ROLE OF ANTIBODIES TO GLUTAMIC ACID DECARBOXYLASE IN TYPE 1 DIABETES

Relation to other autoantibodies, HLA risk markers and clinical characteristics

EMAD SABBAH

Department of Paediatrics

OULU 2000

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Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 8 of the University Hospital of Oulu, on June 6th, 2000, at 12 noon.

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Docent Ilkka Sipilä
Docent Outi Vaarala

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TO MY FAMILY

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Department of Paediatrics, University of Oulu, FIN-90401 014Oulu, Finland

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Abstract

The purpose of this research was to assess the role of antibodies to glutamic acid decarboxylase (GAD) in children with newly diagnosed type 1 diabetes in relation to other disease-associated autoantibodies and HLA-defined genetic disease susceptibility, to evaluate the role of GAD antibodies (GADA) in relation to clinical characteristics at the diagnosis of type 1 diabetes and to compare the frequency and levels of GADA between adult and childhood onset type 1 diabetes.

The study population comprised altogether 999 children and adolescents with type 1 diabetes, 100 affected adult subjects and more than 370 non-diabetic controls. GADA were measured with a liquid radioligand assay, and a similar assay was used for the analysis of antibodies to the islet antigen 2 (IA-2) molecule. Islet cell antibodies (ICA) were determined with conventional immunofluorescence and insulin autoantibodies (IAA) with a liquid phase radioimmunoassay either in a tube or a plate format (microassay).

GADA were detected at diagnosis in 68 to 73% of the children and adolescents with type 1 diabetes. GADA were more frequent in girls and in those older than 10 years of age at clinical disease manifestation. Subjects testing positive for GADA had higher levels of ICA and IAA than those negative for GADA. Multiple antibodies (≥ 2) were observed more often in girls and in children under the age of 5 years.

Children with the HLA DR3/non-DR4 phenotype had the highest GADA levels, significantly higher than those seen in children with the DR4/non-DR3 combination. The highest prevalence of multiple autoantibodies was seen in subjects heterozygous for DR3/4. When studying HLA DQB1 genotypes those with the DQB1*02/y (y = other than *0302) genotype had the highest GADA levels as expected since DQB1*02 and DR3 are in strong linkage disequilibrium. The same group had the lowest frequency of multiple antibodies among the children younger than 10 years of age.

Patients diagnosed with type 1 diabetes before the age of 20 had a higher frequency of all four autoantibodies analysed than those presenting with clinical disease after the age of 20. The proportion of subjects testing negative for all four antibodies was substantially higher among adults than in those under the age of 20. The smallest age-related difference in antibody frequencies was observed for GADA, and the GADA-positive adult patients had on an average about three times higher antibody levels than the GADA-positive children.

No association was observed between positivity for GADA and the degree of metabolic decompensation at the clinical presentation of type 1 diabetes. No significant differences were either seen between the subjects who tested positive for GADA at diagnosis and those who were negative in serum C-peptide concentrations, metabolic control or exogenous insulin requirement over the first 2 years of observation. The proportion of children in clinical remission was, however, lower among GADA-positive subjects than in GADA-negative patients at 18 months after the clinical manifestation. Positivity for multiple antibodies was associated with accelerated beta-cell destruction and increased exogenous insulin requirements over the 2-year observation period.

The observations that GADA are related to female gender, older age and the HLA-DR3/DQB1*02 haplotype suggest that a strong humoral immune response to GAD may reflect a propensity to general autoimmunity rather than specific beta-cell destruction.

Keywords: humoral autoimmunity, metabolic decompensation, genetic susceptibility, clinical remission

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Oulu, May 2000 Emad Sabbah

Abbreviations

ABBOS	a peptide fragment of 13 amino acids of bovine serum albumin
APCs	antigen presenting cells
APS	autoimmune polyendocrine syndrome
BB (rat)	Bio Breeding
BSA	bovine serum albumin
cDNA	complement strand deoxyribonucleic acid
CMV	cytomegalovirus
CNS	central nervous system
DENIS	the German Nicotinamide Diabetes Intervention Study
DiMe	The Childhood Diabetes in Finland Study
DIPP	the Diabetes Prevention and Prediction study
ELISA	enzyme-linked immunosorbent assay
ENDIT	the European Nicotinamide Diabetes Intervention Trial
EV	enterovirus
GABA	gamma amino butyric acid
GAD65	65 kilodalton isoform of glutamic acid decarboxylase
GAD67	67 kilodalton isoform of glutamic acid decarboxylase
GADA	antibodies to GAD65
GHb	glycated haemoglobin
HbA1c	haemoglobin A1c
HLA	human leukocyte antigen
IAA	insulin autoantibodies
IA-2	islet antigen 2
IA-2A	antibodies to IA-2
IA-2β	islet antigen 2β, phogrin
ICA	islet cell antibodies
ICA69	islet cell antibodies to the 69 kD antigen
IDDM	insulin-dependent diabetes mellitus
IFN	interferon
IL	interleukin
INS	insulin gene region

JDF-U	Juvenile Diabetes Foundation units
kD	kilodalton
MHC	major histocompatibility complex
MODY	maturity-onset diabetes in the young
NOD (mouse)	non-obese-diabetic
PAS	protein A Sepharose
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
PTP	protein tyrosine phosphatase
p69	69kD protein
RBA	radiobinding assay
RIA	radioimmunoassay
RNA	ribonucleic acid
SD	standard deviation
SMS	Stiff-man syndrome
TAP	transporter associated with antigen presentation
TBST	Tris-buffered saline with Tween
Th	T helper cell
TNF	tumour necrosis factor
VNTR	variable number of tandem repeats

List of original papers

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

I Sabbah E, Kulmala P, Veijola R, Vähäsalo P, Karjalainen J, Tuomilehto-Wolf E, Åkerblom HK, Knip M & the Childhood Diabetes in Finland Study Group (1996) Glutamic acid decarboxylase antibodies in relation to other autoantibodies and genetic risk markers in children with newly diagnosed insulin dependent diabetes mellitus. J Clin Endocrinol Metab 81:2455-2459.

II Sabbah E, Savola K, Kulmala P, Reijonen H, Veijola R, Vähäsalo P, Karjalainen J, Ilonen J, Åkerblom HK, Knip M & the Childhood Diabetes in Finland Study Group (1999) Disease associated autoantibodies and HLA-DQB1 genotypes in children with newly diagnosed insulin dependent diabetes mellitus (IDDM). Clin Exp Immunol 116:78-83.

III Sabbah E, Savola K, Ebeling T, Kulmala P, Vähäsalo P, Salmela P, Ilonen J & Knip M. Genetic, autoimmune and clinical characteristics of childhood and adult onset type 1 diabetes. Submitted for publication.

IV Sabbah E, Savola K, Kulmala P, Veijola R, Vähäsalo P, Karjalainen J, Åkerblom HK, Knip M & the Childhood Diabetes in Finland Study Group (1999) Diabetes-associated autoantibodies in relation to clinical characteristics and natural course in children with newly diagnosed type 1 diabetes. J Clin Endocrinol Metab 84:1534-1539.

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1. Introduction

Disorders caused by a self-attack against self-target molecules resulting in interference with specific cells or the whole organ are perceived as autoimmune diseases (Roitte *et al.* 1993). These diseases can be classified as organ-specific or non-organ-specific. Organ-specific autoimmune diseases are characterised by the destruction of specific cells as a sequence of direct infiltration by lymphocytes and other mononuclear cells. Autoimmune diseases share several features: (i) they tend to be associated with genes in the major histocompatbility complex (MHC); (ii) they are commonly characterised by circulating autoantibodies and/or autoreactive T cells; (iii) they can be prevented or delayed by immunosuppressive treatment; (iv) they can be induced by immunisation with a self-antigen; and (v) local infiltration by mononuclear cells can be detected histologically (Rose & Bona 1993). Type 1 diabetes can be considered an organ-specific autoimmune disease on the following grounds:

- It is associated with certain HLA haplotypes (Singal & Blajchman 1973, Nerup *et al.* 1974).
- It is associated with circulating autoantibodies (Bottazo et al 1974) and autoreactive T-cells (Nerup *et al.* 1971).
- The ability of the T cells to transfer diabetes adaptively to BB rats (Like *et al.* 1985) and to NOD mice (Bendelac *et al.* 1987).
- It is characterised by infiltration of the pancreatic islets by lymphocytes and monocytes both in man (Gepts 1981, Foulis *et al.* 1986) and in rodent models (Calafiore *et al.* 1993).
- Several immunosuppressive strategies have been shown to be effective in the prevention of experimental autoimmune disease (Bach 1994, Knip 1998).
- Type 1 diabetes is known to have been transferred from a prediabetic subject to an HLA-identical sibling as a consequence of bone marrow transplantation (Lampeter *et al.* 1993), and similar observations have been made in rodent models (Koevary *et al.* 1983, O´Reilly *et al.* 1991).

2. Review of the literature

2.1. Diabetes mellitus

Diabetes mellitus is a syndrome characterised by inappropriate hyperglycaemia due to an absolute or relative deficiency in insulin secretion and/or a reduction in the effectiveness of insulin action. Chronic hyperglycaemia causes damage to the eye, kidneys, nerves, heart and blood vessels, but its aetiology and pathogenesis are heterogeneous resulting in different strategies for prevention, diagnostic screening and treatment.

2.1.1. Classification of diabetes

Two major types of diabetes mellitus are recognised; type 1 and type 2. Other known types of diabetes comprise malnutrition diabetes, which is common in the developing countries, and secondary diabetes brought about by pancreatic disease, drug toxicity, endocrine disorders, or genetic disease (Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 1997).

In type 1 diabetes absolute insulin deficiency results in an increase in circulating concentrations of glucose and free fatty acids and subsequently leads, through hyperglycaemia and osmotic diuresis, to increased urination, thirst and weight loss. Ketoacidosis is a major feature of untreated type 1 diabetes, and circulatory collapse can result from severe acidosis (pH 7.0 or less). Other specific types of diabetes, such as MODY (maturity-onset diabetes in the young) are due to genetic defects in beta-cell function and/or in insulin action (Herman *et al.* 1994). Gestational diabetes applies to women who develop diabetes during pregnancy.

2.2. Type 1 diabetes

Although clinical differences between type 1 and type 2 diabetes mellitus were first reported more than a century ago (Lancereaux 1880), it was not until the mid-1960´s that

convincing evidence of the involvement of the immune system in the pathogenesis of type 1 diabetes was produced (Gepts 1965). A lot has been learned between the initial findings of Gepts and our current knowledge of the pathogenesis of type 1 diabetes, and the accumulated data support the notion that it is an autoimmune disease.

2.2.1. Epidemiological characteristics

Type 1 diabetes is one of the most common chronic diseases in childhood, and obviously has the most conspicuous geographical variation (Diabetes Epidemiology Research International Group 1988), probably partly on account of differences in the prevalence of the susceptibility genes between populations and partly in response to environmental factors, which play an important role (Atkinson & Maclaren 1994). In Scandinavia (Dahlquist & Mustonen 1994), as in other parts of the world (Diabetes Epidemiology Research Group 1990), population-based registers have been established to assess possible changes in its incidence.

2.2.1.1. Geographical patterns and clustering

The incidence rates in subjects under 15 years of age confirm the vast geographical variation in the incidence of type 1 diabetes world-wide, with rates ranging from 1-2 (per 100,000) in Japan to more than 40 in Sardinia and Finland (Green *et al.* 1992, Tuomilehto *et al.* 1999). The lowest rates are to be found in Asia, followed by Africa and South America, while higher rates have been recorded in Oceania (Australia and New Zealand) and North America, and the highest of all in Europe, with Sardinia emerging as the Mediterranean "hot spot", with an incidence rate of 36/100,000 for the period 1989-95 (Songini 1995). Finland also had an incidence of 36/100,000 for the period 1987-92 (Tuomilehto *et al.* 1999), but recent data point to a figure close to 50/1000,000 in 1998-99 (Reunanen, personal communication). Data for Greece (Dacou-Voutetakis *et al.* 1995) have shown a rate of 6.25/100,000, which is among the lowest in Europe. A "cold spot" with an unexpectedly low incidence (2.45/10,000) has been discovered in Macedonia (Kocova *et al.* 1993).

Spanish reports (Calle-Pascual *et al.* 1993) demonstrate a relatively high incidence inconsistent with the general north-to-south gradient in incidence rates. Interestingly enough, the Spanish-heritage population of Cuba appears to have a lower rate, 1.8/ 100,000 than the Hispanic population of Colorado, 9.7/100,000 (Diaz Onorat 1990). Among Yemenite Jews who migrated to Israel, the incidence of type 1 diabetes has increased dramatically since 1980, from 9.1 to 18.5/100,000, supporting the influence of environmental factors acting on a specific genetic background (Shamis *et al.* 1997).

2.2.1.2. Seasonality of diagnosis and date of birth

Many reports have pointed to an increased incidence of type 1 diabetes during the cooler months in both hemispheres, although this has been observed to be less consistent in children under 5 years of age. Recent data provided by the EURODIAB ACE study have revealed a significant seasonal variation in clinical presentation even in the youngest age group and across all European populations studied, with a winter peak that seems to be particularly characteristic of the Scandinavian regions (Levy-Marchal *et al.* 1995). A recent analysis of data from Finland has shown that a statistically significant seasonal variation could be confirmed only in males, with the most visible pattern reflecting a low number of cases diagnosed in June (Karvonen *et al.* 1996, Szybinski *et al.* 1996). A lack of seasonal variation has recently been reported in Madras, India (Ramachandran *et al.* 1996). Italian data indicate a lack of a significant seasonal variation in the Liguria region (Mazzella *et al.* 1995), while Sardinia shows a nadir in the summer (July) and a peak in the autumn (October) (Muntoni *et al.* 1995).

2.2.1.3. Age and sex distribution

The age distribution of newly diagnosed cases of childhood diabetes has been studied extensively (Karvonen *et al.* 1993). This generally shows a peak for clinical onset between 10 and 15 years of age, with a fairly sharp drop in the late teens. It is probable that children who are susceptible to the disease are exposed to the predisposing factor(s) during their first 15 years of life and that either exposure subsequently decreases or a large proportion of the susceptible individuals have already developed the disease by the time they reach 20 years of age. The incidence of type 1 diabetes may have another peak after the age of 40 years (Melton *et al.* 1983). The excess of males (male:female >1) is most pronounced between 15 and 30 years of age (Blohme *et al.* 1992). The EURODIAB ACE analysis provides reliable information on the variation by age groups and sex ratio among European populations. The risk profile in Sardinia increases until the age of 13 years in both males and females, although the profile is steeper in males. A recent study from Sweden suggested that theobserved in incidence rise may mainly be due to an increase in disease rate among children less than 10 years of age (Dahlquist & Mustonen 1994).

2.2.2. Natural course of type 1 diabetes

Gepts already suggested in 1965 that the process of beta-cell destruction is slow and that it may take years to destroy enough beta cells to result in clinical symptoms (Gepts 1965). This is in line with observations that beta-cell dysfunction can be demonstrated up to 6-7 years before clinical presentation (Mrena *et al.* 1999) and the fact that residual beta-cell function may persist for several years after the diagnosis of clinical disease. Subsequently, it was observed that type 1 diabetes occurred more frequently in patients

with autoimmune diseases and that diabetic patients often had organ-specific antibodies. The identification of islet cell autoantibodies (ICA) (Bottazo *et al.* 1974) in combination with the observation of an association between the disease and specific alleles of the human leukocyte antigen (HLA) system (Singal & Blajchman 1973, Nerup *et al.* 1974) and infiltration of the islets by lymphocytes and macrophages lent strong support to the view that type 1 diabetes is an autoimmune disease. The hypothesis of Gepts regarding a chronic rather than an acute process was strengthened by the finding that circulating autoantibodies could be present long before clinical onset of the disease in relatives of patients with type 1 diabetes (Gorsuch *et al.* 1981).

These findings implied that type 1 diabetes is an autoimmune disease caused by multiple interaction between genetic, environmental and immunological factors. Once initiated, the process is insidious, and it is conceivable that a considerable time may elapse before clinical manifestation. In the model of beta-cell destruction, the process shows two phases of preclinical diabetes proceeding a third phase of clinical diabetes. In phase 1 there is no evidence of beta-cell destruction, and only genetic predisposition is present. Due to unknown factors, possibly environmental or of other kinds, a selective attack on the beta cells starts. The duration of this second phase is unknown. In some individuals this process may come to a halt, or regeneration of the beta cells may occur, but in others the destruction process that leads to clinical symptoms will continue. By that time around 70-80% of the beta cells have been lost. After the initiation of exogenous insulin therapy, a phase called the honeymoon period, characterised by physiologically significant endogenous insulin secretion, is initiated (Sperling 1992). This period is of limited duration, however. The remission phase in most children is partial but may be complete in some adult patients who then remain normoglycaemic without exogenous insulin. Strict metabolic control soon after the diagnosis and male sex favour the remission period, whereas young age at diagnosis is associated with no or short remission periods in children with type 1 diabetes (Käär *et al.* 1984). Attempts have been made to induce remission from newly diagnosed type 1 diabetes by using various forms of immunotherapy like cyclosporin A (Martin S *et al.* 1991) or azathioprine (Harrison *et al.* 1985). These agents induced transient remission in up to 50% of the patients treated. However, permanent remissions could not be achieved.

