TEIJA KIMPIMÄKI

Clinical Significance of Autoantibodies Associated with Type 1 Diabetes in Young Children

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine of the University of Tampere, for public discussion in the small auditorium of Building K, Medical School of the University of Tampere, Teiskontie 35, Tampere, on March 22nd, 2002, at 12 o’clock.

Acta Universitatis Tamperensis 859
University of Tampere
Tampere 2002
CONTENTS

CONTENTS 3
LIST OF ORIGINAL PUBLICATIONS 4
ABBREVIATIONS 5
INTRODUCTION 6

REVIEW OF THE LITERATURE 7
  EPIDEMIOLOGY OF TYPE 1 DIABETES 7
  GENETICS OF TYPE 1 DIABETES 8
  THE ROLE OF ENVIRONMENTAL FACTORS IN THE ETIOLOGY AND PATHOGENESIS OF TYPE 1 DIABETES 9
  AUTOIMMUNITY IN TYPE 1 DIABETES 18
  PREDICTION OF TYPE 1 DIABETES 26
  PREVENTION OF TYPE 1 DIABETES 35

AIMS OF THE PRESENT RESEARCH 39

SUBJECTS AND METHODS 40
  SUBJECTS 40
  METHODS 43

RESULTS 46
  EMERGENCE OF AUTOANTIBODIES (I, III) 46
  RELATION BETWEEN HLA-DQB1 GENOTYPE AND APPEARANCE OF AUTOANTIBODIES (I, III, IV) 47
  RELATION BETWEEN ENVIRONMENTAL FACTORS AND APPEARANCE OF AUTOANTIBODIES (I, II) 50
  TRANSIENT AND FLUCTUATING ANTIBODY PATTERNS (I, III, IV) 51
  GENETIC DISEASE SUSCEPTIBILITY AND AUTOANTIBODIES AS PREDICTIVE MARKERS OF TYPE 1 DIABETES IN YOUNG CHILDREN (I, III, IV) 56

DISCUSSION 58
  INDUCTION OF BETA-CELL AUTOIMMUNITY 58
  THE DEVELOPMENT OF BETA-CELL AUTOIMMUNITY 60
  IDENTIFICATION OF SUBJECTS WITH A HIGH RISK OF TYPE 1 DIABETES 61
  FUTURE PROSPECTS 63

SUMMARY 64

ACKNOWLEDGEMENTS 66

REFERENCES 68

ORIGINAL PUBLICATIONS 93
LIST OF ORIGINAL PUBLICATIONS

In addition to the material contained in the original papers listed below, some previously unpublished data are presented in this thesis.


### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD4</td>
<td>cell surface glycoprotein, usually on helper T cells, which recognizes MHC class II molecules on antigen-presenting cells</td>
</tr>
<tr>
<td>CI</td>
<td>95% confidence interval</td>
</tr>
<tr>
<td>DiMe</td>
<td>Childhood Diabetes in Finland Study</td>
</tr>
<tr>
<td>DIPP</td>
<td>Type 1 Diabetes Prediction and Prevention project</td>
</tr>
<tr>
<td>FPIR</td>
<td>first-phase insulin response</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GADA</td>
<td>antibodies to GAD65</td>
</tr>
<tr>
<td>GAD65</td>
<td>65 kilodalton isoform of glutamic acid decarboxylase</td>
</tr>
<tr>
<td>HLA</td>
<td>human leukocyte antigen</td>
</tr>
<tr>
<td>IAA</td>
<td>insulin autoantibodies</td>
</tr>
<tr>
<td>IA-2</td>
<td>islet tyrosine phosphatase (ICA512) molecule</td>
</tr>
<tr>
<td>IA-2A</td>
<td>antibodies to the protein tyrosine phosphatase-related IA-2 molecule</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell antibodies</td>
</tr>
<tr>
<td>ICA69</td>
<td>islet cell antigen with a molecular weight of 69 kD</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>JDFU</td>
<td>Juvenile Diabetes Foundation Units</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PPV</td>
<td>positive predictive value</td>
</tr>
<tr>
<td>RU</td>
<td>relative units</td>
</tr>
</tbody>
</table>
INTRODUCTION

Diabetes mellitus is a chronic glucose metabolism disorder involving increased mortality and morbidity from a variety of end-organ complications, including accelerated atherosclerosis, retinopathy, nephropathy and neuropathy. Its most common form, type 2 diabetes, is usually diagnosed after the age of 30 years and its expression involves various genotypes, phenotypes and etiologies. Most cases of type 2 diabetes are associated with insulin resistance, obesity and the metabolic syndrome.

Type 1 diabetes is characterized by elevated blood glucose levels induced by selective destruction of the insulin-producing pancreatic beta cells. Most new cases are diagnosed under the age of 15 years. The incidence of childhood type 1 diabetes in Finland is the highest in the world, and has gradually increased more than fourfold since the early 1950s, when the first reliable data were published. Only little is known about the pathogenesis of this multifactorial immune-mediated disease and the etiological events that initiate it. Although genetic factors definitely predispose individuals to the disease, most new cases are diagnosed in children with no affected first-degree relatives (Dahlquist et al. 1989, Tuomilehto et al. 1992), and thus environmental factors are thought to contribute to the development of the disease in interaction with genetic factors. The long preclinical period has facilitated promising strategies aimed at delaying or preventing the onset of clinical type 1 diabetes, but so far our knowledge of the initiating events is limited. An improved knowledge of the preclinical process involved in the disease would be essential in order to identify children from the general population who have a high risk of progression to type 1 diabetes and to develop effective preventive strategies for this lifelong disease.
REVIEW OF THE LITERATURE

Epidemiology of type 1 diabetes

No country has escaped type 1 diabetes, although the disease incidence varies considerably from one country to another (Green et al. 1992, Karvonen et al. 2000). The highest incidences in the world in children <15 years of age, ≥20/100,000 per year have been reported from Finland, Sardinia, Sweden, Norway, Portugal, the U.K. and Canada, and the lowest, <1/100,000 per year from China and South America (Karvonen et al. 2000). Only sparse data are available from Asia, Africa and South America, however. The annual incidence of type 1 diabetes in Finland in 1998 was 48.5/100,000 (Podar et al. 2001), but the figure in Estonia, a geographically and genetically closely related country, is less than one third of this (Karvonen et al. 2000).

An increase in the incidence of type 1 diabetes has been observed in a number of countries in recent times (Patterson et al. 1983, Stewart-Brown et al. 1983, reviewed by Bingley and Gale 1989, Tuomilehto et al. 1999), and this effect has been almost linear in Finland since the 1960’s, so that approximately a four-fold increase took place from 1953 to 1996 (Tuomilehto et al. 1999). The comparison of changes in incidence rates between countries is problematic, since variable ascertainment and methodological differences easily result in biases (reviewed by Bingley and Gale 1989), and this led the World Health Organization (WHO) to start its Multi-national Project for Childhood Diabetes (DiaMond) in 1990, with the initial aim of surveying the incidence of type 1 diabetes worldwide (Karvonen et al. 2000).

Considerable variation has been observed in the incidence rates within individual countries in the case of China, Italy and Portugal (Karvonen et al. 2000), but these observations may be biased by the small numbers of cases concerned and the short observation periods. No conspicuous variation has been seen in the incidence of type 1 diabetes between the provinces of Finland (Tuomilehto et al. 1992), possibly due to the fairly homogeneous population or a consistent distribution of environmental exposure to type 1 diabetes.

A slight male predominance in the incidence of type 1 diabetes has been observed in high-incidence countries (Bloom et al. 1975, Muntoni et al. 1992, Tuomilehto et al. 1992, Gardner et al. 1997, Karvonen et al. 2000), a trend that has been observed to be more obvious after puberty (Blohmé et al. 1992). The highest incidence has been reported in early puberty (Christau et al. 1977, Patterson et al. 1983, Green et al. 1992), but the recent increase has been most conspicuous in the age group under 5 years, even though diagnosis of the disease is still fairly

Genetics of type 1 diabetes

**Human leukocyte antigen (HLA) genes**

Susceptibility to type 1 diabetes is polygenic (Anderson et al. 1983). The class II genes of the major histocompatibility complex (MHC) on the short arm of chromosome 6 either predispose individuals to it or protect them from it (Todd et al. 1987). Human leukocyte antigen (HLA) genes have been estimated to explain more than 50% of the heritability of type 1 diabetes (Rotter and Landaw 1984), and the HLA class II D locus was found to be associated with type 1 diabetes in the early 1980’s (Sachs et al. 1980, Platz et al. 1981). The HLA D region is composed of several subregions, including HLA-DR, DQ and DP, each containing A and B genes, which encode for the α and β chains, respectively (Todd et al. 1988). DR4 and/or DR3 alleles are present in 95% of Caucasian patients with type 1 diabetes, compared with a prevalence of 50-60% in the general population (Rotter and Landaw 1984, Todd et al. 1987). Among the HLA class II loci, HLA-DQ genes that are in linkage disequilibrium with HLA-DR show the closest association with the disease (Böhme et al. 1986), and there is a strong linkage disequilibrium between the various loci in the HLA region, so that DR4, for example, is usually present together with DQ8 and DR3 together with DQ2 (Jenkins et al. 1991).

The DQ2 allele has been found to be neutral in relation to the risk of contracting type 1 diabetes unless it is paired with the high-risk DQ8 allele (Baisch et al. 1990). Almost half of all Finnish patients with type 1 diabetes (44%) carry the high-risk allele combination DQ2/8 (Reijonen et al. 1991). The highest risk among Caucasian populations is associated with heterozygosity for DQB1*02 and *0302, i.e. DR3-DQ2/DR4-DQ8 (Rønningen et al. 1991, Ilonen et al. 1996), whereas DQB1*0302 alone, in the absence of protective alleles such as DQB1*0602 and *0603, is associated with moderate risk (Ilonen et al. 1996). The frequency of the high-risk haplotype DQB1*02/*0302 varies between populations, but does not correlate directly with the incidence of type 1 diabetes (Rowe et al. 1994). DQA genes have also been thought to play an active role in diabetes susceptibility, and the combination of DQA and DQB genes is evidently important in this respect (Owerbach et al. 1988, Jenkins et al. 1991). It has also been shown that the DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 combination is the most specific genetic marker of the disease (Heimberg et al. 1992).

The mechanisms of HLA-conferred disease susceptibility and protection have remained open, although various hypotheses have been proposed. It has been proposed that susceptibility to type 1 diabetes and protection from it may arise from individual affinities in the interaction
between a diabetogenic peptide and various class II molecules (Nepom 1990). Another model suggests that the HLA effect may depend on the failure of the immune system to maintain tolerance to pancreatic beta cells (reviewed by Sheehy 1992).

