 progressed to type 1 diabetes (progressors) and tested
positive for IA Abs in more than one follow-up sample. Each progressor was matched with two IAAb-positive non-progressors for sex, HLA genotype, and IAAb-positive observation time. Thus, 45 children comprised the final study cohort in study II. All of the 36 GAD65Ab-positive children were included in study III. Ten of them presented with clinical type 1 diabetes during the observation period. To compare the GADAb characteristics between the progressors and non-progressors, each progressor was matched with two non-progressors for sex, genotype, and GAD65Ab-positive observation time. Samples taken after the diagnosis were excluded in both study II and III. Samples taken after randomization for the intervention trial were excluded in study II but not in study III. The median age at diagnosis was 2.2 years in the progressors included in studies II and III.

The subjects included in study IV were selected from an initial cohort of 90 children and adolescents with type 1 diabetes diagnosed at the Department of Pediatrics, University of Oulu, in 1983-1986 (197). The 50 subjects in whom GAD65Abs had been detected at least once in the series of samples obtained at the diagnosis of type 1 diabetes and at 2, 5, and 10 years thereafter comprised the study population. The GADAb titers had been observed to peak in 21 of the included patients after the clinical presentation of type 1 (197), which was diagnosed at a median age of 9.2 years.

Twenty subjects included in study V were derived from a series of 68 patients with APECED diagnosed in Finland up to 1988 (282). GAD65Ab-positive patients with APECED were included in study V. One to eight serum samples were available from each patient taken at irregular intervals. The median duration of GAD65Ab positivity was 10.4 years. Type 1 diabetes had been diagnosed in six patients at a median age of 29.7 years. Twenty patients admitted to the Departments of Pediatrics or Internal Medicine, Oulu University Hospital, in 1988-1995 due to newly diagnosed type 1 diabetes were used as a control group in study V (297). The controls were matched with the APECED patients for age, gender, and the levels of GAD65Abs detected in the first GAD65Ab-positive sample among the patients with APECED. Two control subjects were re-matched with each of the six diabetic APECED patients based on their age at the time of sampling closest to the diagnosis of diabetes. When the APECED patients with diabetes were compared with those without it, the first available GAD65Ab-positive sample was used.

More detailed data on subjects are presented in the original articles.
Table 3. Summary of the study populations

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Original cohort</th>
<th>Samples obtained</th>
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<td>Mothers with or without type 1 diabetes and their newborn infants</td>
<td>TRIGR</td>
<td>at first trimester, at delivery, cord blood</td>
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<td>IAbs and IAAbs</td>
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<td>Genetically susceptible children with IAAs</td>
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<td>45</td>
<td>Isotype-specific IAAbs</td>
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<tr>
<td>III</td>
<td>Genetically susceptible children with GAD65Abs</td>
<td>DIPP</td>
<td>sequentially from birth</td>
<td>36</td>
<td>Isotype and epitope-specific GAD65Abs</td>
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<td>IV</td>
<td>GAD65Ab-positive children and adolescents with newly diagnosed type 1 diabetes</td>
<td>90 patients with newly-diagnosed type 1 diabetes</td>
<td>at diagnosis and 2, 5 and 10 yrs thereafter</td>
<td>50</td>
<td>Epitope-specific GAD65Abs</td>
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<td>V</td>
<td>GAD65Ab-positive patients with APECED</td>
<td>68 patients with APECED</td>
<td>follow-up samples taken at irregular intervals</td>
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<td>Isotype and epitope-specific GAD65Abs</td>
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<td></td>
<td>GAD65Ab-positive patients with newly diagnosed type 1 diabetes (controls)</td>
<td>352 patients with newly diagnosed type 1 diabetes</td>
<td>at diagnosis</td>
<td>20</td>
<td>Isotype and epitope-specific GAD65Abs</td>
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</table>

4.2 Methods

4.2.1 Assays for insulin antibodies

Insulin antibodies were measured in studies I and II. In study I, the conventional and a novel microassay were compared with each other. The conventional assay was a modification of that described by Palmer et al. (161). This assay requires 200 μl of serum. In the assay, endogenous insulin was removed from the serum sample by acid charcoal extraction. Thereafter, the sample was incubated with commercial mono-125I(TyrA14)-labeled human insulin in the presence or absence of an excess of unlabeled insulin for 20 hours. After the incubation, antibody-insulin complexes were precipitated with polyethylene glycol (PEG), and the precipitated radioactivity was counted. The antibody level was expressed in nU/ml, where 1 nU/ml corresponds to specific binding of 0.01% of the total counts added. The disease sensitivity of the assay was 26% and specificity 97% based on 140 samples derived from the 1995 Multiple Autoantibody Workshop (298). The assay protocol has been used for years in the Research Laboratory of the Department of Pediatrics, University of Oulu.

The new microassay was a modification from that described by Williams et al. (166). In this assay, a total serum volume of only 20 μl was used. Similarly to the conventional assay, serum samples were initially incubated with mono-125I(TyrA14)-labeled human
insulin in the presence or absence of an excess of unlabeled insulin. In contrast to the conventional assay, endogenous insulin was not removed by acid charcoal extraction. After incubation for 72 hours, antibody-insulin complexes were precipitated with Protein A Sepharose. Protein A binds specifically the antibody classes of IgG1, IgG2, IgG4 with a high affinity but IgG3 only weakly. Unbound activity was washed out thoroughly by repeated washing procedures. The precipitated activity was counted with a scintillation counter. Specific binding was expressed in relative units (RU) based on a standard curve run on each plate. The cut-off limit for antibody positivity was determined (Table 4). The disease sensitivity of the assay was 44% and specificity 100% in the recent 2002 Diabetes Autoantibody Standardization Program workshop. The microassay was initially set up for the present study. The major developmental steps of the microassay compared to the conventional assay included a reduced serum volume, the use of multi-well plates instead of separate tubes, resulting in more automatic procedures, and the automatic processing of raw counts per minute (cpm) into RU.

The basic principles of the assay for isotype and subclass-specific IAAbs were similar to those of the new microassay. The assay was modified from a method described previously by Bonifacio et al. (137). In the isotype assay, protein A Sepharose precipitation was replaced by monoclonal mouse anti-human Ig antibodies (IgG subclasses, IgA, IgE, IgM) linked to streptavidin agarose. No competitive inhibition with unlabeled insulin was performed. The results were expressed as standard deviation scores calculated from the following equation: SD scores (SDS) = \[ \text{delta cpm (subclass specific cpm - unspecific anti-rat IgM cpm)} - \text{mean delta cpm of control subjects} / \text{SD delta cpm of control samples} \]. The threshold for positivity was set at + 3 SDS (Table 4).

More detailed descriptions of the assays are presented in the original articles I and II.