2.3. Aetiology and pathogenesis

The aetiology and pathogenesis of type 1 diabetes are not fully understood. The dramatic increase in the incidence of the disease, especially in Scandinavia but also in many other parts of the world, is unlikely to be explained by increases in the genetic trait in the background population, but rather points to the introduction of novel non-genetic factors or an increase in existing exogenous diabetogenic factors. A concordance rate of only 50% for type 1 diabetes was initially reported among monozygotic twins (Tattersall & Pyke 1972), and a Finnish study arrived at an even lower concordance rate (20% among monozygotic twins as compared with 5% among dizygotic ones) (Kaprio *et al.* 1992). Type 1 diabetes could fit into the general causative models described by Rothman (1986), who states that various risk factors, genetic and non-genetic, in addition to other

complementary factors may result in overt diabetes. The pathogenic models for type 1 diabetes are based on autoimmune destruction of the beta cells (Rossini *et al.* 1993, Bach 1994) which has been initiated many years before clinical onset, as reflected by the emergence of humoral and/or cellular autoimmune activity long before manifestation of the disease (Tran *et al*. 1988, Riley *et al.* 1990). Some of the immune markers typically seen in prediabetic individuals may also disappear, as shown in prospective studies of first-degree relatives (Millward *et al.* 1986, Tun *et al.* 1994). The initiation of the autoimmune process may operate at different levels and affect either the beta-cell antigen itself, the antigen presenting process or the regulation of T and B-cell interaction. The disease could be induced by a trigger that alters antigen presentation by increasing MHC binding of an antigen or changing the suppresser/helper T-cell ratio, or through general activation due to a superantigen. The pathogenic model proposed by Eizirik *et al.* (1993) suggests that environmental risk factors inflict direct damage on the beta cells, resulting in the release of sequestered antigens, which in turn induce autoreactive T cells functioning in combination with specific MHC molecules. With the help of autoimmune mediators such as interleukin (IL)-1, tumour necrosis factor (TNF)-α and other cytokines, beta-cell damage may be accelerated (Spinas *et al.* 1988). This acceleration of the disease process could be explained by increased expression of autoantigens such as GAD on the beta-cell surface in response to an increased insulin secretion rate. Accordingly, environmental risk factors that increase the need for insulin and/or increase systemic levels of cytokines may play an important role at this stage in the pathogenic process.

2.3.1. Environmental factors

According to the Diabetes Epidemiology Research International Group (1987), about 60% of the 2.5-fold increase in the incidence of type 1 diabetes in children over the preceding three decades in Finland could be explained by environmental factors.

2.3.1.1. Viral infections

Viral infections are among the main factors thought to contribute to the environmental influence on the pathogenesis of type 1 diabetes. Hypothetically, a virus may trigger or accelerate an autoimmune process in the beta cells through various mechanisms (Rossini *et al.* 1993) such as direct toxicity (killing the beta cell), formation of a novel antigen, induction of MHC (through the release of autoimmune mediators such as interferon (INF)-α and other cytokines), molecular mimicry or interference with the immune system.

The observation of an increase in the incidence of diabetes in young adults as a complication of congenital rubella virus has been known for quite some time (Mclntosh & Menser 1992), already reported an increase in the number of cases of type 1 diabetes 2-4 years after a mumps epidemic. The Childhood Diabetes in Finland (DiMe) study showed increased IgG class mumps antibody titres in children with newly diagnosed type 1 diabetes (Hyöty *et al.* 1993). The human cytomegalovirus (CMV) has also been

implicated in the development of type 1 diabetes in a case report on an infant infected with congenital CMV (Ward *et al.* 1979). A Finnish study, however, found comparable levels of CMV IgG and IgM antibodies in children with newly diagnosed type 1 diabetes (Hiltunen *et al.* 1995*).* Scandinavian studies have indicated that maternal enteroviral infections during pregnancy may be associated with later development of type 1 diabetes in the offspring (Dahlquist *et al.* 1994, Hyöty *et al.* 1995), and recent longitudinal Finnish studies have shown that initially unaffected siblings who progressed to clinical type 1 diabetes during a prospective observation period had twice as often an enterovirus infection detcted by serological responses to enterovirus antigens than control siblings who did not develop the disease (Hyöty *et al.* 1995). In addition, seroconversion to ICA and GADA positivity was temporally associated with enterovirus infections (Hiltunen *et al.* 1997). A British study has shown an increased frequency of enterovirus (EV) RNA detected by PCR in children with newly diagnosed type 1 diabetes relative to control children (Clement *et al.* 1995).

2.3.1.2. Dietary factors

Cow's milk proteins have been suspected as possible risk factors for type 1 diabetes over the past two decades. Since the first epidemiological study showing that a short duration of breast-feeding was a risk factor for type 1 diabetes (Borch-Johnsen *et al.* 1984), a series of case-control studies have confirmed this association (Mayer *et al.* 1988, Kostraba *et al.* 1993, Virtanen *et al.* 1993). The possibility that this association may be due to the early introduction of cow's milk proteins rather than the protective effect of breast-feeding was supported by experiments with BB rats, since feeding these diabetes-prone rats on a mixture of amino acids instead of intact proteins significantly reduced the incidence of diabetes, whereas the addition of skimmed milk powder to the amino acid mixture increased it (Elliot & Martin 1984). Furthermore, studies employing non-obese diabetic (NOD) mice showed that, although genetically predisposed to diabetes, those that were fed a diet in which the sole amino acid source was hydrolysed casein were protected from the disease (Ellliot *et al.* 1988).

It has consistently been reported that children with newly diagnosed type 1 diabetes have increased levels of various cow's milk antibodies (Savilahti *et al.* 1988, Saukkonen *et al.* 1994). A report from the Swedish nation-wide study confirmed that most cow milk antibody levels tend to be increased among patients with newly diagnosed type 1 diabetes relative to control subjects, and that significant differences exist in the case of cow's milk protein ,BSA IgA antibodies and β- lactoglobulin IgA antibodies (Dahlquist *et al.* 1992). Interestingly, BSA shares an amino acid sequence (ABBOS) with an islet protein p69 and accordingly antibodies against this BSA region have been shown to cross-react with p69 (Karjalainen *et al.* 1992). Antibodies to the ABBOS peptide were also observed to be common among children with recent-onset type 1 diabetes, and it was therefore hypothesised that when introduced early into the leaky, immature gut, the ABBOS peptide may induce an immune reaction that could be boosted by the islet protein, which shares an epitope with ABBOS. This hypothesis has been challenged, however, by the findings that BSA antibodies can also be detected in non-diabetic relatives of subjects

with type 1 diabetes and in individuals with other autoimmune diseases (Atkinson *et al.* 1993). A recent Finnish study has demonstrated that oral exposure to cow's milk formula induced bovine insulin-binding antibodies that cross-react with human insulin and accordingly dietary bovine insulin appears to be an environmental trigger of a primary immune response to a beta-cell specific antigen in healthy children (Vaarala *et al.* 1999).

The first report on a relation between the nitrates and nitrites and the incidence of type 1 diabetes was published in the early 1980´s (Helgason & Jonasson 1981). It has long been known from animal studies that nitrosamine compounds are toxic to beta cells, probably by reducing their nicotinamide adenine dinucleotide (NAD) content, which is preventable by pre-treatment with nicotinamide (Wilander & Gunnarsson 1975, Helgason 1982). It is also well established that the beta-cell cytotoxic nitrosourea compound, streptozotocin, induces insulitis in mice when given in multiple small doses (Kolb *et al.* 1987). The results of a population-based, case-control study showed that the frequency of intake of food containing nitrates and nitrites was associated in a dose-response manner with the risk of type 1 diabetes (Dahlquist *et al.* 1990). A population-based Finnish study (Virtanen *et al.* 1994) also found a relationship between the risk of type 1 diabetes and the intake of food rich in nitrites that are partly converted into nitrosamines in the gastrointestinal tract. These observations are further supported by two ecological studies that showed a correlation between the incidence of type 1 diabetes and the nitrate content of drinking water (Parslow *et al.* 1997, Kostraba *et al.* 1992).

2.3.1.3. Unspecific infections and stress

A Swedish case-control study revealed a dose-response relationship between the total number of infections experienced during the year preceding the clinical onset of diabetes and the disease risk (Blom *et al.* 1991). Recently, both an extensive survey (Therlund *et al.* 1995) and a smaller case-control study (Robinson *et al.* 1989) have shown that psychological stressful life events during the year prior to the onset of their disease were significantly more common in individuals with type 1 diabetes than in matched controls. One has also to remember that type 1 diabetes could be triggered by a combination of environmental risk factors, as indicated by Haverkos (1997)

2.3.2. Genetic features in type 1 diabetes

Two approaches have been used to study genetic features in type 1 diabetes. The first is based on association studies, expressing the results as an estimate of risk by comparing the frequency of a genetic trait between patients and unaffected subjects (Green 1982). The second alternative is to determine linkage within families by following the segregation of a marker with the disease. Approximately 33-50% of monozygotic twins and 5-6% of offspring and siblings of patients with type 1 diabetes develop the disease, as compared with a cumulative incidence of 0.2-0.7% in the general population (Atkinson & Maclaren 1994). Therefore, a predisposing genetic background is required for the development of clinical type 1 diabetes. However, the phenotype of type 1 diabetes does

not exhibit a typical Mendelian inheritance that can be attributed to a single locus, and it is therefore an example of a complex genetic disease; one with a multigenic pattern of inheritance and a strong influence of environmental factors which act by modulating (accelerating or delaying) its clinical manifestation.

The strongest susceptibility to type 1 diabetes is associated with the HLA DQ alleles. In Caucasians, disease susceptibility is more closely associated with DQA1*0501-DQB1*0201/DQA1*0301-DQB10302 than with DRB1*03/DRB1*04 (Michelsen J *et al.* 1990), while the DQA1*0102-DQB1*0602-DRB1*0501 genotype has been observed to be protective with respect to type 1 diabetes. The protective DQB1*0602 allele is probably immunodominant over the susceptible DQB1 alleles (Pugliese *et al.* 1995, Hagopian *et al.* 1995). The association of the DQ alleles with diabetes is related to amino acid residue 57 in the β-chain (Todd *et al.* 1987) and amino acid residue 52 in the α-chain (Khalil *et al.* 1990).

In animal models such as the NOD mouse, the inheritance of diabetes is polygenic, with at least ten factors influencing susceptibility, including MHC (Ghosh *et al.* 1993*).* Similarly, at least three genes are involved in the development of the disease in the BB rat (Jacob *et al.* 1992). As in the NOD mouse, the MHC (or adjacent) genes also play a critical role in conferring susceptibility to the disease in the BB rat. The lymphopenia gene (or an adjacent one), together with a third gene, have been shown to confer susceptibility to diabetes in the BB diabetes-prone (DP) rat.

2.3.2.1. The major histocompatbility complex (MHC)

The function of the Major Histocompatbility Complex (MHC), called the HLA (Human Leukocyte Antigen) complex, is derived from the fact that the HLA gene products play a crucial role in shaping the immunological identification of self and non-self, and that these molecules are involved in subcellular peptide transport. The genes encoding the human MHC are located on the short arm of chromosome 6. Three groups of class 1 genes have been identified, denoted A, B and C, while Class II molecules are clustered in three regions: DR, DQ and DP. Recent genome-wide mapping studies have confirmed that the HLA class II region encodes the most important genetic factors for type 1 diabetes (Davies *et al.* 1994, Luo *et al.* 1995). The role of HLA alleles in type 1 diabetes was first indicated by an association with HLA-B8 and B15, and then with HLA-DR3 and DR4 encoded in the DRB1 locus (Singal & Blajchman 1973, Nerup *et al.* 1974). Subsequently, the DQB1 and DQA1 genes were shown to be more closely associated with type 1 diabetes (Todd *et al.* 1987, Todd *et al.* 1990, Khalil *et al.* 1990). Several authors have demonstrated that both the DQ and DR molecules influence type 1 diabetes susceptibility (Sheehy *et al.* 1989, Sheehy 1992, Kockum *et al.* 1993, Cucca *et al.* 1995).

Several genes related to human type 1 diabetes have been located by gene mapping (or linkage analysis by the affected sib-pair method) (Field *et al.* 1994, Hashimoto *et al.* 1994, Davies *et al.* 1994, Rowe *et al.* 1995, Pozzilli *et al.* 1996b). The *IDDM1* gene(s), localised to the HLA complex on chromosome 6p21.3, may explain 45-50% of the genetic risk of developing the disease. Historically, the associations between HLA class I haplotypes and type 1 diabetes (Singal & Blajchman 1973, Nerup *et al.* 1974) was the

first demonstration of the importance of histocompatibility genes in the pathogenesis of the disease, but it is now known that these associations are due to linkage disequilibrium of the class I genes with HLA-DR and HLA-DQ (class II) genes (Faas & Trucco 1994, Sanjeevi *et al.* 1996).

The frequency of HLA-DRB1*0301 (DR3) and DRB1*0401 (DR4) is significantly higher in Caucasian patients with type 1 diabetes than in healthy controls (Ronningen *et al.* 1991), but susceptibility to type 1 diabetes seems to be more strongly dependent on specific HLA-DQ haplotypes, such as DQA1*0302-DQB1*0302 (DQ8) and DQA1*0501-DQB1*0201 (DQ2) (Sanjeevi *et al.* 1995b). In spite of the fact that the marked linkage disequilibrium between the various HLA genes is a complicating factor for the assessment of their association with type 1 diabetes, the association of HLA-DR4 with the disease is most probably a consequence of the linkage disequilibrium of this allele with HLA-DQ8. Interestingly, some studies have failed to show that DQA1*0501-DQB1*0201 (DQ2) is associated with susceptibility to type 1 diabetes when present in genotype combinations other than DQ2/DQ8 (Sanjeevi *et al.* 1996*)*.

The DQB1 allele in HLA-DR15-DQA1*0102-DQB1*0602 (DQ6) confers strong protection from type 1 diabetes, even in genotype combinations with "at risk" haplotypes (Baisch *et al.* 1990, Sanjeevi *et al.* 1994, Reijonen *et al.* 1994). This protection appears to have a dominant character, as the risk is also very low in ICA-positive first-degree relatives of patients who are DQ6 positive (Hagopian *et al.* 1995, Pugliese *et al.* 1995). The DQ6 subtype DQA*0102-DQB1*0604 was nevertheless found to be positively associated with type 1 diabetes in an ethnically mixed population (Penny *et al.* 1993). The DQ7 allele, found initially to be neutral in relation to the type 1 diabetes (Wassmuth *et al.* 1989), was subsequently shown to be associated with protection from disease (Kockum *et al.* 1993, Sanjeevi *et al.* 1994). The most common genotype found in Caucasian patients with type 1 diabetes is DQ2/DQ8 (DQA1*0501-DQB1*0201 /DQA1*0301-DQB1*0302), which is present in up to 35-38% of cases (Nepom 1993, Sanjeevi *et al.* 1994). The disease tends to be associated with different HLA haplotypes among Asian patients than in Caucasians, since the haplotypes associated with susceptibility are HLA DR4/DQ4 and DR9/DQ9 among the Japanese (Aparicio *et al.* 1988, Awata *et al.* 1990) and HLA DR9/DQ9 and DR3/DQ2 among the Chinese (Hawkins *et al.* 1987). The varying HLA associations connected with ethnic background are due to differences in linkage disequilibrium and variable prevalences of the HLA alleles and haplotypes in different populations. For instance, HLA DR3 is very rare in the Japanese population, and the DQ8 haplotype is in linkage disequilibrium with DR8 and not with DR4 as among Caucasians.

The risk of contracting type 1 diabetes in HLA DQ2/DQ8 heterozygous Caucasians is close to 25% among first-degree relatives of type 1 diabetes patients and 4-7% in the general population, while conversely, the risk for DQ8 and DQ2-negative subjects is less than 1% among first-degree relatives and less than 0.05% in the general population (Nepom 1993). About 90% of type 1 diabetes patients are DQ2 and/or DQ8-positive (Sanjeevi *et al.* 1995a).