Other susceptibility genes

Although genetic susceptibility to type 1 diabetes has been thought to be primarily associated with DQ molecules, several other genes, located both inside and outside the MHC region, have been identified as being able to modify this susceptibility (Davies et al. 1994), among which the insulin gene region on chromosome 11p is the best-characterized (Owerbach et al. 1980, Davies et al. 1994).

The role of environmental factors in the etiology and pathogenesis of type 1 diabetes

Twin studies

Only a minority of genetically susceptible individuals develop clinical disease (Deschamps et al. 1992, Rewers et al. 1996). Based on data from the American Diabetes Autoimmunity Study in the Young (DAISY) it was estimated that at least 30% of the general population have some degree of increased genetic risk of type 1 diabetes, but only about 0.5% develop the disease. This suggests that environmental factors play an important role in its pathogenesis. The relatively low concordance rate in monozygotic twins, approximately 20-50%, also supports the importance of environmental factors in the etiology of type 1 diabetes (Kaprio et al. 1992, Kyvik et al. 1995, Verge et al. 1995). Although even identical twins may be genetically different with respect to certain genes (reviewed by Tonegawa 1983), somatic mutations cannot explain this lack of concordance (reviewed by Leslie and Elliott 1994). Monozygotic twin siblings of patients with type 1 diabetes have been reported to have a higher risk of progressing to type 1 diabetes and of developing diabetes-associated autoantibodies than dizygotic twin and non-twin siblings (Redondo et al. 1999), suggesting that islet cell autoimmunity is predominantly genetically determined, but this is contradicted by another report that there is no difference in the observed prevalence of islet cell autoantibodies between unaffected dizygotic and monozygotic twins of patients with type 1 diabetes (Petersen et al. 1997), a finding that was interpreted as providing strong evidence for beta-cell autoimmunity being environmentally rather than genetically determined. The discrepancy between these two twin studies may be due to differences in the populations or in the assays used for the detection of autoantibodies. The nature of the environmental factors that putatively induce the pathogenetic process entailed in the disease remains to be defined, however.
**Geographical distribution**

A coarse polar-equatorial gradient in the incidence of type 1 diabetes between the countries of the world was proposed in the 1980’s, with the highest incidence in northern Europe (Vaandrager et al. 1984, Anonymous 1988), and similarly a close association was observed between the incidence of type 1 diabetes and the average annual temperature. This north-south gradient has been disputed, however, as several exceptions can be observed, the most striking being Sardinia, where the incidence is the second highest in the world (Green et al. 1992), and in any case the correlation between incidence and latitude has later been shown to be weaker than was earlier assumed (Karvonen et al. 2000). On the other hand, the exceptions may be attributed directly to risk factors other than climatic effects (Muntoni et al. 1992).

Seasonal variation in the incidence of type 1 diabetes was first reported in 1926 (Adams 1926), and was later confirmed in a number of surveys (Gamble and Taylor 1969, Christau et al. 1977, Rewers et al. 1987), but not all (Tull et al. 1991, Ramachandran et al. 1996). The highest frequency is regularly observed in autumn and winter, with the lowest in summer. In addition to findings in man, the incidence of the disease has been observed to be reduced at raised temperatures in non-obese diabetic (NOD) mice, a spontaneous model for autoimmune diabetes (Williams et al. 1990). The seasonal variation in the incidence of type 1 diabetes could be due to viral infections. A cold environment could also contribute to the development of the disease by increasing the secretion of counterregulatory hormones, thereby increasing the peripheral need for insulin (Fahlén et al. 1971). The fact that no seasonal variation in incidence has been observed in the youngest age groups in some studies (Patterson et al. 1983, Vaandrager et al. 1984) might be explained by an aggressive process in the development of the disease in young children. Also, the disease process in young children may also be more strongly regulated by non-seasonal or non-environmental factors than that in older children.

The association between population density and the incidence of type 1 diabetes is controversial. The incidence is reported to be higher in urban areas than in rural ones both in Poland and in Novosibirsk, Russia (Rewers et al. 1987, Shubnikov et al. 1992), but it has also been reported that the incidence is higher in sparsely populated rural areas than in urban areas (Patterson et al. 1983). These contradictory findings emphasize the possible importance of socio-demographic and/or environmental factors in the etiology of type 1 diabetes.

**Temporal variation in the incidence of type 1 diabetes**

The incidence of type 1 diabetes in Poland almost doubled within 3 years in the 1980’s (Rewers et al. 1987), an effect which the authors attributed to environmental factors. A similar rapid, epidemic-like increase in the incidence of this disease was also observed in Sweden in
the early 1980’s and in the Virgin Islands (USA) in 1984 (Tull et al. 1991).

Migrant studies

The increased incidence of type 1 diabetes reported in offspring of parents migrating from an area with a low incidence to an area where the incidence is high suggests that environmental factors play an important role in its pathogenesis (Bodansky et al. 1992). When children of Asian origin migrated to Britain, for example, or Polynesians from Western Samoa, with a low incidence of type 1 diabetes, migrated to New Zealand, the incidence of the disease increased to close to that of the host country, with no corresponding change in the incidence rate in the country of origin (Elliott and Martin 1984, Bodansky et al. 1992).

Nutritional factors

Early infant feeding

The role of breastfeeding and early exposure to cow's milk in the etiology of type 1 diabetes has remained controversial. Experiments conducted in 1985 with diabetes-prone rats, which spontaneously develop type 1 diabetes, demonstrated clearly that modification of the dietary protein source could affect the risk of developing autoimmune diabetes (Elliott and Martin 1984, Scott et al. 1985). Cow's milk casein has been thought to trigger diabetes in NOD mice, whereas the early introduction of casein hydrolysate was found to provide protection against the disease (Elliott et al. 1988). These results were later confirmed by Coleman et al. (1990).

An inverse relationship between the duration of breastfeeding and the development of type 1 diabetes was reported for the first time in Norway and Sweden in 1984 (Borch-Johnsen et al. 1984), and ten years later a short duration of breastfeeding was found to be associated with a weak, i.e. a 1.4-fold, risk of type 1 diabetes in a meta-analysis of 13 retrospective studies (Gerstein 1994). However, if the increase in the incidence of type 1 diabetes had been caused only by changes in breastfeeding patterns, a decrease could be expected with time in countries where the frequency of breastfeeding has increased (Dahlquist and Mustonen 1994).

A positive ecological correlation has been observed between cow's milk consumption and the incidence of type 1 diabetes at the population level (Scott 1990, Dahl-Jorgensen et al. 1991). Ecological studies have been criticized as being based on population rather than individual dietary data, and for the fact that possible confounding factors cannot be taken into account (reviewed by Gerstein and VanderMeulen 1996), so that they may overestimate this association. But it is the case that the amount of cow's milk consumed during childhood has been shown to correlate positively with the risk of type 1 diabetes (Verge et al. 1994, Virtanen et al. 1998, Virtanen et al. 2000). Early exposure to cow's milk was first reported to increase the risk of devel-
oping this disease in 1991 (Virtanen et al. 1991). Thus, even though a high intercorrelation exists between age at exposure to cow's milk and duration of exclusive breastfeeding, the early introduction of cow's milk has been observed to be a risk factor for type 1 diabetes independently of the duration of breastfeeding (Virtanen et al. 1993). In one case-control study an association between early exposure to cow's milk and an increased risk of type 1 diabetes was found only in genetically susceptible individuals (Kostraba et al. 1993). Early introduction of cow's milk resulted in a relative risk of 1.6 in the first meta-analysis of retrospective studies, published by Gerstein in 1994, and a risk of similar magnitude was reported in a meta-analysis of 17 case-control studies 2 years later (Norris and Scott 1996). The latter authors concluded that the relatively weak association between dietary exposure in early infancy and the development of type 1 diabetes might be due to biases induced by the methodological limitations of retrospective studies, of which maternal recall was one potential source. The discrepant results may also have been related to whether the duration of total or exclusive breastfeeding was assessed. A prospective cohort study can minimize the probability of recall bias, and three birth cohort studies of first-degree relatives of subjects with type 1 diabetes failed to reveal any relationship of the emergence of diabetes-associated autoantibodies to the age at introduction of cow's milk or the duration of total or exclusive breastfeeding (Norris et al. 1996, Couper et al. 1999, Hummel et al. 2000).

The increased concentrations of cow’s milk antibodies, especially to beta-lactoglobulin and bovine serum albumin (BSA), observed in immunological studies of children with newly diagnosed diabetes support an association between exposure to cow's milk and type 1 diabetes (Savilahti et al. 1993, Saukkonen et al. 1998). It has been proposed that these findings may be due to the existence of enhanced immunological reactivity to cow's milk protein or increased intestinal permeability in subjects with preclinical type 1 diabetes, but the data on T-cell responses to cow's milk proteins have remained controversial (Norris and Pietropaolo 1999), the inconsistent observations having been attributed to differences in assay methodology, data interpretation, or populations examined, as the prevalence of the exposure and the disease in the population may influence the results. Cow's milk has recently been reported to be an environmental trigger for an immune response to insulin in infancy (Vaarala et al. 1999, Paronen et al. 2000).

Breastfeeding has a protective effect against infections via maternally transferred immunoglobulins (Howie et al. 1990) and may thus provide protection from other potential triggers of beta-cell autoimmunity. In addition, the development of oral tolerance by insulin contained in the maternal milk has been thought to contribute to the protective effect of breastfeeding (Kotanko 1997). A weak positive association between early exposure to solid foods and the
risk of type 1 diabetes has also been reported (Kostraba et al. 1993). The protective effect of breastfeeding may thus be due to diminished early exposure to foreign proteins in cow’s milk or solid foods that could induce beta-cell autoimmunity.

**Gluten**

Celiac disease shares genetic markers such as HLA-DR3 and DQA1*0501/DQB1*0201 (DQ2) with type 1 diabetes (reviewed by Sollid and Thorsby 1993). The association between type 1 diabetes and celiac disease was recognized years ago (Walker-Smith et al. 1969), and an increased prevalence of celiac disease has been observed among patients with type 1 diabetes (Not et al. 2001). Observations regarding the role of gluten as an environmental trigger of type 1 diabetes have been contradictory, however. It has been suggested that gluten may be diabetogenic in diabetes-prone rats (Scott et al. 1988), and a long duration of gluten exposure in patients with undiagnosed celiac disease has also been shown to correlate with the prevalence of autoimmune diseases such as type 1 diabetes (Ventura et al. 1999). In contrast, there are some reports of no association between wheat gluten exposure and the incidence of type 1 diabetes (Elliott et al. 1988, Coleman et al. 1990).