### 4.2.2 Assays for GAD antibodies

Antibodies to GAD were analyzed in studies III, IV and V. Antibodies to GAD65 and its epitopes and to GAD67 were quantified with a method principally similar to the microassay for IAAbs (197). Accordingly, 2 μl of serum was incubated with an appropriate antigen labeled with \(^{35}\)S. Antibody-antigen complexes were precipitated with protein A Sepharose, unbound activity was washed off, and the precipitated activity was counted with a scintillation counter. The results were expressed as RU based on a standard curve run on each plate. Contrary to the microassay for IAAbs, the \(^{35}\)methionine-labeled antigens were produced with an \textit{in vitro} coupled transcription-translation system (TNT\textsuperscript{\textregistered} Coupled Reticulocyte Lysate System, Promega, Madison, WI, USA). The assays for GAD65 epitope-specific antibodies and GAD67Abs were initially set up for study III and later also applied in studies IV and V, whereas the assay for GAD65Abs has been used for years in the Research Laboratory of the Department of Pediatrics, University of Oulu. Antibodies to the epitope clusters of GAD65 were determined by GAD65/67 chimeric molecules. The chimeric proteins were GAD65\textsubscript{1.95/GAD67\textsubscript{1.102-593}} for N-terminal antibodies (GAD65-N-Ab), GAD67\textsubscript{1.101/GAD65\textsubscript{96-444/GAD67\textsubscript{453-593}}} for middle-region antibodies (GAD65-M-Ab), and GAD67\textsubscript{1.453/GAD65\textsubscript{445-585}} for C-terminal antibodies (GAD65-C-Ab) (Figures 1 and 2) (139). The
cDNAs for chimeric constructs were kindly donated by Ezio Bonifacio (Milan, Italy). The cut-off limits for positivity in epitope-specific assays and GAD67 assays were determined in our laboratory (Table 4). The disease sensitivity of the GAD65 assay was 82% and specificity 98% in the recent 2002 Diabetes Autoantibody Standardization Program workshop.

The assay for isotype-specific GAD65Abs was initially set up for study III. Similarly to the conventional GAD65Ab assay, 2 μl of serum sample was incubated with labeled GAD65. Thereafter, monoclonal anti-human Ig antibodies were added. After incubation, biotin-specific streptatavidin agarose was added to precipitate immune complexes. Unbound activity was washed off on the filtration plate instead of repeated washings with centrifugations in between. The final activity was counted from the filtration plates. The results were expressed as SDS similarly to the isotype-specific IAAb assay. The isotype-specific GAD65Ab assay was re-modified for study V. Monoclonal anti-Ig antibodies were linked to streptavidin agarose before use. The washing procedure was revised and performed in a similar manner as in the assays for isotype-specific IAAbs. The results were expressed as SDS. Both isotype-specific GAD65Ab assays took part in the serum exchange workshop carried out between laboratories known to measure isotype-specific responses to GAD65 (results presented at the 5th International Congress of the Immunology of Diabetes Society in Chennai, India in 2001). The results of both isotype-specific GAD65Ab assays were highly concordant with the laboratories using methods confirmed to be specific.

More detailed descriptions of the GADAb assays are presented in the original articles III and V.

4.2.3 Other assays

The present studies focused mainly on GAD and insulin antibodies. Previous data on ICAbs, IA-2Abs, and a few clinical parameters were used in some comparisons. Antibodies to islet cells and IA-2 protein were analyzed with the conventional procedures used in the Research Laboratory of the Department of Pediatrics, University of Oulu. ICAbs were quantified by a standard indirect immunofluorescence method using sections of frozen human pancreas from a blood group O donor (152). The sensitivity of the ICAb assay was 100% and specificity 98% in the most relevant standardization workshop (299). Antibodies to IA-2 were analyzed with a method principally similar to that used for GADAbs, with the exception that filtration plates were used (240). The disease sensitivity of the IA-2Ab assay was 62% and disease specificity 100% in the recent 2002 Diabetes Autoantibody Standardization Program workshop. HLA genotyping was based on DNA amplification with polymerase chain reaction, allele-specific triple-label hybridization and time-resolved fluorescence (300, 301). Serum C-peptide concentrations were measured with a commercial Human C-peptide radioimmunoassay kit (Novo Research Institute, Bagsvaerd, Denmark). Other clinical parameters were analyzed by routine laboratory methods.
4.2.4 Statistical analysis

Conventional statistical analyses, such as Spearman’s nonparametric rank correlation test, cross-tabulation, and chi-square statistics or Fisher’s exact test, were applied as appropriate. Mann-Whitney U-test was used for comparing the variables between the two groups. Paired samples were evaluated with Wilcoxon’s rank-sum test in study I. To avoid multiple comparisons, the area-under-the-curve (AUC) approach was used to calculate integrated autoantibody titers over time in the follow-up in studies II and III. Kaplan-Meier life-table survival analysis and log rank statistics were used to assess progression to clinical diabetes in study III. The changes in antibody levels during follow-up were evaluated using two-way analysis of variance for repeated measures in study IV. More detailed descriptions of the use of statistical analyses are presented in the original articles. Statistical analyses were performed using the SPSS statistical software package (SPSS Inc., Chicago, IL, USA) or the Arcus QuickStat Biomedical statistical software (Addison Wesley Longman Ltd, Research Solutions, Cambridge, UK).

### Table 4. Assays for insulin and GAD antibodies

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cut-off limit for antibody positivity</th>
<th>Determination of cut-off limit</th>
<th>Disease sensitivity/ specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conventional IAAbs</td>
<td>68 nU/ml</td>
<td>99th percentile in 102 children</td>
<td>26%/97%</td>
</tr>
<tr>
<td>Microassay IAAbs</td>
<td>1.56 RU</td>
<td>99th percentile in 371 children</td>
<td>44%/100%</td>
</tr>
<tr>
<td>Isotype-specific IAAbs</td>
<td>3 SDS</td>
<td>3 SDS of 44 children</td>
<td></td>
</tr>
<tr>
<td>GAD65Abs</td>
<td>5.35 RU</td>
<td>99th percentile in 373 children</td>
<td>82%/98%</td>
</tr>
<tr>
<td>GAD65-N-Abs</td>
<td>0.86 RU</td>
<td>99th percentile in 104 children</td>
<td></td>
</tr>
<tr>
<td>GAD65-M-Abs</td>
<td>1.51 RU</td>
<td>99th percentile in 104 children</td>
<td></td>
</tr>
<tr>
<td>GAD65-C-Abs</td>
<td>1.59 RU</td>
<td>99th percentile in 104 children</td>
<td></td>
</tr>
<tr>
<td>GAD67Abs</td>
<td>0.91 RU</td>
<td>99th percentile in 104 children</td>
<td></td>
</tr>
<tr>
<td>Isotype-specific GAD65Abs (study III)</td>
<td>3 SDS</td>
<td>3 SDS of 22 children</td>
<td></td>
</tr>
<tr>
<td>Isotype-specific GAD65Abs (study V)</td>
<td>3 SDS</td>
<td>3 SDS of 40 children</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Ligands used for the detection of antibodies directed to the full-length GAD65 molecule, N-terminal, middle, and C-terminal regions of the GAD65 molecule and to GAD67. The GAD65 and GAD67 regions are indicated in white and black, respectively, for each molecule.