Although we cannot yet exclude the possibility that the association of the HLA DQ genes with type 1 diabetes may be due to linkage disequilibrium with a still unknown gene, it is nevertheless highly probable that this association is mediated by the function of the polymorphic class II molecules, which play a critical role in antigen presentation and

T-cell activation. Molecular analysis of the HLA DQ B1 genes showed that two amino acid residues are apparently critical for determining susceptibility to or protection from the disease (Todd *et al.* 1987, Khalil *et al.* 1990). Thus, the absence of an aspartic acid residue in position 57 of the DQβ-chain (non-Asp 57) and the presence of an arginine residue in position 52 of the DQα-chain (Arg 52) confer a high risk of type 1 diabetes. This combination (non-Asp57/Arg52) is present in DQ2 and DQ8, but not in DQ6 or other molecules associated with protection from disease. To explain this phenomenon, it has been hypothesised that DQβ 57 and DQα 52 may be critical in determining which peptides can bind to the HLA molecules (Faas & Trucco 1994). Further research has shown, however, that other amino acid residues (such as DQα Leu69) are also associated with a high risk of type 1 diabetes (Sanjeevi *et al.* 1995b).

Other genes in the class II region that are associated with type 1 diabetes are the transporter-associated genes TAP1 and TAP2, which are located between the DQ and DP regions. These encode heterodimeric protein complexes that enable the transport of endogenous peptides from the cytosol to the lumen of the endoplasmic reticulum (Lotteau *et al.* 1990, Jackson & Petersen 1993). Among the TAP1 and TAP2 polymorphic alleles, TAP2*0101 was shown to be positively associated with type 1 diabetes and TAP2*0201 inversely so (Caillat-Zucman *et al.* 1993, Ronningen *et al.* 1993). It seems, however, that the association between TAP genes and type 1 diabetes can be explained entirely by their linkage disequilibrium with DQ genes.

2.3.2.2. Other susceptibility genes

IDDM2 genes are localised on chromosome 11p15.5, which comprises the insulin gene region. The 5' flanking region of the insulin gene (5'INS) is characterised by a polymorphism comprising a variable number of tandem repeats (VNTR) of 14 bp oligonucleotides. Three 5'INS alleles are known, and these are referred to as class I, class II and class III alleles, in relation to the lengths of the DNA inserts. The 5'INS class I/I genotype is significantly more frequent among patient with type 1 diabetes than among healthy controls (Owerbach & Gabbay 1993, Van der Auwera *et al.* 1993) while the short class III is dominantly protective (Bennett *et al.* 1995). In pancreatic islet cells homozygosity for the class III VNTR was associated with a decreased insulin expression (Vafiadis *et al.* 1997). It was suggested that the class III alleles may reduce the risk of type 1 diabetes by decreasing the levels of circulating insulin, the only known beta cell specific autoantigen. Recent studies on the association of this genetic trait with the expression of both insulin and proinsulin in human fetal and postnatal thymus have indicated that increased expression of insulin may be sufficient to induce negative selection in the thymus resulting in deletion of autorective T lymphocytes (Vafiadis *et al.* 1997, Pugliese *et al.* 1997).

2.4. Autoimmunity in type 1 diabetes

2.4.1. Immunological mechanisms of beta-cell destruction

The mechanisms of beta-cell destruction are still poorly understood. The T cells of the immune system may be directly toxic to the beta cells or they may attract to the pancreas and activate other types of cells such as monocytes, macrophages and eosinophils, all of which could produce beta-cell toxic mediators such as IL-1 or TNF, leading to beta-cell destruction (Neurp *et al.* 1994, Mandrup-Poulsen 1996). Whether the beta-cell damage is due to a state of atrophy (destruction) or reversible inflammation is still unclear, but the latter hypothesis is supported by observations on animal models in which islets from newly diagnosed diabetic NOD mice partially regained their function in the absence of T cells (Strandell *et al.* 1990), and a single injection of a monoclonal antibody directed against T-cell receptors in NOD mice induced rapid normalisation of hyperglycaemia (Sempe *et al.* 1991).

2.4.2. Cellular autoimmunity

The hypothesis that type 1 diabetes is a T-cell mediated disease and that T lymphocytes are major beta-cell aggressors is supported by observations on both animal models and human type 1 diabetes. In animal models, the presence of T cells in the insulitis lesion and their ability to transfer diabetes adaptively to BB rats (Like *et al.* 1985) and to NOD mice (Wicker *et al.* 1986, Bendelac *et al.* 1987, Reich *et al.* 1989) support the crucial role of these cells. In addition, numerous studies have shown a preventive effect of T-cell modulation in experimental diabetes (Seino *et al.* 1993, Hayward *et al.* 1993). The disease can be prevented by neonatal thymectomy (Like *et al.* 1982). There are some reports strongly supporting the role of T lymphocytes in human disease, as well. Several studies have demonstrated the presence of autoreactive T lymphocytes in the peripheral blood of patients with newly diagnosed type 1 diabetes (Harrison *et al.* 1991, Peakman *et al.* 1994), and T-cell infiltration of pancreatic grafts was observed, with a dominance of CD8 T cells over CD4 cells, in transplanted patients who eventually showed a recurrence of type 1 diabetes (Santamaria *et al.* 1992, 1994). Further strong evidence for the involvement of T cells in type 1 diabetes emerged when a woman developed the disease years after receiving a bone marrow transplant from her HLA-identical brother with type 1 diabetes (Lampeter *et al.* 1993). Infiltration of the islets with both T and B lymphocytes and macrophages was detected post-mortem in two infants with recent-onset diabetes (Lernmark *et al.* 1995). A state of insulitis has been demonstrated in the islets of nine patients with recent-onset type 1 diabetes (Imagawa *et al.* 1999). All these observations confirm the role of T cells in the pathogenesis of the disease.

2.4.2.1. T lymphocytes

T lymphocytes can be classified into CD4 and CD8 according to their surface antigen expression. CD4 T cells are perceived considered functionally as so-called T-helper (Th) cells, which can interact with antigen-presenting cells expressing MHC class II molecules. Cytotoxic T cells express the CD8 surface molecule and can interact with cells expressing the MHC class I molecule. CD4 (Th) cells regulate and orchestrate the immune system, and upon activation they can start to secrete cytokines, which recruit and activate other cells: macrophages, Tc cells, natural killer (NK) cells and B cells. The activated cells are attracted to the site of the immune response, which results in infilammatory infiltrates. This combined action leads to the secretion of additional cytokines (lymphokines) that trigger the expansion of certain clones of B and T cells. Eventually, regulatory and suppressive mechanisms will limit and terminate the immune response. CD4 cells can be divided into two functionally distinct subgroups on the basis of their cytokine production: Th1 cells produce predominantly IL-2 and IFN-γ, while the Th2 subsets produce IL-4, IL-5 and/or IL10. The role of CD4+ cells in the pathogenesis of autoimmune diabetes is emphasized by the observation in NOD mice where diabetes could be transferred by CD4+ T-cell clones alone (Haskins & Mcduffie 1990, Shimizu *et al.* 1993).

2.4.2.2. Cytokines and macrophages

The dominance of Th1 type cytokines such as IFN-γ, interleukin 2 (IL-2) and TNF-β has been implicated in type 1 diabetes. Studies in animal models have supported the role of Th1 type cytokines in promoting cell destruction in autoimmune diabetes (Campbell *et al.* 1991, Pilstrom *et al.* 1995, Muir *et al.* 1995, Rabinovitch *et al.* 1996, Chung *et al.* 1997, Zheng *et al.* 1997). In addition, type 2 cytokines (IL4 and IL10) have been suggested to be as protective in NOD mice after oral administration of insulin (Hancock *et al.* 1995) The macrophage-derived proinflammatory cytokines IL-1α, IL-1β, TNF-α and IL-6 have been detected in islets preceding insulitis and the appearance of diabetes in BB rats (Huang *et al.* 1994). IFN-α has been detected in the pancreatic islets of patients with type 1 diabetes (Foulis *et al.* 1987), and IFN-α mRNA has been identified in the pancreas of recently diagnosed cases (Huang *et al.* 1994). In contrast, TNF-α and IL-2 were not found in any pancreas samples. IFN-α has also been detected in lymphocytes infiltrating the islets of subjects with recent-onset type 1 diabetes (Foulis *et al.* 1991*)*. Recent work with animal models (Nicoletti *et al.* 1994, Cailleau *et al.* 1997) supports the view that IL-1 plays a role in the final process leading to beta-cell destruction (Mandrup-Poulsen 1996). Macrophages have also been implicated in the development of type 1 diabetes. They are among the first immune cells to be found in the islets (Walker *et al.* 1988) and can function as antigen-presenting cells (APCs) and exert a cytotoxic effect by releasing cytokines that attract other immune cells. In particular, dendritic cells, which are derived from macrophage precursors, can function as excellent antigen-presenting cells. It is possible that such cells are indeed processing beta-cell antigens in such a way that autoreactive T cells become activated, but it is not known which factors are responsible

for this process. Additional evidence for the role of macrophages in beta-cell destruction has been provided by the finding of the presence of insulin crystals in the macrophages of insulitis lesions, indicating that the macrophages have engulfed beta cells (In't veld & Pipeleers 1988)

2.4.3. Humoral autoimmunity

2.4.3.1. Glutamic acid decarboxylase (GAD)

Properties of GAD. Glutamic acid decarboxylase (GAD) is found in nerves, in which it catalyses the conversion of L-glutamine into γ-amino butyric acid (GABA), the major inhibitory neurotransmitter in the brain. It has been estimated that up to 30% of the neurones are GABA-ergic (Erlander & Tobin 1991). Besides its high concentration in the central nervous system (CNS), GAD is expressed at lower concentrations in the pancreatic islets of Langerhans (Christie *et al.* 1992). GABA was first detected in the pancreatic islets in 1972 (Briel *et al.* 1972) and GAD in 1976 (Okada *et al.* 1976).

Baekkeskov *et al.* (1982) reported that sera from eight out of 10 children with newly diagnosed type 1 diabetes precipitated a protein with a molecular weight of 64,000 (64 kD) from detergent lysates of biosynthetically labelled human islet cells. The subsequent demonstration that 64 kD antibodies were present in BB rats (Baekkeskov *et al.* 1984), NOD mice (Atkinson & Maclaren 1988) and prediabetic humans (Baekkeskov *et al.* 1987) confirmed the importance of this autoantigen for the pathogenesis of type 1 diabetes. Baekkeskov *et al.* (1987) examined 14 ICA-positive first-degree relatives of subjects with type 1 diabetes and reported that 11 had 64 kD antibodies that preceded the clinical onset, and sometimes preceded the detection of ICA. Immune responses to the 64kD antigen were observed to be an early event in the development of type 1 diabetes.

Two GAD isoenzymes, with molecular weights of 65,000 (GAD65) (Karlsen *et al.* 1991) and 67,000 (GAD67) (Michelsen *et al.* 1991, Bu *et al.* 1992) have been identified and characterised. These are encoded by different genes, located on chromosome 10 (Karlsen *et al.* 1991, Bu *et al.* 1992) and chromosome 2 (Michelsen *et al.* 1991, Bu *et al.* 1992), respectively. The exon-intron organisation of the two genes nevertheless suggests that they are derived from a single GAD gene (Bu & Tobin 1994). Both GAD65 and GAD67 are expressed in the CNS of man and animals (Petersen *et al.* 1993). In human islets, however, only GAD65 is expressed at high levels, in contrast to rat islets, where both isoenzymes are present. GAD65 and GAD67 also differ in their subcellular localisation, with the former being predominantly hydrophobic and anchored to synaptic-like vesicles and the latter cytoplasmic, with a preferential perinuclear localisation (Solimena *et al.* 1993). These differences in subcellular localisation have been shown to depend on signals located in the NH2-terminal domains of the two isoenzymes (Solimena *et al.* 1994). Autoantigenic epitopes have been identified in the middle and carboxy-terminal regions of GAD65 by human serum autoantibodies (Kaufman *et al.* 1992, Daw *et al.* 1996) and in the N-terminal region of GAD65 by using human monoclonal ICA (Richter *et al.* 1996). It was observed that an increase in the titre of antibodies to the C-terminal region was associated with conversion to diabetes (Falorni *et al.* 1996). GADA

epitopes in the same sera from nine healthy subjects positive for GADA antibodies who did not progress to clinical type 1 diabetes over an 8-year follow-up period, were localised to the same regions as those observed in patients with type 1 diabetes. However, GADA directed at the C-terminal epitopes were significantly higher in the patients with type 1 diabetes than in the healthy subjects.

The molecular cloning of human GAD65 (Karlsen *et al.* 1991) and the demonstration that this enzyme is identical to the islet 64K autoantigen (Karlsen *et al.* 1992) enabled the development of several assays to detect antibodies to GAD65 in human serum (Rowley *et al.* 1992, Hagopian *et al.* 1993b, Grubin *et al.* 1994, Petersen *et al.* 1994, Aanstoot *et al.* 1994, Savola *et al.* 1998a). Most GADA methods measure the ability of sera to immunoprecipitate GAD. They have been applied as radiobinding assays (RBA), liquid immunoprecipition assays, enzymes-linked immunosorbent assays (ELISA). RBA had stronger predictive characteristics than did ELISA, since the disease sensitivity of GADA in the second workshop was higher when measured with RBAs (76%) than with ELISAs (36%) (Schimidli *et al.* 1994a). The specificity (about 90%) was comparable between various assays.

Function and regulation of GAD in pancreatic islets. Although the presence of GAD and GABA in pancreatic islets has been known since the 1970's (Okada *et al.* 1976), the function of insular GABA still remains unclear (Michalik *et al.* 1993, Gilon *et al.* 1991). Its neuroinhibitory function in the CNS has served as a model for a proposed role in the pancreatic islets. It was initially suggested that GABA produced by the beta cells functioned as a paracrine regulator of glucagon secretion (Rorsman *et al.* 1989). The findings that GABA may be present in synaptic-like microvesicles (SLMV) (Sorenson *et al.* 1991) and that GABA-transported activity occurs in association with beta-cell SLMV (Thomas-Reetz *et al.* 1993) favour a role as a neurotransmitter at the tissue level. GABA may play a role in beta-cell metabolism, and glutamate could be stored as a source of energy for use under stress conditions (Sorenson *et al.* 1991).

GAD was shown to increase in islet cultures in a medium with a high glucose concentration (Kämpe *et al.* 1989). Parallel stimulatory effects of α-ketoisocaproic acid and inhibitory effects of diazoxide on insulin secretion and on GAD expression have been demonstrated several occasions (Björk *et al.* 1992, Hagopian *et al.* 1993a).

Autoantibodies to GAD. The discovery that the enzyme for the inhibitory neurotransmitter GABA, i.e. GAD, is the major target of autoantibodies in both stiff-man syndrome (SMS) (Solimena *et al.* 1988, 1990), a rare neurological disease, and type 1 diabetes (Baekkeskov *et al.* 1990) indicates that both diseases are characterised by a breakdown of tolerance to this antigen and suggests that they share some aetiological features.

The highest diagnostic sensitivity of GADA has been observed at the clinical onset of type 1 diabetes (Schmidli *et al.* 1994b, Falorni *et al.* 1995c, Vandewalle *et al.* 1995, Bonifacio *et al.* 1995a) and typically 70-80% of patients test positive for GADA. The specificity of GADA has been shown to be close to 99%, since these antibodies are present in 1-2% of healthy subjects (Velloso *et al.* 1993, Aanstoot *et al.* 1994, Petersen *et al.* 1994, Grubin *et al.* 1994). The sensitivity is lower in young boys (Schmidli *et al.* 1994b, Falorni *et al.* 1995c), but does not seem to decrease with age as is the case for IAA and IA-2A (Vandewalle *et al.* 1993). The frequency of GADA was 6% among first-degree relatives of affected subjects, and they were found useful for predicting type 1

diabetes in family members (Kaufman *et al.* 1992, Bonifacio *et al.* 1995a, Yu *et al.* 1995, Verge *et al.* 1996). The positive predictive value of GADA for type 1 diabetes may be as high as 50-60% in combination with other islet autoantibodies (Bingley *et al.* 1994, Genovese *et al.* 1994, Hagopian *et al.* 1995). GADA levels decrease somewhat over the first 2 years after diagnosis, but their prevalence is still surprisingly high in the patients with long term disease (Kaufman *et al.* 1992). Another longitudinal study lasting for 6 years after the diagnosis of type 1 diabetes has shown that antibody levels may fluctuate substantially and that close to 60% of patients still test positive for GADA after a disease duration of 6 years (Batstra *et al.* 1997).