**Vitamin D**

Inclusion of cod liver oil in the maternal diet during pregnancy has been observed in Norway to be associated with a decreased risk of type 1 diabetes in the offspring (Stene et al. 2000), an effect which the authors attributed to either the vitamin D or the long-chain n-3 fatty acids present in cod liver oil, or both. Vitamin D supplementation during the first year of life was found to be associated with a decreased risk of type 1 diabetes in a European multicenter case-control study (Anonymous 1999b), and more evidence was obtained recently when data from the Finnish birth cohort study showed that low intake of vitamin D was associated with increased risk of type 1 diabetes (Hypponen et al. 2001). The mechanisms of this protective effect are not known, but vitamin D has been observed to have an immunosuppressive function (Saggese et al. 1989).

**Vitamin E**

It has been suggested that a high dietary intake of vitamin E may reduce the incidence of diabetes in diabetes-prone rats (Behrens et al. 1986) and NOD mice (Hayward et al. 1992). Data from a Finnish case-control study comprising 19 cases of type 1 diabetes and 57 control subjects suggested that a low serum alphatocopherol concentration was associated with an increased risk of type 1 diabetes (Knekt et al. 1999). Vitamin E may have a direct protective effect on the beta cells, or else it may prevent free radicals from causing damage to the beta cells (Slonim et al. 1983, Hayward et al. 1992).
Coffee and tea
The role of coffee consumption in the development of type 1 diabetes has remained controversial. Caffeine has been implicated as a risk factor for type 1 diabetes in utero (Tuomilehto et al. 1990), but its deleterious effect on the pancreatic beta cells was later disputed (Pozzilli and Bottazzo 1991). Data obtained in the Finnish Childhood Diabetes in Finland (DiMe) Study indicated that childhood coffee and tea consumption may be associated with an increased risk of type 1 diabetes (Virtanen et al. 1994b).

Nitrates and nitrites
Increased consumption of smoked/cured mutton containing high concentrations of nitrites around the time of conception has been suspected in Iceland to predispose the offspring to type 1 diabetes (Helgason and Jonasson 1981). Nitrate is transformed in the gut into N-nitroso compounds, the diabetogenic potential of which has been demonstrated in animal experiments (Gunnarsson et al. 1974, Helgason et al. 1982). A population-based case-control study showed a dose-response relationship between the frequency of the intake of food containing nitrosamines and the risk of type 1 diabetes (Dahlquist et al. 1990), and also an association of high dietary protein and carbohydrates with an increased risk of the disease. High concentrations of nitrate in drinking water have similarly been reported to be associated with an increased risk of type 1 diabetes (Kostraba et al. 1992, Parslow et al. 1997), but no association was observed between nitrate or nitrite levels in drinking water and type 1 diabetes in the Finnish DiMe study, where a high dietary intake of nitrite on the part of both the child and the mother, but not of nitrate, seemed to increase the risk of type 1 diabetes in the former (Virtanen et al. 1994a).

Growth
The peak incidence of type 1 diabetes in early puberty observed in most countries has been implicated as being associated with mechanisms of growth, even though such an association has also been disputed (Bloom et al. 1975). No clear pubertal peak in the incidence of type 1 diabetes is detectable in Finland any longer (Tuomilehto et al. 1992, Tuomilehto et al. 1999), but the results of several case-control studies have pointed to a relationship between growth rate and the risk of this disease. Although Leslie et al. (1991) reported that children developing diabetes were short in stature at the time of diagnosis, accelerated linear growth in infancy was later shown to be a risk factor for type 1 diabetes (Hyppönen et al. 1999). Accelerated height gain (Songer et al. 1986, Dahlquist et al. 1991) and increased weight gain (Johansson et al. 1994, Hyppönen et al. 1999) early in life are both associated with an increased risk of type 1 diabetes. Increased growth may be a marker of a physiological mechanism that contributes both to linear growth and to the development of type 1 diabetes, and the increased demands placed on insulin
secretion in subjects with accelerated growth velocity may promote beta-cell stress, as growth hormone is known to enhance peripheral insulin resistance (Amiel et al. 1986). At the same time, hyperfunctioning beta cells may be more susceptible to destructive cytokine activity than beta cells that are less stressed (reviewed by Nerup et al. 1988). Increased insulin secretion has been observed to promote antigen presentation in beta cells, which could thereby increase the risk of islet destruction (Björk et al. 1992). Both increased relative weight and height later in childhood appear to be associated with an increased risk of subsequent type 1 diabetes (Hyppönen et al. 2000).

**Toxins**

There are two animal models for toxin-induced type 1 diabetes (Sheehan and McLetchie 1943, Gunnarsson et al. 1974). The first diabetogenic toxin, alloxan, discovered in 1943, is specific to the pancreatic beta cells, while streptozotocin, a nitrosoamide and wide-spectrum antibiotic, selectively destroys these cells (Bonnevie-Nielsen et al. 1981). Both are thought to exercise their effect by producing free radicals (Slonim et al. 1983).

**Viral infections**

A causal relationship has been shown between type 1 diabetes and a high frequency of infectious diseases (Blom et al. 1991), although also an inverse association between the exposure to infections during the first 6 months of life and subsequent type 1 diabetes has been reported (Pundziūtė-Lyckā et al. 2000). The timing and diabetogenicity of the infective agents may be crucial for the initiation of the pathogenetic process. Congenital rubella has been described in a case report as a cause of insulitis and diabetes (Patterson et al. 1981), and has also been observed to be associated with an approximately 12-20% absolute risk of type 1 diabetes (Menser et al. 1978, Rubinstein et al. 1982). Exposure to mumps, rubella, chickenpox, measles and enterovirus infections may also predispose individuals to the development of beta-cell autoimmunity, as these viruses have been described as inducing the appearance of diabetes-associated autoantibodies (Bodansky et al. 1986, Lönnrot et al. 2000a).

Enteroviruses, particularly coxsackie B viruses, are the main viruses suspected of triggering or promoting beta-cell autoimmunity. Epidemiological studies point to an increased incidence of type 1 diabetes after enterovirus epidemics (Gamble and Taylor 1969), and there is also evidence of a role for enterovirus infections in the initiation of beta-cell autoimmunity in utero. Maternal enteroviral infections during pregnancy have been seen to increase the risk of type 1 diabetes in the offspring (Hyöty et al. 1995, Dahlquist et al. 1995). Recently a case of neonatal type 1 diabetes was described with strong indications of fetal induction of beta-cell autoimmunity associated with a maternal echovirus 6 infection around the end of the first trimester (Otonkoski et al. 2000). Exposure to enterovirus infections
during childhood has been thought to induce and accelerate the autoimmune process entailed in the disease, as children who developed type 1 diabetes later had an excess of enterovirus infections years before presentation with the clinical disease (Hyöty et al. 1995). Further evidence for the role of enteroviruses in the initiation of beta-cell autoimmunity was recorded when enterovirus infections were detected during a 6-month observation period preceding the first emergence of diabetes-associated autoantibodies almost twice as often in autoantibody-positive children than in matched antibody-negative controls (Lönnrot et al. 2000a). It has also been suggested that enteroviruses may be associated with the clinical manifestation of type 1 diabetes. Coxsackie B viruses have been isolated from the pancreas in a fatal case of type 1 diabetes (Yoon et al. 1979), and antibodies to coxsackie B viruses are more prevalent in patients at the diagnosis of type 1 diabetes than in matched control subjects (Banatvala et al. 1985, Frisk et al. 1992). Findings of enterovirus mRNA in patients with newly diagnosed type 1 diabetes are controversial. Clements et al. (1995) reported a frequent occurrence of enterovirus mRNA in serum samples taken from children at the time of diagnosis, whereas no excess of acute enterovirus infection was found in children with newly diagnosed type 1 diabetes in Finnish studies (Hyöty et al. 1995, Lönnrot et al. 2000b). The latter authors did note an increased frequency of enterovirus mRNA in serum samples obtained in the prediabetic phase, however.

The mechanism of virus-induced autoimmunity is still open. Cytolytic and chronic infections of beta cells have been shown to precede the autoimmune process associated with virus-induced beta-cell damage in animal experiments (See and Tilles 1995), and such infections must increase the need for insulin in the peripheral tissues and thereby induce beta-cell stress, possibly precipitating the onset of the disease. Infections could also promote beta-cell destruction by increasing cytokine activity (reviewed by Nerup et al. 1988, Kolb et al. 1995, Seewaldt et al. 2000). Molecular mimicry between microbial agents and autoantigens has also been proposed as playing a potential role in the induction of beta-cell autoimmunity (reviewed by Oldstone 1987, Vreugdenhil et al. 1998), although this has not been convincingly verified (reviewed by Albert and Inman 1999).

**Sociodemographic and psychosocial factors**

Stress has been shown experimentally to affect the functional activity of the immune system (Laudenslager et al. 1983), and severe psychological stress has been reported to be able to depress T-cell function (Bartrop et al. 1977). Stress enhances the secretion of catecholamines, which increases the peripheral insulin requirement (Lager et al. 1986), so that stressful events can precipitate manifestation of the clinical disease (Siemiatycki et al. 1989, Dahlquist et al. 1991). In a report
from Montreal, psychological dys-
functions, e.g. learning problems,
sleeping problems and nightmares,
were shown to be associated with an
increased risk of developing type 1
diabetes (Siemiatycki et al. 1989),
but the retrospective study design
employed an inappropriate control
group, which may have resulted in
recall bias.

It has been suggested that so-
cioeconomic status may be associ-
ated with the risk of type 1 diabetes.
A high incidence of the disease in
Denmark seemed to be associated
with low family socioeconomic
status (Christau et al. 1977), and in a
Swedish survey the father being a
manual worker turned out to be a
risk factor for type 1 diabetes in the
child, although the risk was not asso-
ciated with the father’s education
level (Blom et al. 1989). In contrast,
children from high-income families
in the UK and the USA have been
reported to have an increased risk of
developing type 1 diabetes (Debono
et al. 1983, Mayer et al. 1988). A
low maternal education level has
been observed to be associated with
an increased risk of type 1 diabetes
in the offspring (Blom et al. 1989,
Virtanen et al. 1991), although the
results of a prospective cohort study
pointed to a positive association be-
tween the length of maternal educa-
tion and the disease risk (Virtanen et
al. 1998). The maternal education
level may affect the risk of type 1
diabetes via nutritional habits.

Advanced maternal age at de-
livery has been found in several sur-
veys to heighten the risk of diabetes
in the offspring (Wagener et al.
1983, Blom et al. 1989, Patterson et
al. 1994, Bingley et al. 2000), but not
in all studies (Bock et al. 1994).
High maternal age has even been
proposed as one reason for the in-
creasing incidence of diabetes and
the trend towards a younger age at
diagnosis (Bingley et al. 2000), the
authors suggesting that it could have
an influence on the maturation of the
child’s immune system.