Fig. 2. Autoreactive B-cell epitopes within the GAD65 molecule as reported by Schwartz et al. (187). The GAD65 regions used for the chimeric GAD65/GAD67 molecules are indicated by breaks. Dashed lines represent the N-terminal, solid lines the middle region, and lines with arrows the C-terminal epitopes. One line can cover more than one epitope.
5 Results

5.1 Comparison of two assays for insulin antibodies (I)

Overall concordance between the conventional and new micro IAA assays was 68% when the samples were assigned as positive or negative. Remarkably, 31% of the samples were positive only in the conventional assay. Concordance was perfect in the samples taken in early pregnancy (95%) but substantially lower at delivery (40%) or in cord blood (68%) (Article I/Fig. 3). All the samples testing positive for IAbs only in the conventional assay but not in the microassay had relatively low IAb levels (I/Fig. 3b-c). However, samples with low levels of IAbs in the conventional assay in early pregnancy were also positive in the microassay (I/Fig. 3a).

5.2 Insulin antibodies in pregnant women and in cord blood (I)

Conventional assay. IAbs were not observed in the women without type 1 diabetes in early pregnancy but were, surprisingly, detected in 71% of them at delivery as well as in 32% of their newborn infants (I/Fig. 1). In these women, the levels of IAbs increased highly significantly during pregnancy ($p<0.001$, I/Fig. 2a), but no regular relation was observed in the antibody levels between the maternal samples taken in early and late pregnancy. IAb levels in cord blood were significantly higher than maternal IAb levels in early pregnancy but significantly lower than maternal IAb levels at delivery ($p<0.001$, I/Fig. 2a) and correlated weakly with maternal IAb levels.

Seventy-six percent of the diabetic women were positive for IAbs in early pregnancy, and all of them were at delivery. Of their newborn infants, 71% were IAb-positive (I/Fig. 1). A non-significant increase in IAb levels was observed during pregnancy (I/Fig. 2a), and the titers in the two maternal samples correlated closely. Cord blood IAb levels were significantly lower than maternal IAb levels in early and late pregnancy ($p<0.001$, I/Fig. 2a) and correlated closely with maternal IAb levels.
None of the women turned negative for IAbs during pregnancy, whereas 56 initially negative mothers had low positive titers of IA at delivery. If IAbs were detected in cord blood, the corresponding maternal sample taken at delivery was generally also positive.

**Microassay.** No IAbs were observed in the non-diabetic women or in their newborn infants. Among the affected women, a higher proportion tested positive in early pregnancy (79%) than at delivery (61%), and 61% of their newborn infants were positive for IAbs (I/Fig. 1). In contrast to the conventional assay, the levels of IAbs decreased significantly during pregnancy ($p<0.01$, I/Fig. 2b). The maternal samples obtained at the two time points correlated closely. The IA levels in both maternal samples were significantly higher than the IA level in the newborn infants ($p<0.05$ or less, I/Fig. 2b). Cord blood IA levels correlated closely with early and late maternal IA levels.

Only one mother seroconverted to positivity for IA, while eight became negative during pregnancy. If IAbs were detected in cord blood, the corresponding maternal sample taken at delivery was also positive except in a single case.

### 5.3 Isotype profile of insulin antibodies in young children (II)

Insulin autoantibodies of the IgG1 and IgG3 subclasses dominated the initial response to insulin, being present in 78% and 56% of the 45 children studied, respectively. IgG2-IAAbs were observed initially in 27%, IgA in 13%, and the IgG4 subclass in only 2% of the cases. The initial response comprised significantly more frequently IgG3-IAAbs and more isotypes (2 versus 1) in the progressors than in the non-progressors (II/Fig. 1).

IgG1-IAAbs were seen most frequently (in 96% of children), followed by IgG3 (73%), IgG2 (56%), IgG4 (33%), and IgA antibodies (18%) during the whole observation period. IgG4-IAAbs tended to emerge as the last isotype specificity. The progressors had significantly more frequently IgG3 (100% versus 60%, $p=0.01$) and IgA-IAAbs (33% versus 10%, $p<0.05$) during the follow-up (II/Fig. 1). Both IgG1 and IgG3 responses appeared significantly earlier in the non-progressors ($p<0.05$ or less, II/Table 1), possibly as a consequence of the earlier appearance of IAAbs. The frequency of IgG3-IAAbs and the levels of IgG1, IgG3, and IgA-IAAbs were significantly higher in the progressors in the last sample analyzed (at diagnosis of type 1 diabetes) compared to the temporally corresponding samples in the non-progressors ($p<0.05$, II/Fig. 1 and 2). The progressors had significantly higher integrated levels of IgG1 ($p=0.05$) and IgG3-IAAbs ($p<0.01$), but only the IgG3 antibody levels remained significantly higher ($p=0.04$) after adjustment for the integrated titers of total IAAbs, which tended to be higher in the progressors (II/Table 2).

The temporal profiles of isotype-specific responses to insulin in 10 children are presented in Figure 3. Four of them progressed to clinical type 1 diabetes during the observation period. To note, extremely high levels of IgG3-IAAbs were seen in two progressors (Subjects 1 and 2). IgA-IAAbs were seen initially in two progressors (Subjects 1 and 3) and, after randomisation for the nasal insulin intervention trial, in two non-progressors (Subjects 7 and 10).
Fig. 3. Temporal profiles of subclass-specific IAAbs in 10 children observed from birth. The duration of follow-up is shown in years on the x-axis. IgG1, line with open circles; IgG2, line with closed circles; IgG3, line with closed triangles; IgG4, line with squares, IgA, line with open triangles. Dashed line represents the cut-off limit for antibody positivity (3 SDS). The arrow indicates the time of diagnosis of type 1 diabetes. The black bar represents the duration of the intervention with nasal insulin or placebo. Identical numbering of the subjects has been used in Figure 4.
Fig. 4. Temporal profiles of subclass-specific GAD65Abs in children observed from birth. The duration of follow-up is shown in years on the x-axis. IgG1, line with open circles; IgG2, line with closed circles; IgG3, line with triangles; IgG4, line with squares. Dashed line represents the cut-off limit for antibody positivity (3 SDS). The arrow indicates the time of diagnosis of type 1 diabetes. The black bar represents the duration of the intervention with nasal insulin or placebo. Identical numbering of the subjects has been used in Figure 3.
5.4 Isotype profile of GAD65 antibodies (III and V)

5.4.1 Early response in young children

The initial subclass-specific response to GAD65 comprised IgG1 subclass antibodies in all of the 36 DIPP children. Antibodies of the IgG1 subclass were also dominant in the subsequent samples, being almost always present when GAD65Abs were detected (III/Fig. 2, Table 1). Antibodies of the subclasses IgG2 and IgG3 often appeared together with IgG1 in the first isotype-specific positive sample or soon after the initial IgG1 response (III/Table 1, Fig. 2). The occurrence of the IgG2 subclass response was more stable than that of the IgG3 response, although a transient IgG2 response was observed in many subjects immediately after the initial appearance of the IgG1 response. The IgG2 response lasted longer than one year in eight children and was present in 14 children in their last sample. Antibodies of the IgG3 subclass were observed mostly only during an initial response of 3-12 months, whereafter there was a steep drop in their prevalence (III/Fig. 2). The IgG3 response remained detectable for more than one year in only three children and was present in seven children in their last sample. Antibodies of the IgG4 subclass were clearly the last IgG antibodies to appear (III/Table 1), and only three children had them at the time of the appearance of GAD65Abs. The prevalence of IgG4-GAD65Abs increased progressively, and they were more common than IgG3 antibodies after 12 months and more common than IgG2 antibodies after 15 months (III/Fig. 2). These antibodies were observed to be present for one year or longer in four children and were detected in the last sample analyzed in 11 cases. The levels of antibodies of the IgG subclasses followed the trend observed in their frequencies. Antibodies of the IgA, IgE, and IgM classes were seen rarely and mostly in single samples and at very low levels.