Humoral autoimmunity to GAD and HLA-defined genetic disease susceptibility. A number of attempts have been made to test the hypothesis that there is an association between specific HLA class II genes and GAD antibodies in patients with newly diagnosed type 1 diabetes. An increased frequency of GADA has been observed in patients with certain combinations of HLA-DR or DQ alleles. The prevalence of GADA was significantly increased in type 1 diabetic patients positive for the DR3/DR4 combination as compared with patients having at least one DR antigen other than DR3 or DR4 (Serjeantson *et al.* 1992, Kawasaki *et al.* 1994). In a Swedish study the frequency of GADA positivity was increased in patients who were positive for DRB1*0404-DQ8 relative to those who were DRB1*0404-DQ8-negative, suggesting that DRB1*0404 might be important for the humoral response to GAD (Sanjeevi *et al.* 1994). In Japanese patients with type 1 diabetes of short duration the DRB1*04-DQ4 haplotype was persistently associated with a reduced frequency of GADA, while an increased antibody frequency was seen in those with DRB1*04-DQ8, indicating that the DQB1 locus rather than DRB1 locus is associated with GADA in this population. Patients with type 1 diabetes who were positive for the DR2-DQ6 haplotype, known to be inversely associated with the disease, showed an increased frequency of GADA relative to DR2-positive healthy controls, suggesting that the antibody markers are present in association with type 1 diabetes rather than with a certain HLA-DR allele (Sanjeevi *et al.* 1994).

Studies on the relation between GADA and HLA DQ alleles have shown that the frequency of DQ2 is significantly increased among Australian patients who are positive for GADA as compared with those who are GADA-negative (Serjeantson *et al.* 1993*)*, while the DQ2/DQ8 genotype was significantly increased in GADA-positive Swedish children with disease manifestation after the age of 15 years but not in those with onset at a younger age (Hagopian *et al.* 1995). In Belgian patients with type 1 diabetes GADA were more prevalent in the presence of DQ8, especially in those diagnosed between the age of 20 to 40 years, but not in those with disease manifestation under the age of 10 years (Vandewelle *et al.* 1995). GADA were present in 79% of those carrying DQ2 and/ or DQ8, compared with 57 % of those who were negative for DQ2 and/or DQ8, suggesting that this high risk genotype identifies a subgroup of patients with adult-onset type 1 diabetes (20-40 years) having a high prevalence of GADA (Vandewelle *et al.* 1997).

GAD and other autoimmune diseases. GADA have been detected in many autoimmune diseases. Their frequency is high in patients with the polyendocrine syndrome (Björk *et al.* 1994, Petersen *et al.* 1994, Tuomi *et al.* 1996), and antibodies to both isoforms of GAD were detected in Swedish children with Grave's disease (Hallengren *et al.* 1996). GADA were also present in both Caucasian and Japanese

patients with idiopathic Addison´s disease (Kawasaki *et al.* 1994, Falorni *et al.* 1995b, Tsuruoka *et al.* 1995, Powell *et al.* 1996).

Cellular immune responses to GAD. Atkinson *et al.* (1992) observed that GAD was recognised by T cells in about half of their patients with newly diagnosed type 1 diabetes, Elsewhere CD4 and CD8-expressing T cells have also been successfully stimulated by GAD-derived peptides (Panina-Bordignon *et al.* 1995). Harrison *et al*. (1993) reported an inverse relation between the humoral and cellular immune responses to GAD in high-risk first-degree relatives. T-cell reactivity to both the GAD antigen and the homologous portion of the non-structural P2-C protein of coxsackie virus B has been demonstrated as well (Atkinson *et al.* 1994). The variable results of studies on T cell reactivity to GAD may be explained by variations between the assays or in the antigen preparation used.

Impact of GAD on the pathogenesis of type 1 diabetes. Expermintal studies have been implicated an aetiological role for GAD autoimmunity, either humoral or cellular, in the pathogenesis of beta-cell destruction. NOD mice show early spontaneous T-cell reactivity to GAD, and humoral and cell-mediated autoreactivity to GAD are among the first anti-islet responses, observed initially at 4 weeks of age. This coincides with the appearance of insulitis in these animals (Tisch *et al.* 1993, Kaufman *et al.* 1993). Furthermore, a number of therapeutic approaches, either oral, intrathymic, intraperitoneal, intranasal or subcutaneous administration of GAD or GAD peptides, led to a halt in the disease process in these NOD mice (Tisch *et al.* 1994, McFarland *et al.* 1996, Bellmann *et al*. 1998). The role of GAD as a self-antigen in the pancreatic beta cells in the activation of the autoimmune process has also been confirmed in NOD mice, since GAD-reactive CD4+ Th1 cells induce diabetes in these animals (Zekzer *et al.* 1998). The hypothesis, based on a specific molecular mimicry model involving GAD, was enhanced by the finding of an 18 amino acid peptide with sequence homology between human GAD and the Coxsackie virus P2-C protein (Kaufman *et al.* 1992). This specific region of GAD contains a T cell epitope involved in GAD cellular immunity in human type 1 diabetes (Atkinson *et al.* 1994). Despite the important function of GAD in the production of GABA in the nervous system, it has not been observed to play any role in the development of diabetic neuropathy (Roll *et al.* 1995, Zanone *et al.* 1994).

2.4.3.2. Antigens related to the protein tyrosine phosphatases (PTP)

*Properties of IA-2 and IA-2*β. Islet antigen 2 (IA-2) is a member of the protein tyrosine phosphatase (PTP) family. The IA-2 gene is located on chromosome 7q36 and encodes a 979 amino acid transmembrane protein (Lan *et al.* 1994, 1996). The extracellular and intracellular domains are 576 and 378 amino acids in length, respectively. Blotting analysis has shown that IA-2 is expressed primarily in neuroendocrine cells (Lan *et al.* 1994, Solimena *et al.* 1996, Xie *et al.* 1996). It can be detected in the islets of Langerhans and in many parts of the CNS. Another novel protein tyrosine phosphate is IA-2β, the gene for which has been traced to the same chromosome locus as IA-2. IA-2β has also been shown to be an autoantigen in type 1 diabetes (Lu *et al.* 1996, Notkins *et al.* 1997, Li *et al.* 1997), and there is 75% structural homology between IA-2 and IA-2β**.** The degree of homology is highest in the intracellular domain that is the target of

autoantibodies to PTP-related antigens in patients with diabetes. Almost all subjects with IA-2β antibodies also test positive for (IA-2A) antibodies, implying that the immunodominant epitopes are identical, but a few diabetic sera react uniquely with IA-2β (Wasmeier & Hutton 1996). Studies on the origin of the previously described 40kD and 37kD antigens have revealed that IA-2 is the precursor of the 40kD fragment and IA-2β of the 37kD antigen (Lu *et al.* 1994).

*Autoantibodies to IA-2 and IA-2*β. The biochemical identification of the 40 and 37 kD antigens has facilitated the development of assays similar to those for GADA (Gianani *et al.* 1995). These have been used to confirm the prevalence of the antibodies in patients with newly diagnosed type 1 diabetes, in first-degree relatives (Seissler *et al.* 1996, Verge *et al.* 1996) and in the general population (Bingley *et al.* 1997). The frequency of IA-2A has been shown to be higher than 50% (range 55-75%) in patients with newly diagnosed type 1 diabetes, being highest in those with disease onset before the age of 15 years (Bonifacio *et al.* 1995b, Gorus *et al.* 1997, Roll & Ziegler 1997), and less than 3% (range 0-2.5%) in control subjects (Bonifacio *et al.* 1995a). It is highest in newly diagnosed patients with the HLA DR4 and HLA DQA1*0301-DQB1*0302 genotypes (Gorus *et al.* 1997, Genovese *et al.* 1996, Savola *et al.* 1998a).

IA-2A have also been detected in a small minority of patients with SMS in the absence of type 1 diabetes (Martino *et al.* 1996), but IA-2A have so far remained relatively specific to type 1 diabetes. As for GADA, studies on IA-2A as predictive markers are mainly confined to the probability of disease when detected in combination with other markers such as ICA, GADA and lAA in first-degree relatives (Bingley *et al.* 1994, Christie *et al.* 1997, Savola *et al.* 1998a). There is some evidence that IA-2A persist at a higher frequency up to one year after diagnosis but decreases thereafter (Hawa *et al.* 1997, Savola *et al.* 1998b).

The disease sensitivity of IA-2βA has been observed to be between 35% and 50% in patients with newly diagnosed type 1 diabetes, which is less than that of IA-2A (Wasmeier & Hutton 1996, LaGasse *et al.* 1997).

Cellular immune responses to the IA-2 antigen. There are a few studies on cell-mediated immune responses to the IA-2 antigen. Peripheral blood lymphocytes from patients with type 1 diabetes have been shown to react with the IA-2 antigen (Durinovic-Bello *et al.* 1996, Ellis *et al.* 1998), and T-cell responses to the IA-2 peptide 805-820 were predominantly associated with HLA DR4 in relatives of patients with the disease (Honeyman *et al.* 1998).

2.4.3.3. Islet cell autoantibodies (ICA)

The presence of ICA in the serum of patients with type 1 diabetes was first detected by indirect immunofluorecence in 1974 (Bottazzo *et al.* 1974). International standardisation efforts have reduced the inter-laboratory variation and introduced Juvenile Diabetes Foundation units as a semiquatitive measure for ICA (Gleichmann & Bottazzo 1987). The diagnostic sensitivity of ICA in children and adolescents with newly diagnosed type 1 diabetes is approximately 70%-90%, while 0.1-5% of healthy control subjects test positive for ICA (Karjalainen *et al.* 1986, Schatz *et al.* 1994, Hagopian *et al.* 1995,

Bonifacio *et al.* 1995a, Gorus *et al.* 1997). ICA generally react with all endocrine cells in the islets, but there is considerable heterogeneity in the staining patterns, suggesting that the antibodies have multiple and variable target molecules (Genovese *et al.* 1992, Gianani *et al.* 1992). Persistent ICA positivity seems to predict an increased rate of loss of endogenous insulin secretion after diagnosis (Marner *et al.* 1985, Wallensteen *et al.* 1988, Schiffrin *et al.* 1994). The role of ICA as predictive markers has been more extensively studied than that of any other autoantibodies associated with type 1 diabetes. They can be detected in first-degree relatives of affected patients several years prior to the onset of type 1 diabetes (Gorsuch *et al.* 1981), and the probability of developing disease it has been shown to be directly related to their titre. Relatives having high antibody titres (≥ 80 JDF units) almost always progress to clinical disease, while those with low titres (<20 JDF units) have a substantially lower risk (Bonifacio *et al.* 1990, Riley *et al.* 1990, Bingley 1996). Nevertheless, not all those with very high ICA titres develop type 1 diabetes, and estimates from pooled retrospective data in the ICARUS study indicate that the probability that a first-degree relative with a high titre ICA (>80 JDF units) will develop the diasease within 5 years of testing is approximately 53% (Bingley 1996). As a consequence of the low prevalence of type 1 diabetes in the general population, any immune marker used for disease prediction should have a high specificity. The positive predictive value of ICA in the general population is only 5-6% (Landin-Olsson *et al.* 1992), and accordingly the accurate prediction of clinical type 1 diabetes is possible in only 3-4% of future cases if ICA are used as the only predictive marker (Bingley *et al.* 1993). Furthermore, the presence of ICA does not always indicate loss of beta-cell function (McCulloch *et al.* 1990, Bärmeier *et al.* 1991).

2.4.3.4. Insulin autoantibodies (IAA)

The first molecularly identified autoantigen involved in type 1 diabetes was insulin. This occurred by immunoprecipitation of radiolabelled insulin in patients with newly diagnosed type 1 diabetes before the initiation of insulin treatment (Palmer *et al.* 1983). Although ELISA techniques have been proposed for the analysis of IAA, only RIAs combine adequate diagnostic sensitivity and specificity (Kuglin *et al.* 1990). The diagnostic sensitivity of IAA for type 1 diabetes has been observed to be 30-70% (Soeldner *et al.* 1985, Arslanian *et al.* 1985, Srikanta *et al.* 1986, Landin-Olsson *et al.* 1992), the differences possibly being dependent on the type of assay used (Sochett *et al.* 1989, Vardi *et al.* 1988, Landin-Olsson *et al.* 1992, Gorus *et al.* 1994). The prevalence of IAA among patients with type 1 diabetes is dependent on age at clinical onset, and is highest among young children and lowest among adults (Vandewalle *et al.* 1993, Falorni *et al.* 1996). In addition, some data indicate that the IAA titre correlates directly with the loss of beta-cell function (Vardi *et al.* 1988). Even though the diagnostic specificity of IAA for type 1 diabetes is about 99% (Eisenbarth *et al.* 1992), IAA have a low predictive value for type 1 diabetes, because of the relatively low sensitivity (Palmer 1987). The presence of IAA tends to be associated with the HLA DR4 alleles (Pugliese *et al.* 1994), probably because of linkage disequilibrim with DQ8 (Gorus *et al.* 1994). It should be noted that HLA D02/D08 Positive patients develop the disease more frequently before

age 15 yr. (Knip *et al.* 1986, Hagopian *et al.* 1995). The low diagnostic sensitivity of IAA among adolescents and adult patients limits their use as a single disease marker for the prediction of type 1 diabetes, but their determination can be useful in combination with other autoantibody assays in order to increase the specificity of the predictive assessment. The 2-year analysis of the German BABYDIAB study (Ziegler *et al.* 1999) followed 1353 offspring of parents with type 1 diabetes and observed that IAA were most frequently the first antibodies to appear. The data from this study suggest that screening for IAA at an early age will be a useful strategy for identifying relatives of patients who are at risk for type 1 diabetes in childhood.

2.4.3.5. Other autoantigens associated with type 1 diabetes

ICA69. The 69kD islet cell antigen (ICA69) was first detected in rat insulinoma cells by cross-reactivity with antibodies to bovine serum albumin (Pietropaolo *et al*. 1993). ICA69 was mapped to human chromosome 7p22. There is structural homology between an ICA69-derived peptide and a fragment of bovine serum albumin (BSA) which was been implicated in the pathogenesis of type 1 diabetes (Martin *et al*. 1991, Karjalainen *et al.* 1992). ICA69 can be detected in brain, testis, pancreas and islet cell lines (Mally *et al.* 1996). The frequency of autoantibodies to ICA69 in sera from pre-diabetic individuals and in patients with recent-onset type 1 diabetes is 20-30% (Pietropaolo *et al.* 1993). Autoantibodies to the ICA69 antigen were also reported in patients with rheumatoid arthritis (Martin *et al.* 1995). An inverse correlation between T cell and autoantibody responsiveness to ICA69 was also observed in patients with newly diagnosed type 1 diabetes (Roep *et al.* 1996).

38kD autoantigen. Several studies have suggested that patients with type 1 diabetic sera react with 38kD islet molecules by humoral (Baekkeskov *et al.* 1982, Pak *et al.* 1990) or by T-cell responces (Roep *et al.* 1991) without evidence that these are the same molecule. One of the 38kD autoantigens is an amphiphilic membrane glycoprotein expressed in islet cells and neuronal cell lines. It was discovered by chance when studying the 64kD (GAD) autoantigen. It is present at a relatively low frequency in children with newly diagnosed type 1 diabetes, with prevalences of 20% in recent-onset patients and around 14% in pre-diabetic first-degree relatives (Chang *et al.* 1996). Although the prevalence of antibodies to the 38kD antigen is low in patients with recent-onset type 1 diabetes, it is possible that the combination of GADA, IA-2 and glima 38 antibodies may yield a diagnostic sensitivity well above 90%. (Aanstoot *et al.* 1996). A 38kD autoantigen has also been detected after infecting human islet cells with cytomegalovirus (Pak *et al*. 1990). In animal models such as BB rats, antibodies to this autoantigen were shown to precede the clinical onset of the disease (Ko *et al.* 1991). Roep *et al*. (1990, 1995) have reported T-cell reactivity against a 38kD protein in patients with newly diagnosed type 1 diabetes. Later this protein was identified as an islet mitochondrial protein present both in beta and alpha cells of the pancreas (Arden *et al.* 1996).

2.5. Prediction of type 1 diabetes

Several strategies have been launched for the prediction of type 1 diabetes. Four principal antibody markers are available: ICA, IAA, GADA and IA-2/IA-2βA. It has been demonstrated that ICA are detected in up to 90% of patients with newly diagnosed type 1 diabetes, with only minor variations with respect to age or sex (Bonifacio *et al.* 1995a). IAA are present in the majority of patients diagnosed under the age of 10 years, but are less prevalent in older patients (Vardi *et al.* 1988). GADA are detected in approximately 75% of patients with newly diagnosed type 1 diabetes, and are more prevalent in patients diagnosed after the age of 10 years, while IA-2A are present in about 60% of patients with recent-onset type 1 diabetes after the age of 10 years and are more prevalent in young subjects. GADA and IA-2A are therefore complementary, and one or the other is detected in more than 90% of patients with newly diagnosed disease (Bonifacio *et al.* 1995a, Christie *et al.* 1997, Kawasaki *et al.* 1997).