Combinations of environmental risk
factors

Our knowledge of the possible roles
of putative environmental agents in
inducing and /or promoting the
pathogenetic process involved in
type 1 diabetes has remained limited.
It is also unclear whether single or
multiple exposures to putative envi-
ronmental factors are required for
initiation of the disease process. It
has been suggested that the possible
deleterious effect of nutritional fac-
tors may be cumulative over time
(Scott et al. 1997), and that type 1
diabetes could be induced by a series
of food antigens (Scott and Kolb
1996). The north-south gradient and
the seasonal variation in the inci-
dence of the disease both suggest the
involvement of infectious agents in
its pathogenesis, but the notion of a
viral etiology has also been chal-
lenged (Karvonen et al. 1998). The
risk of developing type 1 diabetes
has been related to month of birth in
studies from Iceland and the UK
(Helgason and Jonasson 1981,
Rothwell et al. 1996), the former
suggesting that the toxic effect of N-
nitroso compounds may explain this
seasonal pattern and the latter study
speaking of intrauterine or perinatal
infections. No association with the month of birth was reported in a study carried out in Denmark, however (Bock et al. 1994). Positive interactions were observed between a high frequency of infections and a high dietary nitrosoamide intake in a Swedish case-control study, where the authors found that different environmental factors were associated with variable risks of children of various ages developing type 1 diabetes, reflecting the complex causal relations underlying this disease (Dahlquist et al. 1991). Interactions between viral infections and other environmental factors, e.g. toxins, have also been proposed as triggers for beta-cell autoimmunity (Toniolo et al. 1980). The etiology of type 1 diabetes may thus be multifactorial, and the triggering or promoting factors may have different mechanisms, either individual or synergistic, by which they initiate or promote beta-cell destruction (reviewed by Leslie and Elliott 1994). The data remain too limited, however, for any conclusions to be drawn regarding the precise causal associations between various environmental factors and type 1 diabetes.

Autoimmunity in type 1 diabetes

Role of genetic and environmental factors

It has been suggested that environmental agents may induce beta-cell autoimmunity during early childhood in genetically susceptible individuals, possibly even in utero in some cases (reviewed by Leslie and Elliott 1994, Petersen et al. 1997), although prenatal induction is probably very rare. The childhood peak in the incidence of the disease and the long prediabetic period both support the idea that the critical period for initiation of the process is early in life. The immune system is immature during fetal life and in early infancy, and at the same time immunological tolerance to various antigens is induced at this period. Several attacks may be needed to cause the disease process to advance to the stage of a significant loss of insulin secreting beta cells (reviewed by Palmer and McCulloch 1991).

HLA genes play an important role in regulating the immune responses directed against putative environmental agents (reviewed by Åkerblom et al. 1997). Evidence for an interaction between genetic and environmental factors has been provided by the observation that the risk of type 1 diabetes associated with early exposure to cow's milk, for example (Kostraba et al. 1993, Pérez-Bravo et al. 1996), or high consumption of cow's milk during childhood (Virtanen et al. 2000), is higher in children with high risk genotypes than in those with low risk genotypes. The seasonality of clinical presentation with type 1 diabetes has also been reported to be associated with HLA susceptibility markers, in that subjects carrying DR4 have a wider seasonal variation in the manifestation of clinical type 1 diabetes than those with DR3 (Ludvigsson et al. 1986). Patients with type 1 diabetes carrying HLA-DR4
have been observed to have a history of viral infections, particularly Coxsackie-B infections, more often than other patients (Eberhardt et al. 1985).

Tuomilehto et al. (1995) hypothesized that the almost linear increase in the incidence of type 1 diabetes in Finland over the past 40 years may have been caused by changes in HLA-conferred genetic disease susceptibility. This is highly unlikely, however, since such a conspicuous increase in the incidence of the disease due to changes in the gene pool would need a much longer time to become detectable (reviewed by Krolevski et al. 1987, Dahlquist and Mustonen 1994). Migrant studies and data on rapid temporal changes in the incidence of type 1 diabetes in some areas show that alterations in incidence cannot be explained entirely by genetic factors. On the other hand, there is still no evidence of causative environmental factors that have intensified in a relatively steady manner over the last few decades.

**Mechanisms of beta-cell destruction**

Type 1 diabetes is caused by destruction of the insulin-secreting beta cells in the islets of Langerhans (Gepts 1965), although little is known about the nature and timing of the initial etiological events. Type 1 diabetes is characterized by a prediabetic period, during which diabetes-associated autoantibodies are detectable in the serum (Gorsuch et al. 1981, Ginsberg-Fellner et al. 1985, Verge et al. 1995). Metabolic changes such as an increase in fasting proinsulin concentrations, a decreased insulin response to intravenous glucose, and changes in fasting glucose concentrations may also be observed several months or even years before the onset of type 1 diabetes (Røder et al. 1994). The duration of the prediabetic period may vary individually (Verge et al. 1995), but it is most often short in subjects whose clinical disease is diagnosed at a young age (Karjalainen et al. 1989, Yamamoto et al. 1998). It has been suggested that the loss of beta-cell mass is not linear and that non-progressive and subclinical beta-cell dysfunction is common (Thivolet et al. 1991). Beta-cell destruction with detectable immunological changes can also be reversible, at least at the early stages of the insulitis process (Yamada et al. 1982, Millward et al. 1986). Observations made in the German BABYDIAB study suggest that beta-cell autoimmunity is more dynamic in infants and young children than in older children, and that the process towards clinical disease is rapid in some cases and more retarded in others (Ziegler et al. 1999). The rate of progression to type 1 diabetes depends on the remaining beta-cell mass and the peripheral need for insulin. Environmental factors may increase the demands for insulin and thereby affect the timing of clinical manifestation (reviewed by Dahlquist 1993). Experiments with mice indicate that about 80-90% of the beta cells are destroyed by the time clinical symptoms of diabetes appear (Bonnevie-Nielsen et al. 1981).
Cellular autoimmunity

Type 1 diabetes is characterized by infiltration of mononuclear cells into the islets and specific beta-cell destruction (Gepts 1965). Local lymphocytic infiltration of the pancreatic islets, termed insulitis, implies an important role for abnormal lymphocytic regulation in the pathogenesis of this autoimmune disease. A novel subtype of type 1 diabetes has been described recently in Japan which was suspected of being idiopathic, as no insulitis was observed in pancreatic biopsies, nor did the patients have any detectable diabetes-associated autoantibodies, although evidence of T-cell lymphocyte infiltration was found (Imagawa et al. 2000). Studies in Caucasians have failed to reveal any clear evidence of a similar subgroup of patients.

It has been suggested that the autoimmune process may be caused by initial beta-cell damage, leading to antigen release and the production of beta-cell cytotoxic cytokines by helper T-lymphocytes (reviewed by Nerup et al. 1988). The trigger for this T-cell autoreactivity has remained unknown, although it has been suggested that the targets of the autoimmune process may be islet cell proteins, i.e. autoantigens. After possible environmental exposure, an antigen is presented to a T-cell receptor by a given HLA molecule (Leslie and Pyke 1991). T cells can only recognize antigens that are associated with class II molecules. Activated T cells expressing the HLA-DR antigen or other markers of activation can be detected in increased levels during the prediabetic period (Faustman et al. 1989), and the formation of a complex between the MHC molecule, the attached autoantigen and the T-cell receptor results in the activation of CD4+ T-helper lymphocytes (reviewed by Sinha et al. 1990). It has been shown in animal models that the development of type 1 diabetes requires the participation of both CD4+ T-helper and CD8+ T-killer cells (Reich et al. 1989). The function of T cells is mainly mediated via cytokines (reviewed by Paul and Seder 1994), and the activation of CD4+ Th1 T cells is known to release predominantly pro-inflammatory cytokines, such as IL-2, interferon-γ and tumour necrosis factor (TNF)-α, and to support macrophage activation, whereas CD4+ Th2 cells release IL-4, IL-5, IL-6, IL-10 and IL-13, and provide help for antibody production (reviewed by Paul and Seder 1994, reviewed by Liblau et al. 1995). Cytokines are important for coordinating the immune response, and a disturbance in the balance between autoreactivity and tolerance can result in autoimmunity (reviewed by Tisch and McDevitt 1996). Th1 cells, which are associated with cellular immunity, are thought to promote the disease, whereas the role of Th2 cells, which are mainly involved in humoral immunity, is controversial (Katz et al. 1995, reviewed by Liblau et al. 1995). It has been proposed that Th1-type and Th2-type cytokines may interact to induce or suppress the destructive process of pancreatic beta cells (reviewed by Kolb 1997). A strong antigen-specific T-cell response and low autoantibody titers would support the hypothesis that a Th-1 type immune response is
typical of beta-cell destruction. In addition to the cytokines, nitric oxide and other oxygen radicals are thought to be intracellular mediators of cell death (Kolb et al. 1995). This led Conrad et al. (1994) to present an alternative hypothesis regarding the mechanisms of beta-cell death, suggesting that other immunostimulatory compounds expressed by beta cells, such as superantigens, might also cause insulinitis. They concluded that it is the local cytokine balance rather than autoreactivity that plays a crucial role in the development of type 1 diabetes.

It was observed in 1984 that activated T lymphocytes are present in the peripheral blood of patients with newly diagnosed type 1 diabetes (Alviggi et al. 1984). Cellular autoimmunity has been seen to be activated most in the prediabetic state and to fade with increasing destruction of the beta cells (Durinovic-Bellò et al. 1996), and it has been suggested that a combination of cellular and humoral immune changes and their tendency to persist may be highly predictive of progression to clinical type 1 diabetes. The data on T-cell reactivity to various diabetes-associated autoantigens in diabetic subjects and non-diabetic controls have remained inconsistent, however. Tun et al. (1994) claimed that an increased proportion of activated T lymphocytes in the peripheral circulation is associated with the presence of insulin autoantibodies or islet cell antibodies and an increased risk of type 1 diabetes. Increased T-cell responses to diabetes-associated autoantigens were detected in newly diagnosed patients with type 1 diabetes and in antibody-positive first-degree relatives as compared with healthy control subjects (Harrison et al. 1992, Durinovic-Bellò et al. 1996). In contrast, Schloot et al. (1997) found no differences in T-cell responses to insulin between patients with newly diagnosed type 1 diabetes and first-degree relatives of subjects with the disease or healthy control subjects. Furthermore, a high frequency of T-cell responses to diabetes-associated autoantigens has been described in healthy control subjects (Schloot et al. 1997, Rharbaoui et al. 1999). Antigen-specific T-cell reactivity has thus been regarded so far as an unreliable indicator of the risk of type 1 diabetes. Workshops aimed at the standardization of T-cell assays have shown that there is a lack of consistency in results regarding the detection of islet autoreactive T cells in human peripheral blood (PBMC), and that a considerable number of laboratories cannot determine T-cell responses to specific antigens reliably and repeatedly (Roep et al. 1999). These discrepancies have been attributed to limitations in assay technology and the quality of antigen preparations. Furthermore, the sensitivity of the peripheral blood mononuclear cell (PBMC) proliferation assay may be somewhat limited. In the case of human subjects one may ask to what extent antigen-specific T-cell responses measured in PBMC reflect the mechanisms operative in the target tissue, comprising the pancreatic beta cells (Roep et al. 1999, reviewed by Wegmann and Eisenbarth 2000).
**Humoral autoimmunity**

**Islet cell antibodies**

Islet cell antibodies (ICA) are detected by an indirect immunofluorescence assay and recognize antigens located in the cytoplasm of the endocrine cells in the pancreatic islets. ICA are mainly polyclonal autoantibodies of the immunoglobulin G (IgG) type (Schatz et al. 1988), and represent immunological markers of an autoimmune process in the pancreatic islets (Del Prete et al. 1977, Gorsuch et al. 1981). Not all the antigens with which ICA react have yet been characterized. ICA were first identified in association with type 1 diabetes in 1974 when they were detected in adult patients with autoimmune polyendocrine disease (Bottazzo et al. 1974). Subsequently they were found in children and adults with newly diagnosed type 1 diabetes (Lendrum et al. 1976, Bottazzo et al. 1980) and also in subjects with normal glucose tolerance (Del Prete et al. 1977). ICA may be detectable in the peripheral circulation several years before the diagnosis of type 1 diabetes (Gorsuch et al. 1981) and their titer falls progressively thereafter (Lendrum et al., 1976, Neufeld et al. 1980).