In general, the humoral immune response to GAD65 appeared to be a dynamic rather than a stable process in young children, as seen in the temporal profiles of the responses in ten children in Figure 4. Three of them progressed to clinical type 1 diabetes during the observation period. In the profiles, the IgG1 response dominated in all subjects. The IgG2 and IgG3 responses appeared at or soon after the initial IgG1 response in Subjects 1, 3, 11, 9, 12 and 13. The IgG3 response emerged in the initial response in Subjects 1, 3, 11, 6, 8, 10, and 12. The IgG4 response was the last Ig subclass to appear in Subjects 5, 8, 9, 12 and 13. Subject 13 was known to have been treated with nasal insulin during the intervention.

5.4.2 Responses in patients with APECED

The humoral immune response to GAD65 was also dominated by IgG1 subclass antibodies in the APECED patients: IgG1 antibodies were observed in 19 of the 20 patients, and their levels were higher than those of any other IgG subclass. IgG4 antibodies were observed in 17 and IgG2 antibodies in 15 patients, whereas IgG3 antibodies were seen in only two patients. An IgG4 response was present or appeared as a
continuous response in 11 cases, whereas the same phenomenon for the IgG2 response was only seen in five patients. Both of the IgG3 responses detected were observed in the first GAD65Ab-positive sample and disappeared later.

5.5 Antibodies to antigenic determinants of GAD65 (III, IV, V)

5.5.1 Early response in young children

The initial humoral immune response to GAD comprised GAD65-M-Abs in 89% of the 36 young children in the first sample positive for epitope-specific GADAbs. The initial epitope-specific response included GAD65-C-Abs in 42% of the children, always together with GAD65-M-Abs, and GAD65-N-Abs in 8% of the cases either alone or together with both GAD65-M-Abs and GAD65-C-Abs. GAD65-M-Abs were later found in all children in whom antibodies to GAD65 epitope clusters were observed. The humoral response spread mainly from the middle region to the C-terminal region, since 12 of the 17 children (71%) with an initial response directed exclusively to the middle region developed subsequently GAD65-C-Abs. If the response spread to the C-terminal region, it occurred rapidly, so that the frequency of GAD65-C-Abs was equal to that of GAD65-M-Abs by 12 months after the initial response to GAD65 (III/Fig. 1). The responses to the N-terminal region were weak and transient and disappeared in all children within 22 months after their appearance.

5.5.2 Response in older children and adolescents at the diagnosis of type 1 diabetes and thereafter

GAD65-M-Abs dominated over the other epitope responses at diagnosis and thereafter in 50 patients, of whom 48 were positive for GAD65Abs at diagnosis and two seroconverted to GAD65Ab positivity after the diagnosis. Thus, 88% of the patients were positive for GAD65-M-Abs at diagnosis and 43% were still positive after 10 years of clinical diabetes, whereas 68% and 27% were positive for GAD65-C-Abs and 4% and 10% for GAD65-N-Abs, respectively (IV/Fig. 1). The prevalence of GAD65Abs, GAD65-M-Abs and GAD65-C-Abs decreased progressively after the diagnosis, whereas the frequencies of GAD65-N-Abs and GAD67Abs remained at a low level (IV/Fig. 1). Throughout the follow-up, GAD65-C-Abs were detected consistently together with GAD65-M-Abs or together with both GAD65-M-Abs and GAD65-N-Abs (96% of GAD65-C-Ab-positive samples), while GAD65-N-Ab was present together with GAD65-M-Abs and/or GAD65-C-Ab (58%) or as the only epitope specificity (42%, IV/Table 1). GAD67Abs were never observed in the absence of GAD65Abs.

The levels of GAD65Abs peaked after the clinical manifestation in 21 of the subjects. In those with declining total GAD65Ab levels, the prevalences of GAD65-M-Abs (from 86% at diagnosis to 26% after 10 yrs) and GAD65-C-Abs (from 66% to 5%) and their
levels decreased progressively after the diagnosis (IV/Fig. 1 and 2). In contrast, the prevalences and levels of GAD65-M-Abs (from 90% to 73%) and GAD65-C-Abs (from 71% to 64%) remained relatively stable in the group with rising GAD65Ab levels (IV/Fig. 1 and 2). As a consequence, a significant difference emerged during the follow-up between the two groups in the levels of GAD65-M-Ab and GAD65-C-Ab ($p<0.001$ and $p<0.01$, IV/Fig. 2). The peak levels of GAD65Abs were associated with the spreading of the response to the C-terminal region in four subjects, to the N-terminal region and to GAD67 in three cases, and to the middle region in two, while no spreading was observed in eleven patients.

Significantly lower exogenous insulin doses ($p<0.05$) and HbA1c levels ($p<0.01$) and higher titres of ICA ($p<0.05$) were observed in the patients with rising levels of GAD65-M-Abs during the observation than in those with decreasing levels of GAD65-M-Abs. The changes in the titers of epitope-specific antibodies during the follow-up were not related to gender, age, HLA DR status, remission, or serum C-peptide concentrations during the follow-up.

### 5.5.3 Response in patients with APECED

Sixteen of the 20 APECED patients were positive for GAD65Abs in all available follow-up samples. Three patients seroconverted to persistent GAD65Ab positivity during the observation period. One patient was positive for GAD65Abs in the first two samples, seroconverted to antibody negativity, and re-converted to positivity later. Antibodies to GAD67 were observed in 16 of the 20 GAD65Ab-positive patients with APECED.

Again, GAD65-M-Abs were observed in almost all (19 of 20) of the GAD65Ab-positive patients with APECED. Antibodies to the C-terminal region were also seen frequently (18 of 20). GAD67 did not completely inhibit the binding of GAD65-M-Abs and GAD65-C-Abs in any of the samples, confirming the presence of specific antibodies to the middle and the C-terminal regions of GAD65. Antibodies to the N-terminal construct, GAD65<sub>1-95</sub>/GAD67<sub>102-593</sub>, were observed in 15 of the 20 patients, in nine of whom specific binding to the N-terminus of GAD65 persisted after GAD67 inhibition. If an epitope-specific response appeared during the follow-up, it tended to remain.