The most effective screening test is likely to be a combined GADA/lA-2A antibody test, at least in subjects older than 10 years of age. The advantages of this are that both antibodies can be screened for in the same test (Bonifacio *et al.* 1995b, Wiest-Ladenburger *et al.* 1997) and that the analysis can be performed on whole capillary blood samples equally well as on serum (Bazzigaluppi *et al.* 1999). GADA and IA-2A have been shown to be 88% and 91% predictive of type 1 diabetes, respectively, in a 12-year follow-up study of non-identical twins (Hawa *et al.* 1997). In another follow-up study lasting 5 years the positive predictive values for GADA and IA2A in first-degree relatives were 52% and 81%, respectively (Verge *et al.* 1996). These positive predictive values could be increased if only high-titre autoantibodies were considered.

A second alternative is based on the number of autoantibody markers. Tests on patients with newly diagnosed type 1 diabetes show that two or more of the antibody markers are needed for disease development, and only a few cases of recent-onset disease test positive for just one marker . (Bingley *et al.* 1994, Genovese *et al.* 1994, Aanstoot *et al.* 1996, Verge *et al.* 1996, Christie *et al.* 1997, Gorus *et al.* 1997, Roll *et al.* 1997). The presence of at least two markers is associated with a relatively high probability of disease (50% or more), and this may be even higher within the subsequent 5-7 years in those with three or four antibody markers (Bingley *et al.* 1994, Verge *et al.* 1996, Christie *et al.* 1997, Gorus *et al.* 1997).

The subsequent measurement of IAA and ICA in those selected on the basis of elevated levels of GADA and/or IA-2A in the screening test will provide a useful means of defining type 1 diabetes probability. IAA are essential for assessing the risk of type 1 diabetes in young individuals, but the additional benefit of measuring ICA early in life is not clear. Vice versa, the benefit of measuring IAA in individuals aged 15 years or older is questionable because of their low prevalence in this age group, and here ICA are still essential. Whether ICA should be considered as an additional marker has been questioned (Verge *et al.* 1996) since in some cases they are due entirely to the presence of GADA (Genovese *et al.* 1992, Marshall *et al.* 1994).

The tools available for prediction of type 1 diabetes include genetic markers, autoantibody markers (ICA, IAA, GADA, and IA-2A) and metabolic assessment of beta-cell function (Landin-Olsson *et al.* 1992, Palmer 1993, Bingley 1996). The ideal marker of prediabetes would be negative in the general population and positive in all subjects

who develop type 1 diabetes (100% sensitive and 100% specific), and should display a titre that correlates with the risk (the higher the titre, the higher the risk of developing type 1 diabetes or the shorter the time before its clinical presentation). No perfect marker exists, however, although e.g. high-titre ICA are more predictive of the development of type 1 diabetes than low-titre ICA (Bonifacio *et al.* 1990, Bingley *et al.* 1994).

The hope that type 1 diabetes could be prevented has been the motivation for testing for islet cell autoantibodies, and in this respect ICA (Ganda *et al.* 1984, Bleich *et al.* 1990, Chase *et al.* 1991, Vardi 1991, Schatz *et al.* 1994, Bonifacio *et al.* 1995a), GADA (Luhder *et al.* 1994, Verge *et al.* 1996,) and 1A-2A (Bonifacio *et al.* 1995a, Verge *et al.* 1996) have all been shown to be predictive in relatives of patients. Furthermore, individuals who test positive for more than one marker have an even higher risk of developing type 1 diabetes in the short term. ICA and GADA are early markers of beta-cell autoimmunity, whereas IAA and 1A-2A may predict a more rapid progression to clinical type 1 diabetes. In at least one case of type 1 diabetes that become manifest in infancy, IAA was the first islet autoantibody, being detected by 6 months of age. This was followed by the later appearance of ICA and GADA, between 6 and 9 months, and by presentation with type 1 diabetes at the age of 14 months (Martikainen *et al.* (1996). The best combination and sequence of markers is yet to be agreed upon. Ziegler *et al.* (1990) reported on the basis of a study of 800 relatives of patients that high-titre ICA (at least 40 JDF units) predicted a risk greater than 50% over 5 years of follow-up. Schatz *et al.* (1994) demonstrated that ICA positivity and low FPIR also predict the development of type 1 diabetes. IAA denote an increased risk of development of the disease in relatives under the age of 20 years independent of the risk conferred by ICA (Krischer *et al.* 1993). The risk of unaffected siblings of patients with type 1 diabetes expressing ICA is linked to the number of HLA haplotypes shared with the diabetic sibling. The risk of a HLA-identical non-diabetic sibling testing positive for ICA is about 10%, that in HLA-haploidentical siblings 5.3%, and that in non-identical siblings 2.4% (Lipton *et al.* 1992).

According to Schott *et al.* (1994), measuring GADA in addition to ICA and IAA may not substantially increase the number of marker-positive non-diabetic subjects identified as being at increased risk of developing type 1 diabetes. Thus, GADA increased the predictive value of autoantibody screening for diabetes but did not increase its sensitivity. Positivity for more than one marker does increase the absolute risk of progression to clinical type 1 diabetes, however (Bingley 1996). In an important study by Verge *et al.* (1996), 2000 relatives of patients with type 1 diabetes were screened for GADA, IA-2A and IAA. Irrespective of FPIR, at least one of the three autoantibodies was found in 98% of the 50 relatives who eventually developed type 1 diabetes during the follow-up and two or more in 80%. Viewed prospectively, relatives with two or more autoantibodies had a 39% risk of developing the disease within 3 years and a 68% risk of doing so within 5 years. When an individual tested positive for all three antibodies the risk of progression to type 1 diabetes was estimated to be close to 100%.

Since approximately 90% of the subjects with type 1 diabetes have no affected first-degree relatives at diagnosis, screening should be expanded to the general population. One feasible strategy would be to screen very early in life for high-risk HLA alleles such as DR3/DR4 heterozygosity, DQBI*02 and DQB1*0302 and low-risk alleles such as DQB1*0602 (Deschamps *et al.* 1992). Individuals who are heterozygous for DQB*02 / *0302 may have a risk for type 1 diabetes as high as 1 in 10. In the DAISY (Diabetes

Autoimmunity Study in Youth) project, more than 9000 cord blood samples were tested, of which about 2% came form high-risk DR3/DR4, DQB1*0302 subjects (Rewers *et al.* 1996). The Finnish DIPP study (Hahl *et al.* 1998), which started in 1994, is targeting the general population. All infants born in three university hospitals in Finland are screened for HLA DQB1 markers from cord blood samples and families with a baby carrying HLA DQB1*02/0302 or the *0302/x genotype are invited for immunological surveillance with blood samples taken at intervals of 3-12 months up to the age of 10 years. Subjects who test positive for at least two autoantibodies in two consecutive samples are invited to participate in a randomised intervention trial aimed at assessing whether it is possible to delay or prevent progression to clinical diabetes by daily administration of intranasal insulin.

2.6. Prevention of type 1 diabetes

Prevention has recently become the target of diabetes research. Two aims have been identified, the first is to recognise individuals with a prediabetic process (Winter & Maclaren 1985, Drash 1995) and the second to prevent insulitis and beta-cell necrosis. The identification of beta-cell antigens that are involved in the autoimmune process has resulted in the hypothesis that interruption of this process might prevent type 1 diabetes. Accordingly, it might be possible to suspend antigen presentation by injecting monoclonal or polyclonal mouse-human hybrid antibodies directed against class II MHC molecules **(**Boitard *et al.* 1988). These molecules could be loaded with irrelevant peptides to exclude self-peptides from being presented to the T cells. Nicotinamide was already shown to be an anti-diabetic compound in the 1940's (Lazarow 1947), and this could serve as a free radical scavenger and inhibitor of the DNA repair enzyme polymerase (ADP-ribose) (Dib *et al.* 1987). More recently, nicotinamide has been reported to play a role in preserving residual beta-cell function in patients with newly diagnosed type 1 diabetes (Pozzilli *et al.* 1996a). The European Nicotinamide Intervention Trial (ENDIT) was initiated in 1994 to assess whether daily administration of a high dose of oral nicotinamide can reduce the risk of progression to clinical type 1 diabetes in high-risk first-degree relatives of affected children (Greenbaum *et al.* 1996). The results of the trial should become available during the first half of the year 2003. The German Nicotinamide Trial (DENIS) was terminated in 1988 after it failed to detect an 80% reduction in the incidence of type 1 diabetes through the use of nicotinamide (Lampeter *et al.* 1998).

The most promising immunoprevention strategies involve "tolerization". In such cases, the autoantigen is injected or ingested in an attempt to develop a "suppressive" or "tolerant" immune response (Atkinson *et al.* 1990, Daniel & Wegmann 1996, Muir & Ramiya 1996, Zhang *et al.* 1997). Feeding of insulin to NOD mice has been found to delay and reduce the incidence of autoimmune diabetes (Zhang *et al.* 1991). Treatment with insulin was chosen for the Diabetes Prevention Trial 1 (DPT-l) with relatives of patients with type 1 diabetes as the target population to assess whether parental insulin treatment is able to delay type 1 diabetes in relatives with a disease risk of more than 50%, while oral insulin is used in relatives having a disease risk in the range 25-50% (Rabinovitch & Skyler 1998). A small pilot study in humans showed that subcutaneous

insulin treatment had a preventive effect in high-risk first-degree relatives (Keller *et al.* 1993). Intensive insulin treatment for the first year after the diagnosis of type 1 diabetes has been shown to prolong endogenous insulin secretion (Shah *et al.* 1989).

3. Aims of the present research

The specific aims of this work were:

1. To evaluate the relation between GADA and other autoantibodies, such ICA, IAA, IA-2A in children with newly diagnosed diabetes.
2. To assess the relation between GADA and HLA-defined genetic disease susceptibility in children with newly diagnosed diabetes.
3. To compare the frequency and levels of autoantibodies (GADA, ICA, IAA and IA-2A), genetic risk markers and clinical characteristics in patients with recent-onset type 1 diabetes diagnosed before and after the age of 20 years.
4. To study the association of GADA and other autoantibodies at diagnosis with the clinical characteristics and natural course of type 1 diabetes over the first 2 years of overt disease.

4. Subjects and methods

Table 1. Populations studied in papers I-IV.

Paper	n	Subjects	Age	Topic
I	747	Children with newly diagnosed type 1 diabetes (DiMe study)	8.4 years (range, 0.8-14.9)	GADA in relation to ICA, IAA and HLA DR alleles
II	631	Children with newly diagnosed type 1 diabetes (DiMe study)	8.4 years (range, 0.8-14.9)	GADA in relation to ICA, IAA, IA-2A and HLA DQB1 alleles
III	252 children, 100 adults	Children and Adults with newly diagnosed type 1 diabetes	9.5 years (range, 0.6-19.8), 35.3 years (range, 20.2-61.9)	GADA, ICA, IAA, IA-2, HLA markers and clinical characteristics in relation to age at diagnosis
IV	747	Children with newly diagnosed type 1 diabetes (DiMe study)	8.4 years (range, 0.8-14.9)	GADA, ICA, IA-2A and IAA in relation to clinical characteristics and natural course

The subjects in papers I, II and IV comprised 747 children under 15 years of age (631 in paper II) out of a total of 801 probands (93.3%) in whom type 1 diabetes was diagnosed during the recruitment period of the Childhood Diabetes in Finland (DiMe) Study, from September 1, 1986 to April 30, 1989 (Tuomilehto *et al.* 1992). The mean age of the diabetic children was 8.4 years (range 0.8-14.9 years), and 411of them were boys (55%). The blood samples were taken at diagnosis, before the first insulin injection. Sera were stored at -20°C until analysed.

The population in paper III included 352 patients (215 males) with newly diagnosed type 1 diabetes mellitus admitted to the Departments of Paediatrics and Department of Medicine, Oulu University Hospital, between April 1, 1988 and September 30, 1995. Their mean age was 16.8 years (range 0.6 - 61.9 years), and they included 252 children

and adolescents under the age of 20 years, with a mean age of 9.5 (4.7; SD) years, and 100 adults whose mean age was 35.3 (9.7) years. The hospital serves a total population of approximately 300,000, including about 90,000 persons less than 20 years of age.

4.1. Methods

4.1.1. Clinical assessment

The *degree of consciousness* at diagnosis was assessed by the clinician examining the patient at the time of hospital admission. Consciousness was estimated to be either normal or impaired. The *degree of dehydration* was evaluated from clinical signs and the relative weight loss. A weight loss of 8% was considered to represent severe dehydration in children younger than 12 years of age, while the limit for older subjects was 7%. If there was a discrepancy between the degree of dehydration based on clinical signs and that assessed from weight loss, the former criterion was always used. *Diabetic ketoacidosis* was defined as a capillary or venous blood pH of less than 7.30, since data on ketonaemia was available for a limited number of children. *Clinical remission* was defined as a period characterised by a daily insulin dose of less than 0.5 IU/Kg and a glycated haemoglobin (GHb) value lower than the mean + 3 SD for non-diabetic subjects.

4.1.2. Radiobinding assay for GADA

GADA were measured in paper I, II and IV with a modification of the radiobinding assay described by Petersen *et al.* (1994). Recombinant human islet GAD65 cDNA was transcribed and translated *in vitro* according to the manufacturer's instructions (Promega, Madison, WI, USA). Serum samples (final dilution 1:25) were incubated overnight with approximately 30,000 cpm of ^{35}S-methionine-labelled human GAD65 in a total volume of 50 µl Tris-buffered saline with Tween (TBST). Protein-A-Sepharose (PAS,7.5 mg, Pharmacia, Biotech, Uppsala, Sweden) in a total volume of 100 µl TBST was used to isolate the immunocomplexes. The quantity of precipitated immunocomplexes was counted with a scintillation counter. All the samples were analysed in quadruplicate with and without competition from an excess of unlabelled human recombinant GAD65 (1 µg/ well) produced in an expression system using baby hamster kidney cells and purified with Triton X-114. GADA levels were expressed in relative units (RU) representing the specific binding as a percentage of that obtained with a positive standard serum; relative GADA unit = 100 x cpm (unknown sample) - cpm (unknown sample incubated with an excess of unlabelled GAD65) / cpm (positive standard serum) - cpm (positive standard serum incubated with an excess of unlabelled GAD65). The cut-off limit for antibody positivity was set at the 99th percentile in 372 non-diabetic children and adolescents, i.e. 6.6 RU. The interassay coefficient of variation was less than 10%. The disease sensitivity of the present assay was found to be 76% and the specificity 97% based 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge *et al.* 1998).

In paper III we analysed GADA with a radiobinding assay modified from that described by Bonifacio *et al.* (1995b). The recombinant plasmid pGEM3 encoding the whole 65 kD form of glutamic acid decarboxylase (GAD65) protein was multiplied in *Escherichia coli* JM 109 cell lines. The GAD65 protein was transcribed and translated *in vitro* according to the manufacturer's instructions (Promega) in the presence of ^{35}S-methionine. Serum samples of 2 µl were incubated overnight with 20,000 cpm of labelled GAD65 and the immune complex isolated by 10 µl Protein-A-Sepharose after incubation for 1 hour. The samples were washed five times with 750 µl of TBST buffer and activity was measured with a scintillation counter and the results expressed in RU based on a $\log_{10}/\log_{10}$ standard curve. The limit of positivity was set at the 99th percentile in 373 non-diabetic children and adolescents, i.e. 5.35 RU. The disease sensitivity of this assay was found to be 67% and the specificity 100% based on the 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge *et al.* 1998).

4.1.3. Radiobinding assay for IA-2A

IA-2A was analysed with a radiobinding assay modified from that described by Bonifacio *et al.* (1995b). IA-2A was produced *in vitro* by transcription and translation of the purified plasmid with a TNT-coupled reticulocyte lysate system (Promega) in the presence of ^{35}S-methionine according to the manufacture´s instructions. Serum samples of 2 µl were incubated overnight at +4°C in 96 deep well plates with 10,000 cpm of labelled IA-2 protein diluted in 50 µl of TBST. On the next day 5 µl PAS (Pharmacia Biotech) were added to isolate the immune complexes. The activity of the samples was measured with a scintillation counter (1450 Microbeta Trilux, PerkinElmer life since Wallac, Turku, Finland), and the results were expressed in RU. The cut off limit for antibody positivity (0.43 RU) was set at the 99th percentile in 374 non-diabetic subjects. The interassay coefficient of variation was 12% at an IA-2A level of 0.63 RU, 10% at a level of 21.3 RU and 8% at a level of 82.6 RU. The disease sensitivity of our assay was 62% and the specificity 97% based on 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge *et al.* 1998).

4.1.4. Islet cell antibodies

ICA were determined with a standard indirect immunofluoresence assay using sections of frozen human group O pancreas (Bottazzo *et al.* 1974). End point dilution titres were examined for positive samples and the results were expressed in JDF units relative to an international reference standard (Bonifacio *et al.* 1988). The detection limit was 2.5 JDF units. Our laboratory has participated in international workshops on standardisation of the ICA assay, in which its sensitivity was 100%, specificity 98%, validity 98% and consistency 98% in the fourth round.