The majority of patients with type 1 diabetes (73 - 88%) test positive for ICA at diagnosis (Landin-Olsson et al. 1992, Bonifacio et al. 1995, Bingley et al. 1997, Gors et al. 1997, Savola et al. 1998b), and their prevalence in first-degree relatives of patients with this disease varies from 2.6% to 6.9% (Bonifacio et al. 1990, Riley et al. 1990, Thivolet et al. 1991, Schatz et al. 1994, Lévy-Marchal et al. 1995, Seissler et al. 1996, Greenbaum et al. 2000) while that in siblings of affected children is from 4.7% to 12% (Gorsuch et al. 1981, Bonifacio et al. 1990, Deschamps et al. 1992, Krischer et al. 1993, Verge et al. 1996, Gors et al. 1997, Kulmala et al. 1998, Yamamoto et al. 1998). ICA in siblings of children with type 1 diabetes are related to young age (Bingley et al. 1994, Kulmala et al. 1998). The prevalence of ICA has been observed to vary from 0.24% to 4.1% in a series of non-diabetic subjects, thus exceeding the prevalence of clinical type 1 diabetes in the background population (Bruining et al. 1989, Boehm et al. 1991, Landin-Olsson et al. 1992, Rowe et al. 1994, Schatz et al. 1994, Lévy-Marchal et al. 1995, Bingley et al. 1997, Knip et al. 1998, Strebelow et al. 1999, Kulmala et al. 2001). It has been suggested that the differences in ICA prevalence between countries and populations parallel differences in the incidence of type 1 diabetes (Karjalainen 1990). The population size and the duration of follow-up are crucial factors affecting the results regarding the prevalence of ICA (Schatz et al. 1994), and methodological differences may also cause variation. Although a series of international workshops have improved standardization of the assay, the task has remained a cumbersome, labor-intensive one mainly due to differences between the various pancreases used as the substrate (Lernmark et al. 1991).

Positivity for ICA has been observed to be associated with HLA DR3/4 heterozygosity (Pagano et al.
1987, Boehm et al. 1991, Karjalainen et al. 1996) and the DQA1*0301-DQB1*0302 haplotype (Vandewalle et al. 1993). Kulmala et al. (2000b) observed in siblings of children with type 1 diabetes that high titers of ICA were associated with HLA identity with the proband, the DR3/4 phenotype, the DQB1*02/*0302 genotype, and the DR4 and DQB1*0302 alleles.

*Insulin autoantibodies*

Autoantibodies to insulin (IAA), the only beta-cell specific autoantigen identified so far, were first observed in untreated patients with newly diagnosed type 1 diabetes in 1983 (Palmer et al. 1983), and this finding was subsequently confirmed (Arslanian et al. 1985, Wilkin et al. 1985). The reports concluded that IAA serve alongside ICA as markers of ongoing beta-cell destruction.

The prevalence of IAA has been observed to vary from 0.9% to 3.0% among children from the background population (Schatz et al. 1994, Bingley et al. 1997, Strebelow et al. 1999, Kulmala et al. 2001) and from 1.4 to 6.9% among siblings of children with type 1 diabetes (Krischer et al. 1993, Karjalainen et al. 1996, Verge et al. 1996, Gorus et al. 1997, Kulmala et al. 1998, Yamamoto et al. 1998). The relatively high variation in the frequency of IAA among patients with newly diagnosed type 1 diabetes (from 28% to 69%) may be partly due to age variations and methodological differences (Landin-Olsson et al. 1992, Bingley et al. 1997, Gorus et al. 1997, Savola et al. 1998b).

Young children with newly diagnosed type 1 diabetes have been reported to have higher IAA titers than older children and adults (Vardi et al. 1988, Komulainen et al. 1999), and titers have also been reported to be inversely related to age in schoolchildren (Bingley et al. 1997) and in unaffected siblings of children with type 1 diabetes (Kulmala et al. 1998). IAA are thought to reflect a faster rate of beta-cell destruction and more rapid progression to the disease (reviewed by Atkinson and MacLaren 1994).

Atkinson et al. (1986) reported that IAA were more frequent in those first degree relatives of affected patients who carried DR3 and/or DR4, and increased prevalences and concentrations of IAA were later confirmed to be associated with the DR4 allele (Ziegler et al. 1991, Bonifacio et al. 1999). In relation to HLA DQ markers, increased frequencies and high titers of IAA are associated with the DQA1*0301-DQB1*0302 (DQ8) haplotype (Vandewalle et al. 1993, Hagopian et al. 1995, Verge et al. 1996). A population-based prospective study targeting siblings showed that an increased prevalence of IAA was closely associated with the DR4 and DQB1*0302 alleles and the DR3/4 phenotype, and that the highest frequencies of IAA were seen in siblings carrying the DQB1*02/*0302 genotype (Kulmala et al. 2000b).

*Antibodies to GAD65*

A protein having a molecular weight of 64 kD was identified as the target of autoantibodies in patients with
newly diagnosed type 1 diabetes in 1982 (Baekkeskov et al. 1982), and it was subsequently demonstrated that the presence of antibodies to a 64 kD human islet cell protein was associated with spontaneous autoimmune diabetes mellitus in the diabetes-prone rat (Baekkeskov et al. 1984). The protein was later identified as the 65 kD isoform of glutamic acid decarboxylase (GAD) (Baekkeskov et al. 1990), an enzyme which catalyses the conversion of glutamic acid to gamma-aminobutyric acid. There is also a 67 kD isoform of this enzyme (Bu et al. 1992). GAD is produced in most neuroendocrine cells, and high levels of it are expressed in the brain (Roberts and Frankel 1950) and in pancreatic beta cells (Okada et al. 1976). Gamma-aminobutyric acid (GABA) and GAD are also expressed in the adrenal medulla, pituitary, oviduct, erythrocytes and liver (reviewed by Erdö and Wolff 1990). Humoral GAD autoimmunity is not necessarily associated with beta-cell destruction, as antibodies to GAD (GADA) are also detected in nondiabetic polyendocrine patients and in patients with the stiff man syndrome (Solimena et al. 1990, Wagner et al. 1994). GADA may appear years before the clinical onset of type 1 diabetes, and can accordingly be used as predictive markers (Baekkeskov et al. 1987).

The frequency of GADA has been observed to vary from 0.5% to 3.0% among children from the background population (Bingley et al. 1997, Strebelow et al. 1999, Kulmala et al. 2001, Marčiulionytė et al. 2001), from 6.4 to 13.0% among siblings of children with type 1 diabetes (Verge et al. 1996, Gorus et al. 1997, Kulmala et al. 1998, Yamamoto et al. 1998), and from 62 to 84% among patients with the newly diagnosed disease (Bonifacio et al. 1995, Sabbah et al. 1996, Bingley et al. 1997, Gorus et al. 1997, Savola et al. 1998b). GADA persist for years after the diagnosis of type 1 diabetes (Christie et al. 1990, Savola et al. 1998b). Bingley et al. (1997) observed the highest concentrations in schoolchildren aged over 10 years. GADA are also more frequent in girls than in boys (Hagopian et al. 1995, Sabbah et al. 1996).

GADA have been reported to be associated with the HLA-DQA1*0501/B1*0201 (DQ2) allele (Hagopian et al. 1995, Sabbah et al. 1999), and also with the DR3 allele (Genovese et al. 1996, Sabbah et al. 1996, Bonifacio et al. 1999) and the DR3-DQB1*02 haplotype (Kulmala et al. 2000b). The last-mentioned authors also reported an increased GADA frequency among siblings of affected probands in with the presence of the DR4-DQB1*0302 haplotype, and in DR3/4 heterozygous subjects (Kulmala et al. 2000b).

IA-2 antibodies

Antibodies against 37kD/40kD tryptic fragments of the 64 kD protein were first described by Christie et al. (1992), and at almost the same time the antigen ICA512 was identified as a target of antibodies present in sera from patients with type 1 diabetes (Rabin et al. 1992). Data subsequently emerged to demonstrate that the 40 kD protein is related to the
islet protein tyrosine phosphatase-related IA-2 molecule, being identical to ICA 512 (Payton et al. 1995), whereas the 37 kD fragment was identified as derived from a closely related protein IA-2β (Lu et al. 1996). IA-2 and IA-2β are proteins that are members of the protein tyrosine phosphatase (PTP) family and are expressed in pancreatic islets and in the brain (Passini et al. 1995). Antibodies to the IA-2 molecule (IA-2A), which are directed against the intracellular portion of the protein (Lampasona et al. 1996), have been implicated as specific markers of beta-cell destruction (Savola et al. 1998a), and have been reported to be associated with rapid progression to type 1 diabetes (Christie et al. 1994), an association which has been confirmed elsewhere (Bingley et al. 1994, Genovese et al. 1996).

The prevalence of IA-2A has been observed to vary from 0.2% to 2.4% among children from the background population (Bingley et al. 1997, Strebelow et al. 1999, Kulmala et al. 2001, Marčiulionytė et al. 2001) and from 1.5 to 5.3% among siblings of children with type 1 diabetes (Verge et al. 1996, Gorus et al. 1997, Kulmala et al. 1998, Yamamoto et al. 1998), while the majority of patients with a newly diagnosed disease (from 54% to 86%) test positive (Bonifacio et al. 1995, Bingley et al. 1997, Gorus et al. 1997, Savola et al. 1998a). IA-2A have been reported to be more frequent in young patients with newly diagnosed disease compared with older ones (Bonifacio et al. 1995, Genovese et al. 1996), and especially in young children (Bingley et al. 1994, Kulmala et al. 2000a).