### 5.6 GAD antibodies and progression to type 1 diabetes (III)

Antibodies to GAD65 appeared earlier in progressors than non-progressors (median age 1.1 versus 1.5 year, $p<0.05$). No significant difference was seen in the appearance of epitope-specific antibodies between the groups (III/Fig. 3). Spreading of the response to the C-terminal region was seen in both groups. Progressors had three or more IgG responses in their initial GAD65 response more often than non-progressors ($p<0.05$, III/Fig. 4), and multiple IgG subclasses in the initial GAD65Ab response predicted more rapid progression to clinical Type 1 diabetes ($p<0.05$, III/Fig. 5a). The late appearance of the IgG4 response was especially associated with non-progression, and in a life-table analysis progression to clinical type 1 diabetes was less common among those with an
IgG4 response ($p<0.05$, III/Fig. 5b). There were no statistically significant differences in the antibody frequencies or integrated antibody levels based on AUC-matched observation time between the groups. However, non-progressors had a trend towards elevated AUC levels of GAD65Abs ($p=0.082$), GAD65-M-Abs ($p=0.055$) and IgG1-GAD65Abs ($p=0.091$) (III/Fig. 6a and 6b). As a curiosity, the levels of IgG4-GAD65Abs correlated with those of IgG2 and IgE antibodies among non-progressors ($r_s=0.46-0.52$; $p<0.05$), whereas no significant correlation was seen among progressors.

5.7 GAD antibodies in APECED and type 1 diabetes (V)

There were no significant differences in the distribution of isotype or epitope-specific GAD65Abs between the GAD65Ab-positive patients with APECED and GAD65Ab-positive patients with type 1 diabetes except for the higher prevalence and levels of Ig2-GAD65Abs in the latter ($p<0.05$, V/Fig. 1). Similarly, no significant differences were observed in epitope and isotype-specific responses between the APECED patients with type 1 diabetes and those with type 1 diabetes alone (V/Fig. 2). Antibodies to GAD67 were more frequent in APECED patients than in patients with type 1 diabetes alone ($p<0.01$) and in APECED patients with type 1 diabetes than in patients with type 1 diabetes alone ($p<0.05$) (V/Fig. 1 and 2). The distribution of epitope and isotype-specific antibodies did not differentiate APECED patients with type 1 diabetes from those without diabetes (V/Fig. 3). No apparent changes occurred in the epitope or isotype profile at the time of diagnosis of type 1 diabetes in patients with APECED. Antibodies to GAD67 were observed in APECED patients irrespective of whether or not type 1 diabetes was present (V/Fig. 3).
6 Discussion

6.1 Insulin antibodies

Special attention has been paid to the role of insulin in the pathogenesis of human type 1 diabetes. First, only the insulin-producing beta-cells are selectively destroyed in type 1 diabetes. In addition, insulin is the only beta-cell-specific autoantigen identified so far. Recent reports have shown more convincingly than before that IAAbs appear as the first or one of the first autoantibodies in young children, being the first sign of autoimmunity to beta-cells in about 90% of cases (18, 19, 136). In two birth cohort studies, the German BabyDiab Study (136) and the Finnish DIPP study (18), IAAbs were observed in all children who progressed to overt type 1 diabetes during maximal follow-up times of 8 years and 2.5 years, respectively. Consistent results were also reported by the American DAISY study (167). High IAAb titers are also known to be associated with the development of type 1 diabetes at young age (164). All these data support the view that insulin is likely to be the primary antigen in the pathogenesis of human type 1 diabetes, at least in young children.

The important role of IAAbs poses demands to the assays for IAAbs. Before the publication of the new microassay for the detection of IAAbs by Williams et al. in 1997 (166), all assays sensitive for IAAbs were based on radio-binding assays, required relatively large serum volumes (up to 600 μl of serum) (298, 302), did not allow easy automation for large-scale screening, and utilized PEG for the precipitation of antigen-antibody complexes (161). In the new microassay, only 20 μl of serum is required, automatization of the assay procedures is possible, and the immune complexes are precipitated with protein A Sepharose is specific for IgG, while PEG precipitates nonspecifically all complexes with a sufficiently high molecular weight. In the original paper of Williams et al. (166), slightly higher sensitivity was achieved by the micro-assay than by the conventional assay. Later, highly concordant results were reported from the two assays by Naserke et al. (303). IAAb titers have been reported to be higher in cord blood than in older children and in non-diabetic pregnant women at delivery (135, 278) when using the conventional assay protocol. However, the elevated IAAb levels in cord blood have been implicated to reflect non-IgG-mediated anti-insulin activity unrelated to
type 1 diabetes, since this activity was not bound by protein A or G (279). This is important in the detection of IAAbs from cord blood, especially if maternal autoantibodies play any role in the development of type 1 diabetes (150, 304).

6.1.1 Humoral anti-insulin activity in pregnancy and cord blood (I)

In the present study, the conventional IAAb assay and the new microassay yielded discrepant results on IAAbs in pregnant women at delivery and in cord blood. The conventional assay revealed a higher frequency and higher levels of IAAbs in cord blood and in maternal samples obtained at delivery but not in samples taken in early pregnancy. This discrepancy is not due to insensitivity of the microassay, since the women with low IAb levels in the conventional assay in early pregnancy did test positive for IAbs in the microassay. The elevated cord blood IAb levels observed only when using the conventional assay in the present study confirm the postulation that non-Ig-mediated anti-insulin activity is present in cord blood (279).

In addition, these observations demonstrate for the first time that normal pregnancy also induces the appearance of non-Ig-associated anti-insulin activity into the maternal circulation during pregnancy. This was evident based on the elevated IAb levels in the mothers at delivery according to the conventional assay but not according to the microassay. The undefined anti-insulin activity may be associated more closely with the maternal state than with the fetus, since it was observed more often in non-diabetic maternal samples at delivery (71%) than in cord blood samples (32%). Another survey reported anti-insulin activity to be detectable in 96% of the cord blood samples of infants of healthy mothers (279), which is three times higher than the present frequency of 32%.

Pregnancy has a fundamental impact on the production of hormones and proteins. It is also known that the placenta is not only a barrier between the mother and the fetus but also acts as a selective transmission pathway for various substances (275). In addition, the placenta synthesizes specific proteins. Thus, the anti-insulin activity could be of maternal, placental, or fetal origin. It is known that the concentrations of various insulin-like growth factor binding proteins (IGFBP) capable of binding insulin increase during pregnancy in both maternal and fetal circulation (305, 306). In a preliminary assessment of an independent series of newborn infants, we did not find any correlation between the conventionally assayed IAAb levels and the IGFBP-1 or IGFBP-3 concentrations in the cord blood of newborn infants.

The first report on the presence of anti-insulin activity in cord blood did not provide any explanation for the observation (279). Another study indicating increased cord blood IAAb in infants of non-diabetic mothers linked the observation to the physiological phenomenon of increased protein concentration in cord blood (135). The paper reporting higher neonatal IAAb levels compared to corresponding maternal levels implied that the IAAbs were intrinsic to the immune repertoire of the fetus (278). They suggested, alternatively, that less IAAbs are masked by free endogenous insulin, or that low-avidity IAAbs produced in utero are abundant in cord blood. It is unlikely that the microassay would be less sensitive in the detection of low-avidity antibodies than the conventional assay, since IAbs were observed at similar frequencies in early pregnancy in women
affected by type 1 diabetes by the microassay and the conventional assay. The omission of acid charcoal extraction from the micro-assay hardly explains the difference observed between the two assays. It has been reported that there is no difference in sensitivity regardless of whether or not charcoal extraction is included in the conventional assay (298), supporting the view that the natural levels of endogenous insulin are too low to affect the frequency of antibody positivity in the radiobinding assays. It has been reported that the endogenous free insulin bound by IAAbs could result in some false negative results when the microassay format is used (307). In that study, plasma samples were used, whereas serum samples were used in the present study. Decreased IAb levels were observed in diabetic mothers during pregnancy in the present study, irrespective of whether or not the samples were pretreated with acid charcoal to remove endogenous insulin in the microassay. This is in accordance with several reports showing that IAb concentrations do not increase during pregnancy (271-273) but rather decrease as a consequence of hemodilution (268). In addition, there is no obvious reason why normal pregnancy would induce the production of autoantibodies (269, 270).