4.1.5. Insulin autoantibodies

Serum IAA levels were determined in papers I-III with a radiobinding assay modified from that described by Palmer *et al.* (1983). Endogenous insulin was removed with acid charcoal prior to the assay, and free and bound insulin were separated with polyethelene glycol after incubation with mono- ^{125}I (Tyr A 14)-human insulin (Novo Research Institute, Bagsvaerd, Denmark) for 20 hours in the absence or presence of an excess of unlabelled insulin. The IAA levels were expressed in nU/ml, where 1 nU/ml corresponds to a specific binding of 0.01% of the total counts. The interassay coefficient of variation was less than 8%. A subject was considered to be positive for IAA when the specific binding exceeded 54 nU/ml (99th percentile in 105 non-diabetic subjects). The disease sensitivity of this assay was 26% and the specificity 97% based on 140 samples derived from the 1995 Multiple Autoantibody Workshop (Verge *et al.* 1998).

Serum levels of IAA were quantified in paper IV with a radiobinding assay in a microformat modified from that described by Williams *et al.* (1997). Antibody-antigen complexes were precipitated with PAS (Pharmacia Biotech) after incubation of the serum samples with mono-^{125}I-TyrA14-human insulin (Amersham, Little Chalfont, Bucks, UK) for 72 hours in the absence or presence of an excess of unlabelled insulin. The specific binding represented IAA levels expressed in relative units (RU) based on a standard curve run on each plate using the MultiCalc™ software program (PerkinElmer life since Wallac, Wallac). A subject was considered to be positive for IAA, when the specific binding exceeded 1.55 RU (99th percentile in 371 non-diabetic Finnish subjects). The performance characteristics of this assay were compared to that run in Bristol (Williams *et al.* 1997) based on a blinded sample exchange comprising 100 samples. There was a close correlation between the two assays ($r = 0.96$; $p<0.001$), and the concordance rate was 94%. The disease sensitivity of our microassay was 35% and the specificity 100% based on 140 samples derived from the 1995 Multiple Autoantibody Workshop (Verge *et al.* 1998).

4.1.6. Genetic methods

HLA DR typing (paper 1), was performed using conventional HLA serology as described earlier (Tuomilehto-Wolf *et al.* 1989). During the first year of the research T and B-lymphocytes were isolated by means of a “Percoll” centrifugation gradient. Subsequently immunomagnetic beads (“Dynabeads”, Dynal AS, Oslo, Norway) coated with monoclonal antibodies against either class I or class II antigens were used. The beads were added to cooled, citrated blood, and the rosettes formed were isolated with a magnet, washed, resuspended and then directly added to the test plate, each well of which contained 1 µl well characterised HLA antiserum. After 20-min incubation at room temperature, 2 µl rabbit complement was added to each well, and after a second incubation for 20 min, a mixture of acridine orange and ethidium bromide was added. After further incubation for 15 min the reactions were read under a fluorescence microscope with an excitation filter of 470-490 nm. All HLA A, B, C and DR

specificities recognised by the Nomenclature Committee of the World Health Organisation in 1984 were included in the test panel (Nomenclature for factors of the HLA system 1984).

HLA-DQB1 typing (papers II, III) was performed by a recently described method based on time-resolved fluorescence (Sjöroos *et al.* 1995). The second exon of the DQB1 gene was amplified by PCR and the product subsequently hybridised with four lanthanide-labelled, sequence-specific oligonucleotide probes identifying the following alleles known to be significantly associated with either susceptibility to or protection against type 1 diabetes: DQB1*0302, *02, *0301 and *0602-03. The index cases were classified into four groups based on their genotype: DQB1*0302/02, *0302/x (x = other than *02), *02/y (y = other than *0302) and other DQB1 genotypes. In addition, DiMe patients who were homozygous for *0302 or *02 were identified based on the parental alleles.

4.1.7. Other methods

Endogenous insulin secretion (paper IV). Random serum C-peptide concentrations were analysed with a radioimmunoassay using antiserum K6 (Novo Research Institute, Bagsvaerd, Denmark) as described earlier (Heding *et al.* 1975). The detection limit was 0.02 nmol/l. We have shown previously that there is a close correlation between random postprandial serum C-peptide concentrations, serum C-peptide levels measured 120 min after a standardised breakfast and 24 hr urinary C-peptide excretion (Karjalainen *et al.* 1988).

Metabolic control (GHb). Standard methods were used for blood HbA1 and HbA1c analysis. To make the results obtained in the DiMe study comparable, they were expressed as standard deviation scores (SDS) above the mean in non-diabetic subjects.

4.2. Statistical methods

Differences in the frequencies of autoantibodies were tested with cross-tabulation and Chi-square statistics. The various groups were compared with the t-test (two-tailed) or parametric one-way ANOVA analysis of variance in the case of normal distribution and the Mann-Whitney U-test or Kruskall Wallis one-way analysis of variance in the case of ordinal data. Logarithmic transformations were performed to normalise skewly distributed continuous variables. Age adjustment (papers I, III, IV) was performed with an analysis of covariance. Proportions were age-adjusted by direct standardisation by reference to the age distribution of the whole diabetic population (Morris & Gardner 1989). The Bonferroni adjustment for multiple comparisons was used when appropriate. Multiple regression analyses were performed in paper IV, with antibody positivity and antibody levels as dependent variables and sex, age and DQB1 alleles as independent variables. In all cases a p value less than 0.05 was considered statistically significant.

5. Results

5.1. Relation of GADA to other autoantibodies in subjects with newly diagnosed type 1 diabetes (I, III)

GADA were detected in 547 of the Finnish children with newly diagnosed type 1 diabetes (73.2%; CI 70.0-76.4%). A higher proportion of the girls tested positive for GADA (259/336, 77.1%; CI 72.6-81.6%) than of the boys (288/411, 69.8%; CI 65.6-74.4%, P=0.04), and subjects aged 10 years or older were found to be positive more often than younger ones (Table 2). The proportions of index cases positive for various combinations of autoantibodies are shown in (Fig. 1) The index cases negative for GADA had lower levels of ICA (median 34 vs. 40 JDF units; P=0.003) and of IAA (median 43 vs. 55 nU/ml; P=0.03) than those testing positive. There was no correlation between GADA and ICA levels (r_s=0.02, n=697), or between GADA and IAA (r_s=0.04, n=636). A modest correlation was seen between ICA and IAA (r_s=0.13; P=0.001; n=664).

Table 2. Prevalence (95% CI) of GADA, ICA and IAA in three age groups of children with newly diagnosed type 1 diabetes.

Antibody, (%)	I < 5 years (n = 165)	II 5.0-9.9 years (n = 291)	III 10.0-14.9 years (n=291)	Statistics
GADA	70.9 (63.9-77.9)	68.7 (63.4-74.1)	79.0 (74.3-83.7)	$X^2_{df=2}$=9.13; *p*=0.01 I vs. II; *p*=0.05 II vs. III; *p*=0.01
ICA	86.7 (81.4-91.9)	90.0 (86.6-93.5)	77.0 (72.1-81.8)	$X^2_{df=2}$=19.62; *p*<0.001 I vs. III; *p*=0.02 II vs. III; *p*<0.001
IAA	72.7(65.9-79.6)	44.7(38.9-50.4)	38.8(33.2-44.5)	$X^2_{df=2}$= 50.77; *p*<0.001 I vs. II; *p*<0.001 I vs. III; *p*<0.001

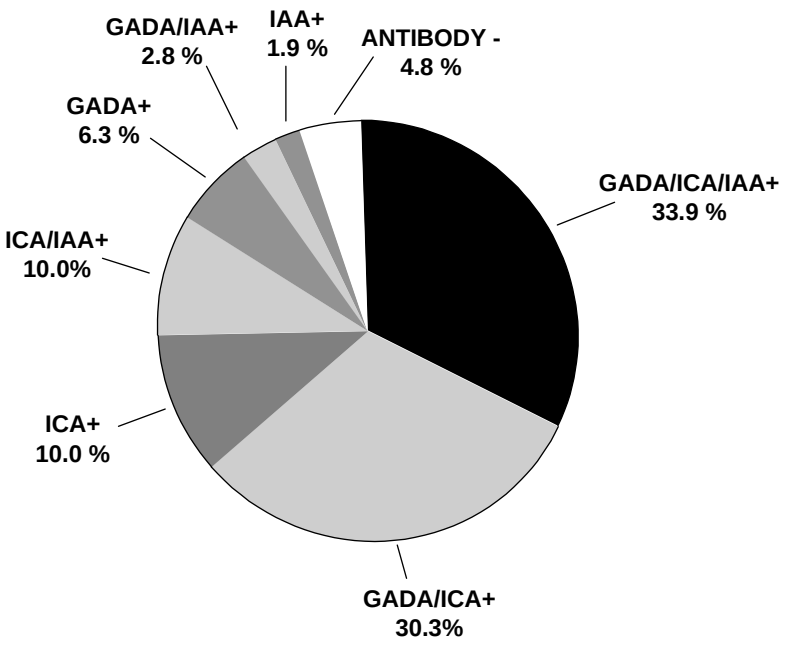

Fig. 1. Proportions of positivity for various autoantibodies in 747 children with newly diagnosed type 1 diabetes.

Multiple autoantibodies (at least two) were observed more often in the girls (81.3%; CI 77.1-85.4%) than in the boys (73.5%; CI 69.2-77.8%, P=0.02) and index cases younger than 5 years of age were more often positive for multiple autoantibodies than those in the age group 5.0-9.9 years or those older than 10 years (83.0%; CI 77.2-88.8%, 77.3%; CI 72.5-82.2%, 73.2%; CI 68.1-78.3% respectively, Chi-square for trends 5.71; P=0.02).

In paper III the children were shown to have a higher frequency of all four autoantibody specificities analysed. The adult patients most frequently tested positive for GADA, while ICA were the most common single autoantibodies in the children, as shown in Table 3. When the patients were divided into age groups at 10-year intervals, there was an overall decreasing frequency of antibodies with age up to 39 years (Fig. 2). The highest frequency of ICA, IAA and IA-2A could be seen in the patients younger than 10 years of age, while those aged 10-19 years. had the highest prevalence of GADA. Only 4% of the children had no detectable antibodies, whereas 30% of the adults tested negative for all four antibodies (Fig. 3). Close to 70% of the children but only one third of the adults tested positive for three or more antibodies. The adult patients who were positive for GADA had higher antibody levels than the GADA-positive children [median 92.8 (interquartile range 34.1-166.2) RU vs. 31.2 (11.8-106.3) RU; P< 0.001].

Table 3. Frequency of disease-associated autoantibodies in children and adults with newly diagnosed type 1 diabetes. Data are n (%).

Antibody	Children (n = 252)	Adults (n = 100)	*p*-value
ICA	212 (84.1)	45 (45.0)	<0.001
IAA	137 (54.4)	20 (20.0)	<0.001
GADA	171 (67.9)	51 (51.0)	0.005
IA-2A	200 (79.4)	48 (48.0)	<0.001
MAA	176 (69.8)	34 (34.0)	<0.001

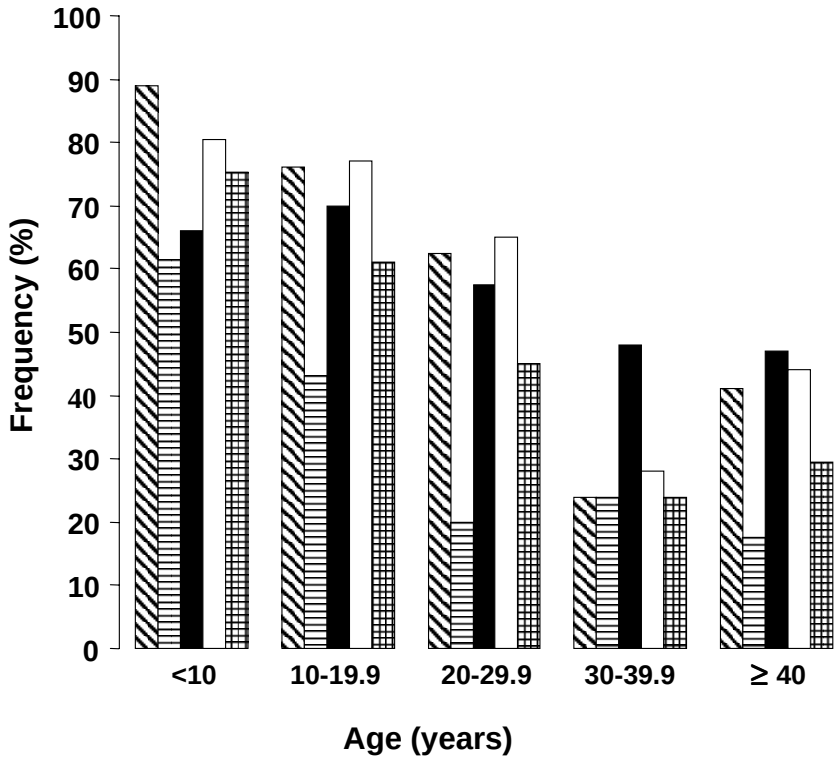

Fig. 2. Frequency of ICA (▨), IAA (▤), GADA (■), 1A-2A (□), and multiple (≥ 3) antibodies (▦) in 352 subjects in relation to age at diagnosis of type 1 diabets mellitus.

Fig. 3. Number of autantibodies at clinical presentation of Type 1 diabetes mellitus in 252 children and adolescents (A) and in 100 adults (B). No (□), one (▨), two (▤), three (▦) and four (■) antibodies.

5.2. Relation of GADA to genetic risk markers (I, II, III)

5.2.1. Relation of GADA and other antibodies to HLA DR phenotypes (I)

It was found in paper I that there were no significant differences in the prevalence of GADA among the newly diagnosed children when classified according to their HLA-DR status (Table 4). The DR2-positive probands tested positive for GADA as often as the other subjects [71.4% (CI 50.4-92.5%) vs. 73.6% (CI 70.4-76.9%]. When the levels of GADA were compared between the phenotypes, DR4/nonDR3 cases were observed to have the lowest levels, while those with DR3/nonDR4 had the highest (Fig. 4).

Table 4. Frequency (95% CI) of GADA and multiple antibodies (at least two antibodies) in 719 children with newly diagnosed type 1diabetes according to their HLA-DR status.

Antibody, (%)	I DR3/4 (n=156)	II DR4/ non DR3 (n=403)	III DR3/non DR4 (n=68)	IV Others (n=92)	Statistics
GADA	77.6 (71.0-84.2)	71.2 (66.8-75.7)	77.9 (67.8-88.0)	73.9 (64.7-83.0)	$X^2_{df=3}$=2.71; p=0.44
Multiple antibodies	83.3 (77.4-89.3)	77.7 (73.6-81.8)	63.2 (51.5-75.0)	78.3 (69.7-86.9)	$X^2_{df=3}$=11.04; p=0.01 I vs. III, p=0.01 II vs. III, p=0.01 III vs. IV, p=0.01

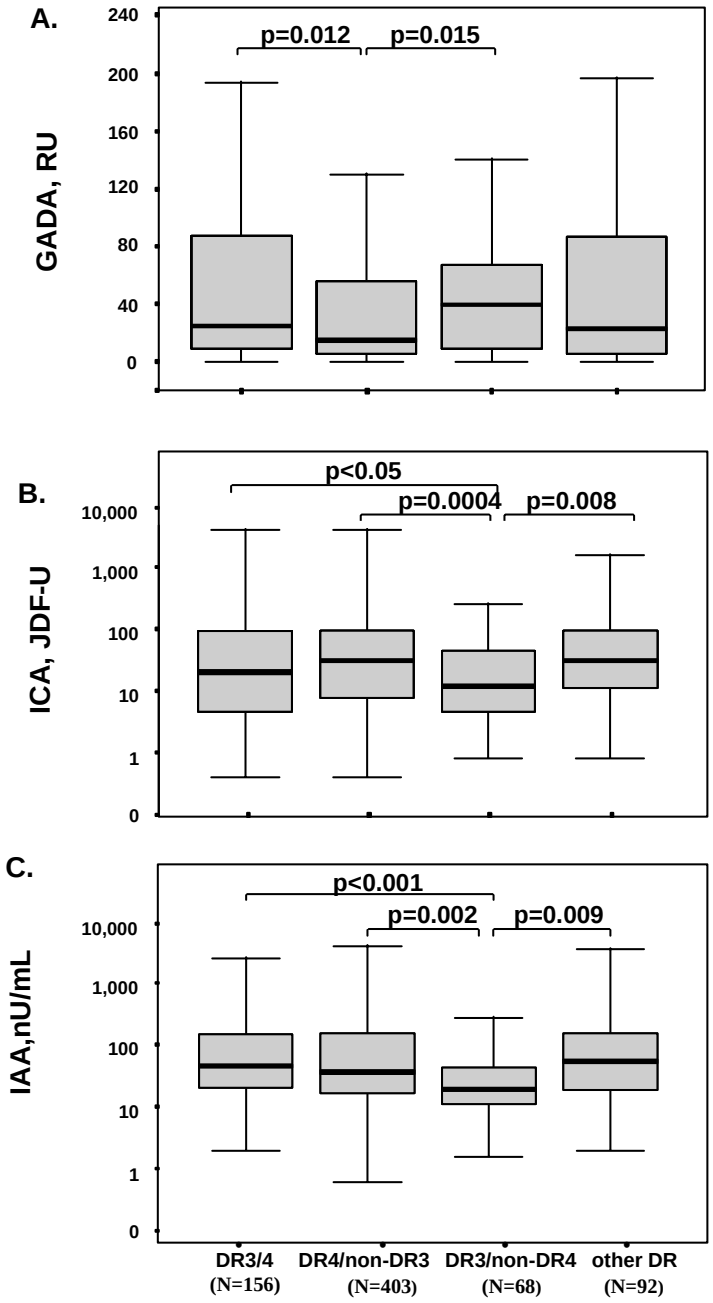

Fig. 4. GADA (A), ICA (B) and IAA levels (C) in 719 children with newly diagnosed type 1 diabetes according to their HLA-DR status. Each box plot represents the median (–) and the 25th and 75th percentiles. The error bars represent the smallest and largrst observed values that are not outliers.