Positivity for IA-2A has been reported to be associated with the DR4 allele and DR3/4 heterozygosity (Genovese et al. 1996, Savola et al. 1998a, Kulmala et al. 2000b), and with the DR4-DQB1*0302 (DQ8) haplotype (Verge et al. 1996, Sabbah et al. 1999, Kulmala et al. 2000b).

**Antibodies to other antigens**

In addition to insulin, glutamate decarboxylase and IA-2/IA-2β, a number of autoantigens have been identified as potential targets for an autoimmune attack on beta cells progressing to type 1 diabetes. These include carboxypeptidase H, a 38 kD antigen, islet cell antigen, with a molecular weight of 69 kD (ICA69), beta-cell glucose transporter, the insulin receptor and sialoglycolipids (reviewed by Atkinson and MacLaren 1993). Glycolipids containing sialic acid were initially suggested as a target antigen for ICA (Nayak et al. 1985), but this was later disputed when ICA were found to be reactive with GAD (Genovese et al. 1992).

Carboxypeptidase H is a glycoprotein expressed in islet secretory granules and neuroendocrine cells that is associated with the production of peptide hormones and neurotransmitters. It catalyses the conversion of proinsulin to insulin (Castaño et al. 1991). The ICA69 molecule has structural similarity to two short regions of BSA and it has been proposed that it may play a role in cow’s milk-induced autoimmunity (Karjalainen et al. 1992). This was
later disputed, however, since the homology between ICA69 and BSA is limited (Pietropaolo et al. 1993), and cellular immunity against BSA seems to be relatively rare and non-specific in patients with type 1 diabetes (Atkinson et al. 1993). Autoantibodies against the 38 kD antigen (Baekkeskov et al. 1982), glucose transporter 2 (GLUT-2) (Johnson et al. 1990) and the insulin receptor (Maron et al. 1983) have been detected in patients with type 1 diabetes, but none of these humoral immune responses is frequent and the predictive characteristics of the autoantibodies are not defined.

**Prediction of type 1 diabetes**

Many factors may combine to determine the risk of developing type 1 diabetes, including family history of the disease, genotype, age, positivity for diabetes-associated autoantibodies and first phase insulin response (FPIR). Feasible screening strategies and surrogate markers of the clinical disease have been evaluated in order to identify subjects at high risk of developing type 1 diabetes and to reduce the duration of intervention trials aimed at preventing or delaying its onset.

**Genetics**

The risk of type 1 diabetes has been observed to be higher in the offspring of affected fathers than in those of affected mothers (Warram et al. 1984, Dahlquist et al. 1989, Tuomilehto et al. 1995), in line with the finding that children of diabetic fathers have signs of beta-cell autoimmunity earlier and more often during the first years of life than do children of diabetic mothers (Roll et al. 1996, Verge et al. 1996). The mechanism that accounts for this difference is not known, although genetic factors and aspects associated with the intrauterine environment have been implicated (Warram et al. 1984). It is also possible that maternal insulin treatment during pregnancy may help the fetus to tolerate insulin-specific immune responses and thereby reduce the risk of childhood diabetes, as recently suggested by Paronen et al. (2000), who observed a reduced T-cell response to insulin in infants of mothers with type 1 diabetes as compared with infants of affected fathers.

The sibling relationship is associated with about a 4-8% absolute risk of developing type 1 diabetes during one’s lifetime (Tarn et al. 1988, Reijonen et al. 1994), with estimated values of 10-20% for HLA-identical siblings, 4-9% for HLA-haploidentical siblings and 0-1% for HLA-nonidentical siblings (Platz et al. 1981, reviewed by Eisenbarth 1986, Tarn et al. 1988, Deschamps et al. 1992). Siblings who belong to families with multiple cases of type 1 diabetes have a higher risk of developing the disease than those with only one affected first-degree relative (Riley et al. 1990, Deschamps et al. 1992, Krischer et al. 1993), because they more often carry a genotype associated with high risk (Ilonen et al. 1996).
The analysis of genetic susceptibility to type 1 diabetes provides a possibility for identifying individuals with an increased genetic risk of developing the disease. Genetic screening is also feasible in order to exclude subjects with protective HLA haplotypes from intervention trials. The absolute risk of contracting type 1 diabetes by the age of 15 years in the Finnish population is about 0.7%, while that conferred by the high-risk HLA-DQB1*02/*0302 genotype is approximately 7% and that associated with the moderate-risk genotype DQB1*0302/x (where x = other than *02, *0301, or *0602) is close to 2.5% (Ilonen et al. 1996). A strong HLA-conferred risk is more frequent among children who present with clinical diabetes at a young age (Mustonen et al. 1985, Karjalainen et al. 1989, Vandewalle et al. 1993, Pugliese et al. 1995, Ilonen et al. 1996). These DR3/DR4 heterozygous patients are characterized by rapid beta-cell destruction and poor metabolic control after clinical disease manifestation (Schenker et al. 1999). The HLA-DR4 allele has been reported to be associated with female gender and more severe clinical disease presentation (Eberhardt et al. 1985, Ludvigsson et al. 1986), and it has been suggested that subjects carrying the high-risk allele DR4 or the DQB1*02/*0302 genotype experience early induction of the destructive disease process in the pancreatic islets and early appearance of diabetes-associated autoantibodies (Komulainen et al. 1998, Schenker et al. 1999). The HLA-DQB1*z/z genotype (z ≠ DQB1*02 or *0302) has also been reported recently to be associated with severe metabolic decompensation at the time of diagnosis of the disease (Komulainen et al. 1998). The reason for these partly contradictory findings is unclear, but the authors suggested that the development of type 1 diabetes requires an especially aggressive immune attack in subjects with decreased genetic disease susceptibility. Lower frequencies of ICA, GADA, IA-2A and multiple antibodies have been observed in unaffected siblings carrying the protective DR2 and DQB1*0602-3 alleles than in other siblings of children with type 1 diabetes (Kulmala et al. 2000b). Although the DQB1*0602 allele is associated with a low disease risk, it does not completely prevent the appearance of diabetes-related autoantibodies, indicating that the protective effect associated with it is a partial one (Pugliese et al. 1995, Kulmala et al. 2000b).

Age and progression to type 1 diabetes

The risk of first-degree relatives of subjects with type 1 diabetes developing the disease is inversely correlated with age (Deschamps et al. 1992, Bingley et al. 1994, Yamamoto et al. 1998), and young age is an independent risk factor for the development of diabetes in children with affected first-degree relatives (Cantor et al. 1995, Bingley 1996), although the predictive value of young age (<10 years) has been observed to be relatively low (9%) (Yamamoto et al. 1998).
Autoantibodies

Appearance of autoantibodies

Autoantigens recognized by circulating antibodies can be detected in the prediabetic period, so that diabetes-associated autoantibodies currently represent the best markers of beta-cell autoimmunity and destruction in man. Bonifacio et al. (1999) observed that IAA concentrations rose rapidly in the early preclinical phase of type 1 diabetes and reached a peak close to the time of diagnosis. First-degree relatives of affected subjects having at least one detectable autoantibody were found to have a higher risk of developing clinical type 1 diabetes than those who had no antibodies (Bingley et al. 1994, Kulmala et al. 1998, Yamamoto et al. 1998), so that the estimated positive predictive value (PPV) attached to the occurrence of at least one autoantibody has been reported to be 46%, the sensitivity 98% and specificity 98.5% among such relatives over a follow-up period of 5 years (Verge et al. 1996).

A recent Swedish study concluded that islet autoantibodies were detectable in a cord blood sample more frequently than expected in children who later developed type 1 diabetes, and the authors suggested that the autoimmune process could have been initiated during fetal life (Lindberg et al. 1999). Diabetes-associated antibodies that are detectable in cord blood are in most cases transferred transplacentally from antibody-positive mothers to their offspring, since immunoglobulin G antibodies are actively transported over the placental barrier (Pitcher-Wilmott et al. 1980, Hämäläinen et al. 2001). These antibodies usually disappear during the first 3-6 months of life, and at the latest by the age of 15 months (Ziegler et al. 1993, Hämäläinen et al. 2000). The first signs of beta-cell autoimmunity may appear early in life, as the first IAA in children of mothers with type 1 diabetes were observed at the age of 6 months (Martikainen et al. 1996). In the prospective German BABYDIAB Study the first IAA in offspring of diabetic parents were detected at the age of 9 months (Ziegler et al. 1993, Roll et al. 1996, Ziegler et al. 1999), and the frequencies of various autoantibodies in offspring of affected parents between 2 and 5 years of age were similar to those detected in older relatives of patients with type 1 diabetes, so that the authors suggested that the first signs of beta-cell autoimmunity appear before the age of 5 years.

Persistence of autoantibodies

Diabetes-associated autoantibodies sometimes fluctuate from positivity to negativity, and may occur transiently. An Australian study of newborn infants of first-degree relatives with type 1 diabetes showed transient low titers of autoantibodies to be common (Colman et al. 2000), although transient antibody positivity was relatively rare and restricted to subjects other than those carrying the high-risk genotypes (DR3/4, DQ2/8) in the American Diabetes Autoimmunity Study in the Young, which included first-degree relatives of subjects with type 1 diabetes and individuals from the general popula-
tion (Yu et al. 2000b). In both of these reports transient antibody positivity was restricted to one autoantibody. Any of the four antibody types, ICA, IAA, GADA and IA-2A, may disappear, although transient beta-cell autoimmunity was particularly rare among those with multiple autoantibodies in a Finnish population of unaffected schoolchildren (Kulmala et al. 2000a) and IAA have been observed to disappear more often than ICA among unaffected schoolchildren (Lévy-Marchal et al. 1995). This disappearance effect is mainly restricted to low-titer autoantibodies (Colman et al. 2000, Kulmala et al. 2000a), and the reason for such fluctuations remains to be defined. It does mean, however, that the timing of sampling is critical for risk assessment based on positivity for various autoantibodies and their combinations. A sample taken at or close to the time of diagnosis of the diabetic proband has been proposed as the most useful for evaluating the risk of type 1 diabetes in siblings (Kulmala et al. 2000b), while follow-up studies suggest that the screening of autoantibodies in sequential samples improves their predictive characteristics (Yamamoto et al. 1998). Diabetes-associated autoantibodies persist for years after diagnosis and may still be detectable 10 years later (Savola et al. 1998b). The significance of such antibodies is an open question.