6.1.2 Insulin antibodies in prediabetic young children (II)

The DIPP study made it possible to generate new and unique data on the detailed characteristics and natural course of the humoral immune response to insulin from the first appearance of autoantibodies to the clinical presentation of type 1 diabetes in very young children with genetic disease susceptibility. The initial humoral immune response to insulin comprised IgG1 antibodies in all children and IgG3 antibodies especially in those who progressed to type 1 diabetes later. In subsequent samples, IgG1 antibodies were present in almost all children, IgG3 antibodies in three-fourths, IgG2 antibodies in about half, and IgG4 antibodies in one-third of the children, the distribution being accordant with the previous reports on the isotype distribution of IAAbs in a small group of IAAb-positive children (137, 171). IgG3 responses were observed more often in the present study than in other reports, probably as a consequence of the more frequent sampling and the young age of the children observed.

The frequent appearance of IgG3-IAAbs was mainly due to the high prevalence of IgG3 antibodies in progressors. IgG3 antibodies were more frequent and present at higher levels in progressors than in non-progressors throughout the observation period. None of the isotype-specific IAAbs were observed to be associated with the development of type 1 diabetes in the German BabyDiab Study (137). However, the sampling intervals were much longer, and the number of diabetic children with IAAbs was low in that report. A broad initial Ig isotype response to insulin was associated with the development of type 1 diabetes in the present study, being consistent with a previous report on the islet autoantibody characteristics in first-degree relatives of patients with type 1 diabetes from the Oxford and Munich family studies (171).

Interestingly, IgA-IAAbs were observed more frequently during the follow-up and were present at higher levels in the last sample analysed in progressors than in non-progressors. This could be interpreted to reflect the role of the gut immune system in the development of beta-cell autoimmunity. It has been hypothesized that the regulatory
defects in the tolerization to the dietary bovine insulin present in cow’s milk results in the development of an immune response targeting human insulin and leading to beta-cell autoimmunity (54). Exposure to cow’s milk has been shown to induce humoral immune responses to bovine insulin that correlate with the autoimmune IgG1 and IgG2 responses to human insulin in patients with type 1 diabetes (308).

6.2 GAD antibodies

GAD has been proposed to have a unique and central role in triggering diabetes and even to be “the single autoantigen for diabetes” (309, 310). These claims are based mainly on NOD mice studies. In contrast, evidence from human studies argues for the view that the humoral immune response to GAD would reflect a less harmful process than the humoral immune responses to the other main autoantigens, i.e. insulin and IA-2. First, the levels of IAAbs and IA-2Abs have been reported to be higher in progressors than in non-progressors, whereas no such association was observed for GAD65Abs (156, 171). In addition, high titers of GAD65Abs have been shown to correlate inversely with T-cell responses to GAD in patients with type 1 diabetes (95). The humoral immune response to GAD65 also tends to remain positive longer after the diagnosis than the responses to insulin and IA-2 and even to appear for the first time after the onset of clinical disease in some cases (196, 197). In addition to type 1 diabetes, GADAbs have been documented in many other autoimmune diseases and neurological disorders, often at higher concentrations than in type 1 diabetes (286, 291, 311). However, GAD65Ab-positive patients affected by these diseases rarely develop autoimmune type 1 diabetes. Considering all the above observations, there is a need to characterize in detail the humoral immune response to GAD in man.

6.2.1 GAD antibodies in prediabetic and diabetic subjects and patients with APECED (III, IV, and V)

The present study provides detailed characterization of the humoral immune response to GAD65 in GAD65Ab-positive individuals with preclinical and clinical diabetes as well as in patients with APECED. Especially, new data can be presented on the dynamics of the immune response to GAD.

The humoral immune response to GAD was confirmed to be composed predominantly of IgG1 subclass antibodies and antibodies targeting the middle region of GAD65 (137, 139, 216). Other IgG subclasses and antibodies to the C-terminal region were often also present, while other isotype-specific antibodies, i.e. IgA, IgM, and IgE, were rarely detectable, which is consistent with the previous reports (137, 139, 171, 211, 220). The early phase of the humoral autoimmune response to GAD65 is a highly dynamic process reflected by isotype switching from the IgG1 to the other IgG subclasses and rapid epitope spreading from the middle region of GAD65 to the C-terminal region. The short sampling intervals made it possible to observe that IgG3 antibodies were characteristic of
the initial 12-month response. IgG2 antibodies often appeared together with IgG3 antibodies as a component of the early response but tended to remain positive for a longer time than IgG3 antibodies. IgG4 antibodies appeared clearly as the last IgG subclass, being initially nearly undetectable. Rapid epitope spreading from the middle region to the C-terminal region was reliably documented for the first time in more than 40% of the young children. This phenomenon had been observed previously only in isolated individuals (139). The dominance of GAD65-MAbs over GAD65-C-Abs continued to be apparent after the diagnosis. Responses to the N-terminal region were not observed equally frequently as earlier reported (139, 211), probably due to differences in the ages of the subjects studied and the chimeric molecules used. In general, the distribution of epitope-specific GAD65Abs was relatively uniform in young children, whereas conspicuous individual variation was seen in the IgG subclass responses, except those to IgG1.

The present study shows that the rising GAD65Ab levels after the diagnosis are related to the persistence of both the middle-region and the C-terminal GAD65Abs. The rising levels of GAD65Abs and GAD65-M-Abs were related to higher ICAb titers, which confirms that GAD65Abs persisting after the clinical manifestation of type 1 diabetes may reflect a continuous immune response to the remaining islets. High levels of GAD65-M-Abs were related to a reduced requirement for exogenous insulin and lower HbA1c levels. It remains open, however, whether the reduced HbA1c levels and daily insulin dose are a consequence of the production of endogenous insulin from residual beta-cells, since changes in the GAD65Ab levels were unrelated to the serum C-peptide concentrations, as also shown earlier in patients with type 1 diabetes (197, 199, 312). Previous reports on a positive relationship between the levels of GAD65Abs (313) or GAD65-C-Abs (314) and the insulin requirement and between the levels of GAD65Abs and impending complete beta-cell failure (315) in patients with adult-onset diabetes do not seem to be applicable to type 1 diabetes presenting in childhood. A cellular immune response to GAD65 has been reported to occur in GAD65Ab-positive adult onset diabetes but not in GAD65Ab-positive patients with type 1 diabetes with residual beta-cell function (316).