5.2.2. Relation of GADA and other antibodies to HLA DQ genotypes (II, III)

There were no significant differences between the various HLA DQB1 alleles and genotypes in the proportions of children testing positive for GADA in paper II (Table 5), but cases carrying the *02/y genotype had a decreased frequency of IAA and IA-2A and tested less often positive for multiple autoantibodies than those with the other genotypes. The prevalence of IA-2A was particularly low in the 11 patients who were homozygous for DQB1*02 [27.3% (CI 4.1-58.6%) vs. 71.7% (95 CI 62.6-80.7%) among those with the heterozygous *02/y genotype; P<0.001].

*Table 5. Frequency (95% confidence interval; CI) of ICA, IAA, GADA, IA-2A and multiple autoantibodies (MAA; ≥ three antibodies) in children with newly diagnosed type 1 diabetes by HLA DQB1 genotypes. x other than DQB1*02, y other than DQB1*0302.*

Antibody, (%)	I DQB1*02/0302 (n = 152)	II DQB1*0302/x (n = 306)	III DQB1*02/y (n = 110)	IV Other DQ (n = 63)
ICA	82.9 (76.8-88.9)	85.6 (81.7-89.6)	78.2 (70.3-86.0)	88.9 (80.9-96.8)
IAA	54.6 (46.6-62.6)	49.0 (43.4-54.6)	33.6 (24.7-42.6) *	58.7 (46.2-71.2)
GADA	75.7 (68.8-82.6)	71.9 (66.8-78.0)	77.3 (69.3-85.2)	65.1 (52.9-77.1)
IA-2A	81.6 (75.3-87.8)	93.8 (91.0-96.5)	67.3 (58.4-76.1)***	88.9 (80.9-96.8)
MAA	75.0 (68.0-81.9)	75.2 (70.3-80.0)	55.5 (46.0-64.9) ****	73.0 (61.7-84.3)

* $p < 0.01$ (compared with the three other genotypes); ** $p < 0.001$ (compared with DQB1*0302/x; *** $p = 0.05$ or less (compared with the three other genotypes); **** $p = 0.02$ or less (compared with the three other genotypes)

The children carrying the DQB1*0302/x genotypes had higher ICA levels than those with DQB1*02/y (Fig. 5), but lower GADA levels than those carrying *02/0302 or *02/y. Cases who were positive for *02/y had lower IAA and IA-2A levels than the other three genotypes (Fig. 6). The probands carrying the protective DQB1*0602 or *0603 alleles had an increased frequency of ICA (Table 6) and higher antibody levels than the remaining patients (71 vs. 36 JDF units; P=0.007).

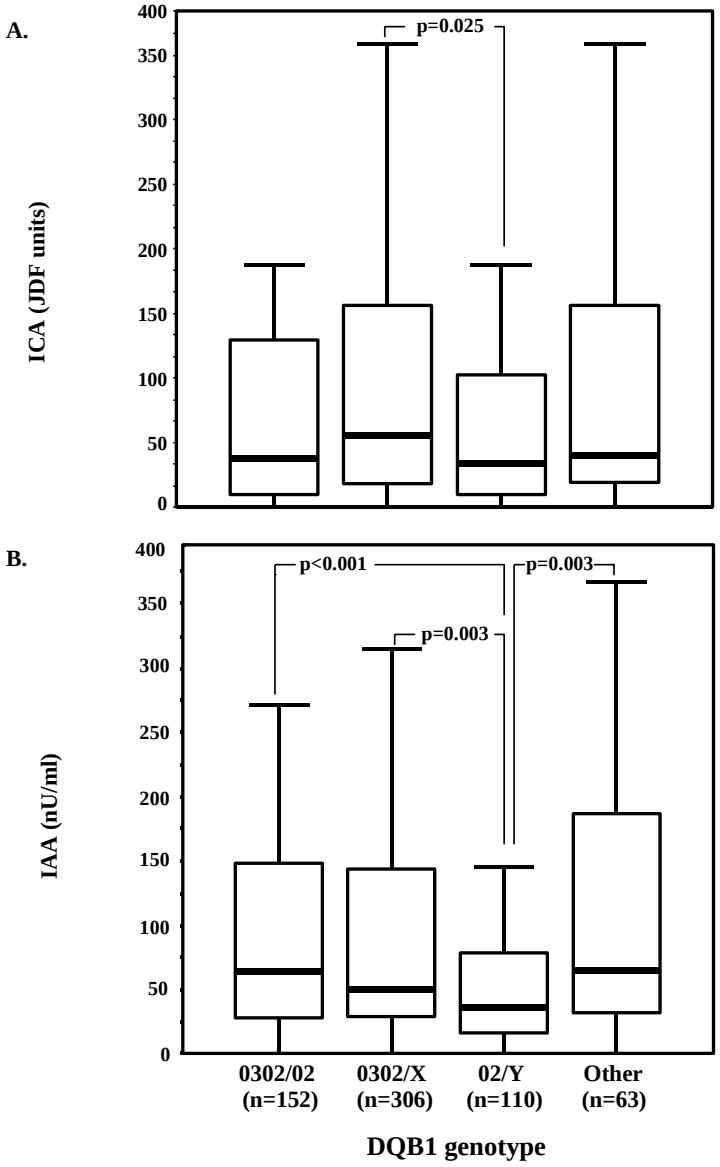

Fig. 5. ICA (a) and IAA (b) levels in 631 children with newly diagnosed type 1diabetes according to their HLA-DQB1 genotypes. Each box plot represents the median (–) and the 25th and 75th percentiles. The error bars represent the smallest and largest observed values that are not outliers.

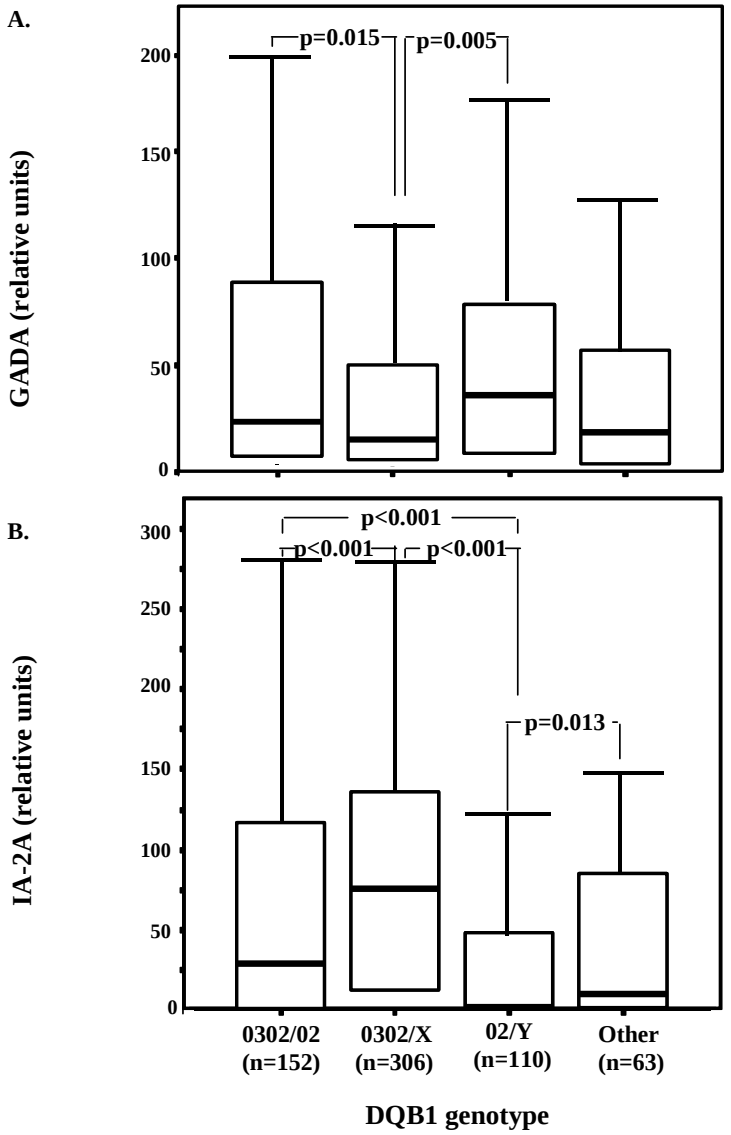

Fig. 6. GADA (a) and IA-2A (b) levels in 631 children with newly diagnosed type 1 diabetes according to their HLA-DQB1 genotypes. Each box plot represents the median (–) and the 25th and 75th percentiles. The error bars represent the smallest and largest observed values that are not outliers.

*Table 6. Frequency (95% CI) of ICA, IAA, GADA, IA-2A and multiple autoantibodies (MAA; ≥ three antibodies) in children with newly diagnosed IDDM who were positive for HLA-DQB1*0602 or*0603.*

Antibody, (%)	DQB1*0602 or *0603 (n = 43)	Other subjects (n = 588)
ICA	95.3 (88.2-100.0)*	83.2 (80.4-86.5)
IAA	56.1 (40.2-72.0)	48.3 (44.1-52.2)
GADA	73.2 (59.0-87.3)	73.4 (69.7-76.9)
IA-2A	87.8 (77.6-98.3)	85.4 (82.5-88.2)
MAA	74.4 (60.8-88.0)	71.3 (67.6-74.9)

* p =0.06 (compared with others)

In a multiple regression model with sex, age and HLA-DQB1 alleles as independent variables, GADA levels were associated with female sex (P<0.001) and age (P<0.001), but not with HLA-DQB1*02. ICA were inversely related to age (P<0.05). There were also inverse relations between IAA and both age (P<0.001) and HLA-DQB1*02 (P<0.05).

In paper III the children with the DQB1*02/y genotype were seen to have a lower frequency of IA-2A than those carrying the DQB1*02/0302 or *0302/x genotypes (Table 7), and the prevalence of IA-2A was also conspicuously low in the adults with the DQB1*02/y genotype, significantly lower than in those carrying the DQB1*0302/x genotype.

*Table 7. Frequencies of ICA, IAA, GADA, IA-2A, and multiple autoantibodies (MAA; ≥ three antibodies) by DQB1genotypes in subjects with newly diagnosed type 1 diabetes under and over the age of 20 years at diagnosis. x other than DQB1*02, y other than DQB1*0302.*

Antibody, (%)	I DQB1*02/0302	II DQB1*0302/x	III DQB1*02/y	IV Other DQ genotype
< 20 years	(*n* = 44)	(*n* = 101)	(*n* = 33)	(*n* = 10)
ICA	79.5	88.1	75.8	100
IAA	61.4	57.4	51.5	40.0
GADA	72.7	67.3	72.7	40.0
IA-2A	86.4‡	89.1§	60.6‡, §	90.0
MAA	70.5	78.2	66.7	70.0
> 20 years	(*n* = 5)	(*n* = 35)	(*n* = 10)	(*n* = 9)
ICA	60.0	71.4‡, #	30.0‡	33.3#
IAA	0.0	31.4	20.0	22.2
GADA	80.0	68.6	60.0	44.4
IA-2A	60.0	71.4ϕ,χ	20.0χ	33.3ϕ
MAA	60.0	57.0	30.0	22.2

‡P = 0.04, §P = 0.001, #P = 0.04, P = 0.02, ϕP = 0.02, χP = 0.02

A similar difference in the frequency of ICA could be seen between these two genotypes among the adult patients. The few individuals who carried the protective DQB1*0602-3 alleles, nine in the total population, had a decreased frequency of IA-2A and multiple antibodies (Table 8).

*Table 8. Frequency (95% CI) of ICA, IAA, GADA, IA-2A and multiple autoantibodies (MAA; ≥ three antibodies) in subjects with newly diagnosed Type 1 diabetes according to HLA DQB1*0602 or*0603 protective alleles.*

Antibody, (%)	DQB1*0602 or *0603 (n = 9)	Other subjects (n = 238)	*p*-value
ICA	55.6 (15.0-96.0)	79.0 (73.8-84.2)	NS
IAA	33.3 (5.1-72.0)	49.6 (43.1-56.0)	NS
GADA	33.3 (5.1-71.8)	68.5 (62.6-74.4)	NS
IA-2A	33.3 (5.1-71.8)	78.6 (73.3-83.8)	0.006
MAA	22.2 (8.9-56.7)	69.3 (63.7-75.6)	0.009

A lower level of IA-2A was seen among the children with the DQB1*02/y genotype than in those positive for DQB1*0302/x (median 25.6 RU vs. 105.5 RU; P=0.002). There was also a significant difference in IA-2A levels between the children with *0302/x and those who were heterozygous for *02/0302 (median 109.8 RU vs. 43.8 RU; P=0.04). Similar differences could be observed among the adults, with the highest IA-2A levels in the patients carrying DQB1*0302/x being significantly higher than the levels in the other patients (median 109.8 RU vs. 6.8 RU; P=0.04).

5.3. GADA and other antibodies in relation to clinical characteristics and natural course of type 1 diabetes (III, IV)

The clinical and biochemical characteristics of the index cases of paper IV who were positive and negative for GADA at diagnosis are presented in Table 9. The patients positive for GADA were older than the negative ones, and the proportion of boys among them was lower. There were no significant differences between the GADA-positive and negative subjects in clinical or metabolic characteristics at diagnosis, but children with IA-2A had lower serum C-peptide concentrations than the antibody-negative ones [mean 0.19 ± 0.14 (SD) nmol/l vs. 0.25 ± 0.18 nmol/l; P=0.003].

No significant differences between the probands who tested positive for GADA at diagnosis and those who were negative were seen in subsequent serum C-peptide concentrations, daily insulin doses or GHb levels during the follow-up period of 2 years. The proportion of children in clinical remission was nevertheless lower among the GADA-positive subjects at 18 months (2.4% vs. 9.5%; P=0.02). Those who had detectable IA-2A initially had lower serum C-peptide levels at 24 months [0.06 ± 0.07

(SD) vs. 0.11 ± 0.18 nmol/L; P=0.045], and they needed more exogenous insulin at 18 months (0.69 ± 0.21 vs. 0.57 ± 0.24 IU/kg/day; P=0.005) and 24 months (0.73 ± 0.21 vs. 0.64 ± 0.21 IU/kg/day; P<0.001).

Table 9. Clinical and biochemical characteristic of type 1 diabetes at diagnosis in cases who were positive or negative for GAD autoantibodies (GADA). Data are n (%) or means ± SD. p-values were obtained after age adjustment.

Characteristic	GADA + (n = 547)	GADA - (n = 200)	*p*-value
Age (yr)	8.6 ± 3.8	7.9 ± 3.6	0.03
Males (%)	288 (52.7)	123 (61.5)	0.04
Diminished consciousness	70 (12.8)	19 (9.5)	0.24
Severe dehydration	71 (13.9) (n = 511)	1 (11.1) (n = 189)	0.40
Ketoacidosis	115 (22.6) (n = 509)	35 (19.1) (n = 183)	0.41
pH	7.34 ± 0.11 (n = 510)	7.35 ± 0.10 (n = 183)	0.16
Blood glucose (mmol/L)	21.4 ± 9.3 (n = 518)	21.2 ± 10.1 (n = 192)	0.78
Glycated hemoglobin (SDS)	13.1 ± 5.8 (n = 397)	12.6 ± 6.0 (n = 142)	0.71
Serum C-peptide (nmol/L)	0.19 ± 0.14 (n = 543)	0.21 ± 0.17 (n = 198)	0.37
Exogenous insulin dose Over the first 24h ($IU \cdot kg^{-1}$)	1.19 ± 0.66 (n = 452)	1.18 ± 0.71 (n = 175)	0.85

Both the children with three or more antibodies and those with one or two antibodies were significantly younger at clinical manifestation than those who tested negative for all four antibodies (Table 10). No significant differences were seen among the three groups in the clinical characteristics or in the degree of metabolic decompensation at diagnosis but the children testing positive for multiple autoantibodies needed higher doses of exogenous insulin than the others at 12, 18 and 24 months (Fig. 7 A) and had lower serum C-peptide concentrations during the second year after diagnosis (Fig. 7 B). A lower proportion of the patients who were positive for multiple autoantibodies were in clinical remission at 12 months (12.4 vs. 22.6%; P=0.008) and at 18 months (2.2% vs. 9.6%, P=0.004) than of the remaining children.