**ICA in the prediction of type 1 diabetes**

The predictive value of ICA differs considerably between family members of patients with type 1 diabetes and healthy children from the background population. In the absence of other islet antibody specificities, ICA are only weakly predictive of clinical disease in first-degree relatives of affected subjects (Bingley et al. 1994, Verge et al. 1996, Gardner et al. 1999), whereas they constitute the most sensitive single antibody marker among unaffected siblings of children with type 1 diabetes, as they identify approximately 80% of those who will progress to clinical disease (Kulmala et al. 1998, Yamamoto et al. 1998). ICA have been reported to have a PPV of 40-59% for type 1 diabetes among unaffected siblings of children with the disease (Deschamps et al. 1992, Knip et al. 1998, Kulmala et al. 1998, Yamamoto et al. 1998). Persistently positive ICA are substantially more predictive of subsequent disease among first-degree relatives than intermittently positive ICA (Tarn et al. 1988). ICA positivity alone nevertheless has a low predictive value (4-7%) for the development of type 1 diabetes in the general population, as its frequency greatly exceeds the number of affected cases (Karjalainen 1990, Landin-Olsson et al. 1992, Lévy-Marchal et al. 1995, Bingley et al. 1997, Knip et al. 1998). In contrast to these findings, however, a survey carried out in Florida reported that the predictive value of ICA was equally high among schoolchildren as among first-degree relatives of subjects with type 1 diabetes (Schatz et al. 1994).

According to a German study, increased titers of ICA are associated with strong genetic susceptibility to
type 1 diabetes in the background population (Boehm et al. 1991), whereas a Finnish survey indicated no significant difference in the distribution of HLA-DQ	extsubscript{B}1 alleles and genotypes between ICA-positive and ICA-negative schoolchildren (Kulmala et al. 2000b). The risk of progression to type 1 diabetes is closely related to the ICA titer, in that the higher the antibody titer is, the higher is the risk of progression to disease in the background population (Karjalainen 1990), and in relatives of patients (Bonifacio et al. 1990, Riley et al. 1990, Deschamps et al. 1992, Bingley et al. 1994, Kulmala et al. 1998). The PPV was reported to increase from 35% to 75% in first-degree relatives of patients with type 1 diabetes when ICA increased from 5 JDFU to 20 JDFU (Thivolet et al. 1991), although that of an ICA titer of $\geq$20 JDFU has in any case been reported to be considerably higher among sibs of children with type 1 diabetes than in the general population (Knip et al. 1998). Bonifacio et al. (1990) observed that the PPV for progression to type 1 diabetes within 10 years among first-degree relatives of affected patients varied from 40% at an ICA threshold of 4 JDFU to 100% at an ICA threshold of 80 JDFU. High ICA titers ($\geq$80 JDFU) have been reported to be inversely associated with age (Bingley et al. 1994), and persistently high titers are highly predictive for future diabetes in unaffected siblings of children with type 1 diabetes irrespective of their HLA genotype (Karjalainen et al. 1996). A considerable proportion of subjects with newly diagnosed type 1 diabetes have an ICA titer lower than 80 JDFU, however, and accordingly the increased predictive value and specificity of high ICA titers is associated with a decrease in sensitivity (Deschamps et al. 1992, Bingley et al. 1994).

The prediction of type 1 diabetes can be improved by combining markers of genetic susceptibility and autoantibodies. A prospective survey in France demonstrated that ICA-positive siblings who were heterozygous for DR3/4 had a high risk of developing type 1 diabetes, the cumulative incidence after 8 years of follow-up being 70%, while that in ICA-negative siblings carrying the DR3/4 haplotype was 5% (Deschamps et al. 1992). DR3/4 heterozygosity has also been observed to be associated with rapid progression to diabetes in young ICA-positive siblings of subjects with type 1 diabetes (Deschamps et al. 1992). An increased risk of progression to type 1 diabetes associated with a combination of ICA positivity and DR3 and/or DR4 alleles has also been reported among unaffected schoolchildren (Schatz et al. 1994). The presence of DR3/4 or DQ	extsubscript{B}1*02/*0302 in addition to ICA increases the predictive value of IAA, GADA and IA-2A, although such a combination reduces the sensitivity (Yamamoto et al. 1998, Kulmala et al. 2000b).

**IAA in the prediction of type 1 diabetes**

IAA are common in young children developing type 1 diabetes (Arslanian et al. 1985, Vandewalle et al. 1993, Bingley 1996, Roll et al.
1996, Bonifacio et al. 1999, Komulainen et al. 1999, Ziegler et al. 1999, Colman et al. 2000, Yu et al. 2000a). They have been observed to be among the first autoantibodies to appear (Roll et al. 1996, Bonifacio et al. 1999, Ziegler et al. 1999, Yu et al. 2000a) and are thereby valuable predictive markers of diabetes in very young children.

The sensitivity of IAA for identifying siblings of children who progress to clinical type 1 diabetes has been observed to be relatively low, 25-58% (Kulmala et al. 1998, Yamamoto et al. 1998), and they have been reported to be associated with an approximately 30% risk of type 1 diabetes within a period of 8 years among siblings of children with the disease (Kulmala et al. 1998). IAA positivity alone has a low predictive value for disease development in the general population, 5-6% (Landin-Olsson et al. 1992, Bingley et al. 1997), but high titers confer a higher risk of progression than lower titers (Ziegler et al. 1989).

**GADA in the prediction of type 1 diabetes**

GADA positivity has been shown to have a sensitivity of 58-69% for type 1 diabetes among siblings of affected children (Kulmala et al. 1998, Yamamoto et al. 1998), and to be associated with a 42-52% risk of disease manifestation within 5-8 years among first-degree relatives of affected subjects (Kulmala et al. 1998, Verge et al. 1996). This marker alone has been estimated to entail a low risk of progression to type 1 diabetes, 7%, in schoolchildren over an interval of 10 years (Bingley et al. 1997).

**IA-2A in the prediction of type 1 diabetes**

Like GADA, IA-2A have been reported to have a sensitivity of 58-69% for type 1 diabetes among siblings of affected children (Kulmala et al. 1998, Yamamoto et al. 1998). Although IA-2A have been observed to have a high PPV for the disease, 55-70%, in these siblings (Kulmala et al. 1998, Yamamoto et al. 1998), they are usually the last or one of the last autoantibodies to appear in preclinical type 1 diabetes (Roll et al. 1996). Irrespective of the fact that they may be a less sensitive disease marker than ICA, they have been observed to be highly predictive of future manifestation among first-degree relatives of affected subjects (Bingley et al. 1994, Gorus et al. 1997), with a calculated risk of approximately 80% within 5 years (Verge et al. 1996). IA-2A positivity alone has been estimated to entail a low risk of progression to type 1 diabetes, 6%, among schoolchildren within a period of 10 years (Bingley et al. 1997).

As older children may have had diabetes-associated autoantibodies transiently at an earlier age, it is difficult to make accurate assessments of the predictive value of the various autoantibody specificities on the basis of cross-sectional data. In addition, the predictive characteristics are dependent on the performance of the autoantibody assays, the cut-off limits used, cohort size, the duration of the observation period and age het-
heterogeneity, and one should therefore be cautious when comparing the prevalences of autoantibodies with risks of progression to type 1 diabetes.

Antibody combinations and the risk of developing type 1 diabetes

Risk assessments can be improved by combining various islet cell-specific autoantibodies (Bingley et al. 1994, Roll et al. 1996, Verge et al. 1996, Kulmala et al. 1998). Positivity for both ICA and IAA is associated with a higher risk of developing type 1 diabetes than positivity for ICA alone (Srikanta et al. 1986, Ziegler et al. 1989, Krischer et al. 1993, Bingley et al. 1994, Schatz et al. 1994), although IAA seemed to provide only a modest increase in the PPV relative to ICA among siblings of affected children in the Finnish DiMe study (Knip et al. 1994). Positivity for ICA is combined with other diabetes-associated autoantibodies more often in sibs than in children from the background population (Knip et al. 1998).

Verge et al. (1996) reported that combined screening for IAA, GADA and IA-2A is highly predictive of future type 1 diabetes in first-degree relatives of affected subjects irrespective of ICA status. Since ICA reflect antibodies to a number of islet antigens, including GAD and IA-2 (Genovese et al. 1992, Myers et al. 1995), the combination of GADA and IA-2A has been proposed as a replacement for ICA in screening. Bonifacio et al. (1995) were the first to report that the combination of GADA and IA-2A identified more than 90% of future cases of type 1 diabetes among first-degree relatives, although it was ICA that constituted the most sensitive single screening test. In a German survey, combined screening for IA-2A and GADA identified more than 80% of first-degree relatives of patients who tested positive for ICA and more than 90% of those who had an ICA titer of 20 JDFU or more (Seissler et al. 1996). It has in any case been proposed that GADA and IA-2A complement each other, as they differ in their associations with HLA risk markers, gender and age (Savola et al. 1998a), and this combination has been reported to have an even higher sensitivity than ICA alone (Savola et al. 1998a). Several other authors have also reported that combined testing for GADA and IA-2A exhibits high sensitivity and specificity for type 1 diabetes (Bingley et al. 1997, Dittler et al. 1998, Kulmala et al. 1998), and this has consequently been proposed as the first line of screening for beta-cell autoimmunity both among first-degree relatives of subjects with type 1 diabetes (Bingley et al. 1999) and in the general population (Bingley et al. 1997, Strebelow et al. 1999). A report from the BABYDIAB Study, observing children from birth, does not support the notion that the combination of GADA and IA-2A could replace the ICA assay, as it points to a relatively low sensitivity for this combination in young offspring of diabetic parents (Ziegler et al. 1999). Most of previous studies had been concerned with first-degree relatives of patients with type 1 diabetes who were older than 5 years of age. As previously
mentioned, IAA are prevalent in children with newly diagnosed type 1 diabetes in that age group, indicating that these should be included together with GADA and IA-2A in the first line of screening, at least in young children (Dittler et al. 1998).

Association of the number of antibodies with progression to type 1 diabetes

The risk of individuals having a single detectable autoantibody developing type 1 diabetes has been reported to be low among first-degree relatives of affected subjects (Verge et al. 1996, Kulmala et al. 1998) and among children representing the general population (Strebelow et al. 1999), but progression is not excluded (Kulmala et al. 1998). The majority of such antibody positivity may nevertheless represent non-progressive beta-cell autoimmunity (McCulloch et al. 1990).

Approximately 90% of newly diagnosed subjects with type 1 diabetes have been reported to have multiple autoantibodies, i.e. two or more (Savola et al. 1998a), and positivity for multiple autoantibodies has been reported to be highly predictive of future disease among first-degree relatives of affected subjects (Bingley et al. 1994, Verge et al. 1996, Kulmala et al. 1998, Yamamoto et al. 1998, Ziegler et al. 1999) and particularly among young children with an increased genetic risk (Roll et al. 1996). In an American study the PPV of positivity for at least two autoantibodies among first-degree relatives of affected subjects over a 5-year follow-up period was reported to be 68%, sensitivity 80% and specificity 100% (Verge et al. 1996). Positivity for multiple autoantibodies is also highly predictive of future type 1 diabetes in the background population (Bingley et al. 1997). On the other hand, the appearance of multiple autoantibodies is affected by the HLA genotype, as their prevalence is higher among those with a high-risk genotype than among those with other genotypes (Colman et al. 2000, Kulmala et al. 2000b). Although the PPV is highest among genetically susceptible subjects testing positive for multiple autoantibodies, the sensitivity is reduced by combining genetic risk and autoantibody status.