It was observed that there are similarities but also differences in the humoral immune response to GAD in different groups of GAD65Ab-positive individuals. IgG1-GAD65Abs dominated in all GAD65Ab-positive subjects. An IgG2 response was more often detected in patients with type 1 diabetes than in those with APECED. As mentioned above, IgG3 antibodies to GAD65 were characteristic of an early immune response in young children, as seen in study III. This was also supported by the observation in study V that only the youngest patient from the group of 20 subjects with isolated type 1 diabetes tested positive for IgG3-GAD65Abs (his age was 3.4 yrs), and out of the two APECED patients testing positive for IgG3 antibodies, one was the youngest child in the APECED group (positive at the age of 4.3 years). The specificity of epitope-specific GAD65Abs was surprisingly similar in the different subject groups. The present study indicates that the isotype and epitope characteristics of GAD65Abs are similar, irrespective of whether or not type 1 diabetes is diagnosed in GAD65Ab-positive patients with APECED. As reported previously (286), a strong cross-reactive humoral response to GAD67 was observed only in patients with APECED, and this was the only conspicuous difference between the diabetic patients with APECED and the patients with type 1
diabetes alone. The high GAD67Ab levels may indicate that immunization to GAD67 occurs independently of GAD65 in patients with APECED, possibly reflecting a different pathogenetic mechanism (289). This is supported by the fact that the HLA-conferred susceptibility to type 1 diabetes has not been observed in patients with APECED (285).

6.2.2 GAD antibodies and progression to type 1 diabetes (III)

None of the epitope-specific responses to GAD65 were found to be associated with progression to type 1 diabetes in the present study. This is in accordance with the previous reports of prospectively observed individuals: The responses to GAD65 epitope clusters were not found to be related to the development of type 1 diabetes in prospectively monitored and sequentially sampled young first-degree relatives with at least one family member affected by type 1 diabetes in the German BabyDiab study (139), in the Finnish DiMe study (317), or in the combined cohort of Bart’s Oxford and the Munich family studies (171). In the first two of the above studies, chimeric constructs identical with the present ones were used. In contrast, one research group reported that patients with newly diagnosed type 1 diabetes can be discriminated from healthy GAD65Ab-positive children by their increased reactivity to the C-terminal region of GAD65 (211) and from GAD65Ab-positive adults, first-degree relatives of patients with type 1 diabetes (including both children and adults) (213), and patients with slow-onset autoimmune diabetes (214) by their reduced reactivity to the N-terminal region of GAD65 or to GAD67. All these findings have been obtained in cross-sectional study cohorts. The authors of these studies concluded that patients with type 1 diabetes are characterized by GAD65-specific antibodies recognizing restricted epitopes on the molecule, whereas the occurrence of cross-reactive GAD65/67Abs with a broad epitope-binding profile is typical of other conditions. No immunological explanation was presented for the association of reduced reactivity with the development of type 1 diabetes. The authors did not confirm the specificity of the epitope responses by GAD67 inhibition. It may be possible that the quality of the humoral response to GAD varies between children and adults. The differences in chimeric constructs may also contribute to the discrepancy. A larger N-terminal portion of GAD65 was used to detect high levels of GAD65-N-Abs in non-diabetic individuals (aa 1-243 versus aa 1-100 in the present chimera). Neither of the two chimeras is restricted exactly to the amino acid residues reported to be included in the N-terminal domain of native GAD65 (aa 1-200) (187).

No statistically significant differences were observed between progressors and non-progressors in the levels of the various isotypes. Life-table analyses revealed that a narrow IgG subclass-specific response in the first GAD65Ab-positive sample and the emergence of an IgG4 response to GAD65 in subsequent samples are associated with a reduced risk of progression to type 1 diabetes. No isotype switching or specific isotype distribution within the GAD65Ab response was observed to be associated with progression to type 1 diabetes in young children in the German BabyDiab Study (137), in first-degree relatives of patients with type 1 diabetes in the Oxford and Munich family studies (171) or in the cohort of non-diabetic twins with affected identical pairs (220). In contrast, elevated levels of IgE and IgM-GAD65Abs (217) or IgG2 and/or IgG4-
GAD65Abs (216) have been reported to characterize non-diabetic GAD65Ab-positive relatives of patients with type 1 diabetes. However, the specificity of the assays employed in those two studies was not confirmed in the recent workshop on the analysis of isotype-specific GAD65Abs. A broad isotype response to insulin and IA-2 but not to GAD65 was reported to be associated with progression to overt type 1 diabetes in older first-degree relatives of patients with type 1 diabetes in the combined series of the Oxford and Munich family studies (171), which is inconsistent with our results on young DIPP children.

6.3 Th1/Th2 paradigm and risk assessment

One might expect that the analysis of antigen-specific T-cell responses would be the most useful approach to study the polarization of immune responses between Th1 and Th2-like immunity. Unfortunately, the lack of direct access to the target tissue in type 1 diabetes, i.e. the pancreatic islets, is a serious obstacle on the way toward meaningful T-cell studies in this disease. The proportion of beta-cell autoreactive T-cells is presumably relatively low in the peripheral circulation even in subjects with preclinical type 1 diabetes, and it is therefore not surprising that it has turned out to be challenging to find any differences in antigen-specific T-cell responses between subjects with preclinical or clinical type 1 diabetes and control subjects. Moreover, T-cell studies are laborious and complicated to perform. The measurement of cytokines from either peripheral T-cell preparations or whole blood may be too non-specific a method to study events in the pancreas. An alternative approach is to analyze the quality of the humoral immune response to specific antigens, especially in large-scale population-based studies.

Beta-cell destruction is considered to be mediated through a Th1-dominated destructive autoimmune process. The presence of IgG1 and IgG3 subclass antibodies has been hypothesized to reflect Th1-related immunity (89, 216). An IgG3 response is considered to be induced most efficiently by virus infections (318). It has been proposed that persistent infection of beta-cells with viruses modulates the Th1/Th2 cytokine balance and induces MHC protein expression in a manner that results in the loss of self-tolerance and immunization to self-antigens (28, 31). The present study showed that an IgG3 response was frequently present as a component of the initial response to insulin and GAD65. In fact, the strongest initial IgG3 responses to insulin and/or to GAD65 were observed in progressors. This may indicate that immunization to a new autoantigen is probably induced by a virus infection and occurs in a Th1 cytokine milieu. It has been shown in NOD mice that the Th1 response dominates the early induction of diabetes, while the Th2 response arising later slows down the autoimmune process (319). The production of antibodies of the IgG3 subclass has been reported to be most pronounced especially in the early phase of Lyme Borreliosis, which is characterized by a Th1-type immune response with abundant production of IFN-\( \gamma \) (89). A continuous Th1-dominated response may result in rapid aggressive beta-cell destruction. In the present study, the levels of IgG3-IAAbs were higher in progressors than in non-progressors during follow-up, supporting the view of a relationship between Th1 dominance and active beta-cell destruction. The present study indicated that high levels of IgG1 antibodies may reflect
the dominance of this isotype within the humoral immune response in general rather than indicate a bias of the immune response.