Table 10. Clinical and biochemical characteristics of type 1 diabetes at diagnosis in cases who were positive for multiple (≥ 3 antibodies-AB), 1 or 2 antibodies and in those with no detectable antibodies. Data are n (%) or means ± SD. AB, antibodies; F, F-ratio; df, degree of freedom. p-values were obtained after age adjustment.

Characteristic	I ≥ 3 AB (n = 542)	II 1-2 AB (n = 188)	III No AB (n = 17)	*p*-value
Age (yr)	8.2 ± 3.8	8.8 ± 3.5	11.3 ± 2.6	$F_{df=3}$ = 6.54; $p < 0.001$ I vs. III; $p < 0.001$ II vs. III; $p = 0.002$
Males	296 (54.6)	104 (55.3)	11 (64.7)	$\chi^2_{df=2}$.= 0.69; $p = 0.71$
Diminished consciousness	70 (12.9)	15 (8.0)	4 (23.5)	$\chi^2_{df=2}$.= 5.48; $p = 0.07$
Severe dehydration	70 (13.8) (n = 507)	19 (10.7) (n = 177)	3 (18.8) (n = 16)	$\chi^2_{df=2}$.= 1.54; $p = 0.46$
Ketoacidosis	120 (23.8) (n = 504)	26 (15.1) (n = 172)	4 (25.0) (n = 16)	$\chi^2_{df=2}$.= 5.82; $p = 0.06$
pH	7.34 ± 0.10 (n = 396)	7.35 ± 0.11 (n = 128)	7.29 ± 0.16 (n = 11)	F = 0.21; $p = 0.65$
Blood glucose (mmoI/L)	21.5 ± 9.6 (n = 542)	21.2 ± 9.0 (n = 188)	25.0 ± 13.1 (n = 17)	F = 1.35; $p = 0.25$
Glycated hemoglobin (SDS)	12.9 ± 3.2 (n = 385)	13.1 ± 3.4 (n = 128)	12.6 ± 2.6 (n = 11)	F = 2.24; $p = 0.14$
Serum C-peptide (nmol/L)	0.19 ± 0.14 (n = 396)	0.21 ± 0.15 (n = 128)	0.33 ± 0.26 (n = 11)	F = 2.30; $p = 0.10$
Exogenous insulin dose during the first 24h ($IU \cdot kg^{-1}$)	1.23 ± 0.65 (n = 396)	1.09 ± 0.61 (n = 128)	1.20 ± 0.95 (n = 11)	F = 0.00; $p = 0.96$

Fig. 7. Mean serum C-peptide concentrations (A) and daily insulin doses (B) over the intial 2 yr of type 1 diabetes in children with multiple autoantibodies (■) and in other subjects (□). * P=0.005; ** P<0.001, after adjustment for age and multiple comparisons.

The proportion of males was higher among the adult patients in paper III than among the children (Table 11), and the adults had a longer symptomatic period before diagnosis and had experienced less infections over the 3 months before diagnosis. The relative weight of the children had declined more than that of the adults. There was no significant difference in blood glucose concentrations or serum C-peptide levels at diagnosis, but the children had a higher mean haemoglobin A1c (HbA1c) value than the adult patients, and the males had higher HbA1c values than the females among the adults (median 11.3 vs. 10.0 %; P=0.04). The adult patients had a higher pH and higher bicarbonate and base excess concentrations at diagnosis than the children.

Table 11. Clinical characteristics of children and adults with newly diagnosed type 1 diabetes. Data are n (%) or medians (interquartile range).

Characteristic	Children (n = 252)	Adults (n = 100)	*p*-value
Males (%)	145 (57.5)	70 (70.0)	0.04
Duration of symptoms (wk)	2 (1-4)	5.5 (4-24)	<0.001
Relative body weight (%)	93.0 (87.0-100.5)	96.5 (87.5-110.0)	<0.001
Number of preceding infections	1 (0-2) (n = 195)	0 (0-1) (n = 99)	<0.001
Blood glucose (mmol/L)	20.7 (14.9-25.7) (n = 251)	20.5 (17.8-25.8) (n = 100)	NS
Glycated haemoglobin, HbA1c (%)	12.9 (10.8-15.5) (n = 219)	10.2 (9.0-12.7) (n = 74)	<0.001
Serum C-peptide (nmol/L)	0.14 (0.10 - 0.21) (n = 225)	0.19 (0.11-0.27) (n = 47)	<0.001
pH	7.37 (7.31-7.40) (n = 250)	7.41 (7.39-7.43) (n = 93)	0.02
Bicarbonate (mmol/L)	22.0 (14.2-25.0) (n = 243)	24.5 (22.0-26.7) (n = 91)	0.002
Base excess (mmol/L)	-2.7 (-10.1-0.6) (n = 250)	0.55 (-1.7-2.1) (n = 92)	0.001
Ketonuria (%)	168 (80.0) (n = 210)	78 (79.6) (n = 98)	NS

6. Discussion

6.1. GADA in patients with newly diagnosed type 1 diabetes (I, III)

Our data show that girls more frequently test positive for GADA than boys and older children more often than younger ones. Thus these autoantibodies have characteristics different from both ICA and IAA which are more common among young diabetic children and have not shown any consistent sex preference in children. In agreement with other reports (Schmidli *et al.* 1994b, Bonifacio *et al.* 1995a, Falorni *et al.* 1995c, Vandewalle *et al.* 1995), a high sensitivity was observed for GADA at the clinical onset of type 1 diabetes, with 73.2% of the newly diagnosed patients in the present series testing positive for these antibodies. In our children with newly diagnosed type 1 diabetes the sensitivity of ICA alone was 84 %, and it increased by 9% when GADA was added and by about 5% when IAA was added, while the sensitivity of at least one positive antibody tests out of three was more than 95%. It may be possible to increase the predictive value of GADA by developing epitope-specific assays, since several epitopes associated with type 1 diabetes or the stiff-man syndrome have been identified (Richter *et al.* 1993).

The patients in paper III diagnosed between the ages of 10 and 19 years had the highest frequency of GADA, whereas the adult patients had the highest GADA levels. Others has been observed a higher frequency of GADA in adult patients diagnosed before the age of 40 than in children and adolescents (Vandewalle *et al.* 1995). Taken together, these observations indicate that very young patients tend to mount a weak antibody response to GAD, and that the intensity of the GAD response increases with age. These observations support that GADA are a less sensitive marker of type 1 diabetes than ICA in children younger than 10 years of age and accordingly cannot be employed alone as an optimal screening tool in that age group. Only a combination of autoantibody assays for GADA, IAA and IA2A seems capable of replacing the ICA test in young children.

6.2. Relation of GADA to other autoantibodies and association between autoantibodies and age at diagnosis (I, III)

Data on the relation between GADA and other autoantibodies have been conflicting. In one study GADA were found only in ICA-positive subjects with type 1 diabetes (Martino *et al.* 1991), whereas no close relation was observed by Roll *et al.* (1994) in first-degree relatives. Verge *et al.* (1994) observed a close association between GADA and ICA in their survey of 273 children with newly diagnosed type 1 diabetes. Among ICA positive first-degree relatives, 78% were GADA-positive up to 4 years before the clinical onset of diabetes (Zimmet *et al.* 1994). Although a significant qualitative association was noted between these two antibodies in the present patients, no significant correlation was obseved between GADA levels and ICA titres after excluding the double negative cases. This observation, in combination with the finding that about one fifth of the ICA positive subjects (20.2%) tested negative for GADA, indicates that GAD does not necessarily constitute the major antigen for ICA in subjects with newly diagnosed type 1 diabetes.

The children and adolescents in paper III definitely had a more frequent and stronger humoral immune response to various beta-cell antigens than the adult patients did, even though the adults who were positive for GADA had higher antibody levels than the GADA-positive children. The higher prevalence of IAA in young patients is well documented (Karjalainen *et al.* 1986, Vardi *et al.* 1988, Vandewalle *et al.* 1995), and in the present series the IAA frequency was seen to drop from 62% in those under the age of 10 years to 20% in the adult patients. The latter group showed no further decrease in the prevalence of IAA with increasing age, however. According to our results ICA were significantly more often detectable in the children than in the adults in the present population which is in line with a German study reporting an ICA frequency of 83% among patients diagnosed with type 1 diabetes before the age of 40 but only 46% among older patients (Lohmann *et al.* 1997).

6.3. Relation of GADA and other autoantibodies to genetic risk factors (I, II)

There is linkage disequilibrium within the MHC region, particularly between the alleles of the HLA loci. Accordingly certain allele combinations are found more often than would be expected on the basis of their allele frequencies at the population level. As a consequence of this phenomena DR4 and DQB1*0302 are always present together, while a majority of those carrying DQB1*02 also have DR3. The result on the relation between GADA, other autoantibodies and HLA DR alleles showing high IAA and ICA levels in DR4-positive index cases supports previous observations of an association between these two antibodies and the DR4 allele (Vexiau *et al.* 1988, Ziegler *et al.* 1991). In contrast to ICA and IAA, GADA were found at the highest levels in index cases carrying DR3. This indicates that GADA expression is regulated genetically in a way different from that of ICA and IAA. Multiple antibodies were more frequently found in DR3/4 heterozygous patients. It is known that there is an over-representation of DR3/4 heterozygous subjects among young children with newly diagnosed type 1 diabetes as compared with

adolescents and adults with recent-onset disease (Karjalainen *et al.* 1989). These observations support the concept that a strong genetic susceptibility is associated with aggressive, rapidly progressing beta-cell destruction, as reflected by marked ICA and IAA responses and clinical manifestation of type 1 diabetes at a young age, while a weaker genetic predisposition results in a slower destructive process and disease presentation in adolescence or later.

The findings in paper II indicate that the HLA-DQB1 genotype may have a modifying influence on the expression of disease-associated autoantibodies, but there is no clear evidence of a simple linear relation between genetic disease susceptibility and the intensity of the humoral immune response to beta-cell antigens, although we observed a decrease in the prevalence and levels of IAA from the high risk *0302/02 genotype down to the low risk *02/Y genotype. This trend was diffracted by a higher frequency and increased levels of IAA in children carrying neutral or protective genotypes. Patients with strong genetic disease protection had both an increased prevalence and high levels of ICA, suggesting that the genetic disease protection can be broken by a strong autoimmune reaction.

According to our observations and those of others (Hagopian *et al.* 1995, Genovese *et al.* 1986) there are differences in the autoantibody profile between those carrying the DR4/DQB1*0302 haplotype and cases with theDR3/ DQB1*02 haplotype/Y genotype, the former being characterised by increased IAA and IA-2A concentrations but low GADA levels, whereas low IAA and IA-2A levels and increased GADA concentrations are typical for the latter cases. The most novel observation in the present survey is the conspicuously low frequency and levels of IA-2A in the few patients who were homozygous for DQB1*02 indicating that this trait carries relatively strong protection against an antibody response to the IA-2 molecule.

6.4. Relation of autoantibodies to clinical characteristics of type 1 diabetes (IV, III)

The results regarding the relation between GADA and clinical characteristics in paper IV indicate that positivity for GADA has no impact on the degree of metabolic decompensation at clinical presentation with type 1 diabetes, nor was any association found between initial GADA positivity and residual beta-cell function after diagnosis. These findings are consistent with those reported in a Swedish survey (Örtquist *et al.* 1997). In contrast, Petersen *et al*. (1994) reported a lower glucagon-stimulated C-peptide response during the first year of clinical disease in young adult patients initially positive for GADA than in antibody-negative ones. This observation suggests that GADA positivity at the diagnosis of type 1 diabetes predicts a more rapid progression to total beta-cell destruction. On the other hand, GADA have been reported to remain detectable for a long period of time after the clinical manifestation of diabetes (Christie *et al.* 1990). The persistence of GADA in affected subjects without any signs of endogenous insulin secretion (Zanone *et al.* 1994, Savola *et al.* 1998b) suggests that their production may be

stimulated at least partly by an antigen other than beta-cell derived GAD, possibly reflecting immune stimulation by small amounts of extrapancreatic GAD or by cross-reactive exogenous antigens.

There are no previous data on the possible relation between IA-2A and the metabolic state at diagnosis or the clinical course thereafter in children with type 1 diabetes. In paper IV the affected children testing positive for IA-2A at diagnosis had lower serum C-peptide concentrations than the IA-2A-negative ones both initially and after an interval of 2 years. This implies that IA-2A may to some extent reflect beta-cell destruction.

The critical issue in paper III is whether the adult patients really have type 1 diabetes. The arguments in favour of this are based on observations that all the adult patients had typical symptoms and signs of the disease before diagnosis, that they had severely decreased endogenous insulin secretion, as reflected in serum C-peptide concentrations similar to those seen in the children and adolescents, that with the exception of 20 subjects with a relative body weight $\geq$ 120%, they were not obese, and that they remained insulin-dependent for at least 12 months after diagnosis. Our results imply that beta-cell destruction progresses more slowly in those presenting with the disease after the age of 20. Accordingly, the age at clinical onset of type 1 diabetes may be determined by the intensity of the beta-cell destructive process, which may be modulated by both genetic and environmental factors.

6.5. Impact of GADA on the prediction of type 1 diabetes

Bingley *et al.* (1994) reported that combined analysis of autoantibodies improves the prediction of type 1 diabetes. Similarly, it has been reported that the combination of GADA with high-titre ICA can increase the predictive value from 66% to 75% in first-degree relatives (Thivolet *et al.* 1992). Aanstoot *et al.* (1994) suggested that the combination of ICA and GADA could increase the positive predictive value for type 1 diabetes in the general population from 50% for ICA alone to 67%. In our population the sensitivity of ICA alone was 84 %, and it increased by 9% when GADA was added and by about 5% when IAA was added, given that the sensitivity of at least one positive antibody test out of the three was more than 95%. It may be possible to increase the predictive value of GADA by developing epitope-specific assays, since several epitopes associated with type 1 diabetes or the stiff-man syndrome have been identified (Richter *et al.* 1993). Epitope-specific antibodies associated with protection against type 1 diabetes have also been described (Ujihara *et al.* 1993).

7. Conclusions

The following conclusions can be drawn from the present results:

1. Humoral autoimmunity against GAD among patients with newly diagnosed type 1 diabetes is more common in females and subjects older than 10 years of age. GADA are a less sensitive marker of type 1 diabetes than ICA in children younger than 10 years of age and accordingly cannot be employed alone as an optimal screening tool in that age group.
2. The genetic regulation of GAD autoimmunity appears to be different from that of ICA and IAA, since HLA DR3/nonDR4 subjects had increased GADA levels. HLA DQB1*0302, the most important single susceptibility allele for type 1 diabetes, is associated with a strong antibody response to IA-2 and insulin, while GAD-specific humoral autoimmunity is linked to the DQB1*02 allele. IA-2A may represent beta-cell specific autoimmunity, while GADA seems to represent a propensity to general autoimmunity.
3. Strong genetic susceptibility is associated with aggressive, rapidly progressing beta-cell destruction, as reflected in strong ICA and IAA responses and clinical manifestation of type 1 diabetes at a young age, whereas a weaker genetic predisposition results in a slower destructive process and disease presentation in adolescence or later. A strong antibody response to GAD65 seems to be characteristic of the latter disease variant.
4. Positivity for GADA has no impact on the degree of metabolic decompensation at clinical presentation with type 1 diabetes, while IA-2A may to some extent reflect beta-cell destruction.
5. Positivity for multiple diabetes-related autoantibodies is associated with accelerated beta-cell damage, resulting in rapid progression to clinical diabetes and leading to total beta-cell destruction after the diagnosis.

6. Clinical manifestation of type 1 diabetes before the age of 20 years is associated with a strong HLA-defined genetic disease susceptibility, an aggressive humoral immune response to various beta-cell antigens, a higher frequency of preceding infections and a shorter duration of symptoms and more severe metabolic decompensation at diagnosis.
7. The age at clinical onset of type 1 diabetes is determined by the intensity of the beta-cell destruction process, which is modulated by both genetic and environmental factors.

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