A Th2-dominated autoimmune response is considered to reflect non-aggressive beta-cell autoimmunity. Such a response may dominate in individuals in whom detectable autoantibodies are present for 10 years or more before the clinical diagnosis of type 1 diabetes. In the present study, the Th2-related IgG4 subclass appeared clearly as the last IgG subclass in the response to insulin and GAD65, especially in the GAD65Ab-positive children who remained non-diabetic during the observation. This could be interpreted as a change of the immune response from initial Th1-biased immunity to less invasive Th2 immunity. This is in accordance with the finding that the Th2-related subclasses of GAD65Abs and IAAbs increase after the induction of the Th2-type response in NOD mice (319). This finding may be associated with the observation showing reduced production of IgG2 and IgG4-specific antibodies to tetanus vaccine in young islet autoantibody-positive children at risk for type 1 diabetes compared to children without detectable autoantibodies (96). The elevated IgG4-GAD65Ab levels hardly reflect the increase of overall IgG subclass concentrations during normal childhood, since one would then expect to see a similar increase in the other subclass responses to GAD65. However, IgG4-GAD65Abs did not discriminate non-progressors from progressors; only a retrospectively performed life-table analysis indicated that progression to clinical type 1 diabetes was significantly less common among those with an IgG4 response during the observation period. To note, the cumulative integrated antibody values (AUC) applied in the analysis of IgG4-GAD65Ab levels may not provide any information on possible late changes in antibody titers, e.g. the emergence of an increasing IgG4 response in the non-progressors. The significant correlation between the levels of IgG4 and the levels of both IgG2 and IgE-GAD65Abs observed only in non-progressors, all of which are proposed to reflect Th2-biased immunity, may reflect co-stimulation of the production of Th2-related immunoglobulins. Other studies involving reliable isotype-specific GAD65Ab assays have failed to show that the isotype-specific response to GAD65 would predict future type 1 diabetes, possibly due to infrequent sampling (137, 171, 317).

The present results suggest that the analysis of epitope-specific GAD65Abs may not facilitate the prediction of disease development. All prospective studies using chimeric GAD molecules are consistent with this observation (139, 171, 317). Epitope spreading within the GAD65 molecule was documented convincingly in the present study. It occurred rapidly and was not related to disease progression, which is contrary to the observation showing that T-cell epitope spreading within GAD is associated with the stage of disease development in NOD mice (223). However, the possibility that the responses to more restricted epitopes within the GAD65 molecule, based on the use of monoclonal epitope-specific antibodies, for instance, would provide additional predictive information cannot be excluded. In contrast to the experience of GAD65 epitopes, humoral reactivity to multiple IA-2 epitopes has been reported to be related to more rapid progression to overt type 1 diabetes when compared to a response restricted to a single epitope (140, 171).

It has been reported recently that the risk of type 1 diabetes can be stratified on the basis of antibody titer and isotype and epitope-specific responses in autoantibody-positive relatives of patients with type 1 diabetes (171). In that report, the highest risk was associated with high-titer IA-2Abs and IAAbs, the presence of multiple IgG subclass
responses to IA-2 and insulin and responses to multiple IA-2 epitopes. The 5-year diabetes risk associated with various combinations of these markers ranged from 63 to 89% compared to a risk of 48% achieved based on the conventional multiple antibody positivity. The present study confirms that a broad IgG subclass response to insulin in the initial antibody-positive sample predicts rapid progression to clinical type 1 diabetes. No association was observed between GADAb titers or multiple epitope/isotype responses to GAD65 and progression to diabetes. The present study confirms that high titers of GAD65Abs are not associated with an increased risk for type 1 diabetes in young children. Rather, GAD65Ab titers tended to be higher in non-progressors. However, autoantibody positivity for GAD65 is known to increase the risk of future type 1 diabetes (156). A broad initial isotype-specific response to GAD increased the risk for overt disease according to the present study. To summarize, the analysis of isotype-specific antibodies to islet cell antigens, particularly insulin and IA-2, seems to increase the specificity of risk assessment.

The present study design made it possible to detect even minute changes in autoantigen-specific humoral responses, because of the frequent and sequential sampling schedule starting from birth. The data showed that frequent sampling is needed for detailed characterization of the dynamic immune response, since isotype and epitope-specific responses seemed to appear sequentially at short intervals, and the isotype responses, especially the IgG3 response to GAD65, often lasted for less than one year. This may explain, for instance, why IgG3 responses have been observed infrequently in other studies using longer sampling intervals. The administration of intranasal insulin to autoantibody-positive humans at risk for type 1 diabetes has been reported to be associated with an increase in the antibody response and a decrease in the T-cell response to insulin, whereas no effect has been observed on FPIR or the levels of GAD65Abs and IA-2Abs (320). This indicates that mucosal administration of an antigen may specifically switch a Th1 response to a Th2 response. The initiation of exogenous insulin treatment either subcutaneously or intravenously in patients with newly diagnosed type diabetes has been reported to promote a Th2-linked IgG4 response to insulin, but no induction of isotype-specific responses to GAD65 or IA-2 has been observed (170). Samples taken after randomization for the nasal insulin trial were excluded from the analysis of IAAbs, but were included in the study of GAD65Abs in the present series. This implies that the treatment with intranasal insulin did not affect the IAAb results presented in this study. No immediate changes were observed in the epitope or isotype-specific responses to GAD65 after the initiation of the intervention trial.
7 Conclusions

The observations of the present study provide the following conclusions:

1. Methods utilizing PEG as a precipitating agent are not reliable for the detection of insulin autoantibodies in pregnant women and newborn infants. Uncharacterized non-Ig insulin-binding activity is induced by pregnancy and is present in both the maternal and the fetal circulation at birth.

2. The humoral immune response to insulin and GAD65 is a highly dynamic process with isotype and epitope spreading in young children with genetic susceptibility to type 1 diabetes. The responses to both insulin and GAD65 are dominated by the IgG1 subclass, while other IgG subclass-specific antibodies are observed to a lesser extent. An initial humoral autoimmune response to GAD65 starts as a response to the middle region of GAD65 and spreads rapidly to the C-terminal region.

3. The strong IgG3 response to insulin in those who progressed rapidly to overt type 1 diabetes may reflect the role of destructive Th1-polarized immunity in the development of type 1 diabetes. The initial humoral autoimmune response to insulin and especially to GAD65 often comprises an IgG3 response that may reflect a Th1-polarized response at the time when an immune response to a new autoantigen is induced.

4. Young children who progress to clinical type 1 diabetes are characterized by a broad initial isotype response to insulin and to GAD65. A strong IgG1 and IgG3 response to insulin is associated with rapid progression to overt type 1 diabetes. None of the epitope or isotype-specific antibody responses to GAD65 may be applicable to the discrimination of progressors and non-progressors among GAD65Ab-positive young children. An emerging IgG4 response tended, however, to characterize the children who remained non-diabetic over the first few years of humoral GAD65 autoimmunity.

5. The increase in GAD65Ab titers after the diagnosis of type 1 diabetes is related to persistent immune reactivity to the middle and C-terminal region of the GAD65 molecule, and the rising levels of antibodies to the middle region of GAD65 are associated with higher levels of ICA and a decreased requirement for exogenous insulin, probably reflecting the presence of residual beta-cell mass.

6. The profile of the isotype and epitope-specific response to GAD65 is highly similar in patients with type 1 diabetes and patients with APECED. Only GAD65Abs of the
IgG2 subclass are associated more closely with isolated type 1 diabetes. It is not possible to discriminate APECED patients affected by type 1 diabetes from unaffected ones by means of epitope and isotype-specific GAD65Abs.
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