Young age is also a risk factor for the appearance of multiple autoantibodies. Young children with newly diagnosed type 1 diabetes have been reported to have all four autoantibodies more often than older children (Komulainen et al. 1999), and unaffected schoolchildren who had multiple autoantibodies (>2) were observed to be younger than those who tested positive for a single autoantibody (Kulmala et al. 2000a).

The risk of developing type 1 diabetes increases with the number of autoantibodies detectable, so that three or more have been reported to be associated with a risk of 66-100% among siblings of affected children (Kulmala et al. 1998, Yamamoto et al. 1998). Bingley et al. (1994) reported that the risk of progression to type 1 diabetes among first-degree relatives increased from less than 10% when ICA alone were detectable to 88% when at least three autoantibodies were detectable. Posi-
tivity for all four autoantibodies is a late sign of an autoimmune process in the pancreatic beta cells and is highly predictive of the future development of diabetes in first-degree relatives of affected subjects (Bingley et al. 1994, Kulmala et al. 1998). Screening strategies aim at identifying as early as possible all those individuals who will eventually develop the clinical disease, and in this respect the appearance of all four autoantibodies may be too late sign of beta-cell autoimmunity to enable effective intervention to be initiated with the aim of preventing or delaying the onset of the disease. The risk of disease manifestation is also associated with presence of persistent autoantibodies, and first-degree relatives of affected subjects having at least two autoantibodies persistently have been shown to have a high risk of progression (Gardner et al. 1999, Yu et al. 2000a).

**Measures of insulin secretory capacity**

The specificity of risk assessments in children with signs of beta-cell autoimmunity may be further improved by combining markers of genetic susceptibility, disease-associated autoantibodies and FPIR to intravenous glucose. The earliest metabolic abnormality during the preclinical disease process is a decrease in FPIR to intravenous glucose (Srikanta et al. 1983, Ganda et al. 1984), which has been shown to be highly predictive of the development of type 1 diabetes in ICA-positive first-degree relatives (Srikanta et al. 1985, Robert et al. 1991, Bingley 1996), although a normal FPIR does not exclude the possibility of rapid progression to the clinical disease (Robert et al. 1991). Impairment of beta-cell function has also been demonstrated in a few genetically susceptible relatives testing negative for ICA (McCulloch et al. 1990). The decrease in FPIR before diagnosis was first suggested to be linear, and it was reported that the time to diagnosis could be predicted on this basis (Srikanta et al. 1984). The finding was later challenged, however, by the observation that the disease process may be less predictable, especially in its early phases and among adults (McCulloch et al. 1990, Thivolet et al. 1991). It has been shown in several instances that prediction of the duration of the preclinical period is not feasible on the basis of FPIR (Robert et al. 1991, Thivolet et al. 1991, Knip et al. 1994), and in any case, standardization of the intravenous glucose tolerance test protocol and the insulin assay used would be critical in order to be able to compare FPIR results within and between individuals (Bingley et al. 1992).

Both ICA and IAA have been observed to be closely associated with a decreased FPIR in relatives of patients with type 1 diabetes (Krischer et al. 1993). High titers of ICA are inversely correlated with FPIR (Bingley 1996), and positivity for at least two antibodies combined with a low FPIR has been found to be highly predictive of future diabetes among first-degree relatives of affected subjects (Verge et al. 1996). Low FPIR levels are associated with considerable loss of beta-cell mass (Veijola et al. 1995) and the preclini-
cal process in such subjects may have advanced to a late phase at which it is too late to intervene. Data on the association of FPIR with autoantibodies in the general population are sparse, but a low FPIR has been observed to be primarily associated with positivity for multiple autoantibodies among unaffected schoolchildren (Strebelow et al. 1999, Kulmala et al. 2000a).

Both HLA-identity and the DQB1 high-risk genotype have been observed to be associated with a decreased early insulin response and increased risk of developing type 1 diabetes in siblings of affected children (Veijola et al. 1995). The DQB1 alleles associated with a reduced risk of type 1 diabetes nevertheless do not provide protection from a decreased FPIR and the development of type 1 diabetes (Greenbaum et al. 2000, Kulmala et al. 2000a).

Prevention of type 1 diabetes

Animal experiments

A series of experiments have been conducted to show the effect of specific cytokines or their antagonists in preventing or delaying type 1 diabetes (reviewed by Tisch and McDevitt 1996), and the use of specific antigens has also provided promising results. Prophylactic insulin treatment of diabetes-prone BB rats was observed to result in a 50% reduction in diabetes (Gotfredsen et al. 1985), and insulin in its biologically active form has been reported to prevent insulitis. Atkinson et al. (1990) showed that a low-dose subcutaneous prophylactic insulin therapy reduced the frequency of pancreatic insulitis and diabetes in NOD mice, which spontaneously develop autoimmune diabetes with many immunological and pathological similarities to human type 1 diabetes. Considerable interest has been focused on the possibility of oral administration of a target antigen to inhibit autoimmune diseases (reviewed by Thompson and Staines 1990), and orally administered insulin has been found to reduce the severity of lymphocyte infiltration into the pancreatic islets and to successfully prevent autoimmune diabetes in animals (Zhang et al. 1991, Ploix et al. 1998). It has been suggested that insulin therapy may induce or prolong natural tolerance of endogenous insulin in the prevention of type 1 diabetes, and it may also place the pancreatic beta cells "at rest" and protect them from immune destruction by decreased expression of the autoantigens (Atkinson et al. 1990, Zhang et al. 1991, Keller et al. 1993). Although antigen-specific immunotherapy has been observed to be a promising strategy for preventing type 1 diabetes, the combination of various diabetes-associated autoantigens has been regarded as a more effective approach (reviewed by Tisch and McDevitt 1996, Cetkovic-Cvrlje et al. 1997). The timing and route of administration may be critical for the development of immunological tolerance, as peptides may also accelerate the pathogenesis of autoimmune disease (Blanas et al. 1996).
Several antioxidants have been shown to be useful in preventing autoimmune diabetes in experimental models. Nicotinamide, a watersoluble vitamin B, has been the target of considerable interest, based on the finding that this compound prevents or reduces the incidence of the disease in animal models of immune-mediated diabetes (Yamada et al. 1982). Its primary action may be inhibition of the activation of polyadenosine 5’-diphosphatase (ADP)-ribose synthetase and thereby inhibition of induction of apoptosis and effects on gene expression (Burkart et al. 1999, reviewed by Kolb and Burkart 1999).

**Primary prevention in man**

The increased ability to identify subjects at risk for type 1 diabetes has facilitated strategies to prevent or reduce the incidence of the disease as an ultimate therapeutic goal. Prevention of type 1 diabetes can be implemented at three stages: primary, secondary and tertiary (reviewed by Knip 1998). Primary prevention covers strategies aimed at reducing its incidence by preventing its manifestation in individuals who lack any signs of beta-cell destruction. The identification of etiological risk factors associated with the disease is essential for successful primary prevention, as it may be possible to modify or eliminate the response to these agents in subjects at high risk of progression. As environmental factors are assumed to trigger beta-cell autoimmunity early in life, primary prevention needs to target young children. The “Trial to Reduce IDDM in the Genetically at Risk” (TRIGR) is an intervention trial aimed at assessing whether it is possible to prevent clinical type 1 diabetes in young, genetically susceptible first-degree relatives of affected subjects by eliminating cow’s milk proteins over the first 6-8 months of life (Åkerblom et al. 1993). In a small pilot study comprising 20 infants, avoidance of cow’s milk proteins over the first 9 months of life did not provide total protection against type 1 diabetes (Martikainen et al. 1996), but the results of a second pilot study indicated that this kind of early nutritional intervention is able to reduce the frequency of various diabetes-associated autoantibodies by 40-60% by the age of 2 years (Åkerblom et al. 1999).

**Secondary prevention**

Secondary prevention is aimed at reducing the incidence of the disease in individuals with signs of beta-cell autoimmunity by preventing the progression of the preclinical disease process. Early identification of individuals with signs of beta-cell destruction provides an opportunity for doing this. Intervention at an early stage in the preclinical process should increase the possibilities of successful prevention, but this should be attempted only in the context of clinical studies as defined in the statement by the American Diabetes Association (Anonymous 1999a).

Nicotinamide has been used in clinical trials aimed at preventing or delaying the clinical manifestation of
type 1 diabetes in unaffected first-degree relatives of patients. The German Nicotinamide Diabetes Intervention Study (DENIS) failed to detect the expected 80% reduction in the incidence of diabetes in first-degree relatives at high risk (Lam-peter et al. 1998), whereas an open population-based prevention trial among schoolchildren in the Auckland region of New Zealand reported a reduction of approximately 50% in the cumulative incidence of type 1 diabetes in subjects treated with nicotinamide (Elliott et al. 1996).

The large multicenter European Nicotinamide Diabetes Intervention Trial (ENDIT), targeting ICA-positive relatives of affected patients, which will be completed in 2003 (Pociot et al. 1993), involves randomized subjects who have on average a lower risk of progression to type 1 diabetes than the DENIS participants. Nicotinamide has also been observed to preserve beta-cell function after the diagnosis of type 1 diabetes (Vague et al. 1987), but has been reported in some studies to reduce insulin sensitivity in subjects with impaired beta-cell function and thereby to increase insulin resistance. The therapeutic effect of nicotinamide has thus been challenged (Greenbaum et al. 1996), although the effects on peripheral insulin sensitivity may be due to residues of nicotinic acid in the drug preparation used.

Insulin is the only antigen-specific therapy that has been proposed for preventing diabetes in humans. In a pilot trial of low-dose subcutaneous insulin treatment in relatives of patients with type 1 diabetes, only one of the five children treated with insulin developed diabetes whereas all seven untreated subjects did so (Keller et al. 1993). Thereafter a number of multicenter trials were launched with the aim of preventing or delaying the onset of type 1 diabetes in high-risk subjects by means of prophylactic subcutaneous insulin therapy. The American Diabetes Prevention Trial 1 (DPT-1) is aimed at preventing type 1 diabetes in unaffected first and second-degree relatives with signs of beta-cell autoimmunity by means of either parenteral or oral insulin (Greenbaum et al. 2000). The trial has two arms, one using daily injections of subcutaneous insulin in combination with a short period of intravenous insulin infusion once a year, while the other is based on the use of oral insulin once a day. The preliminary results of the parenteral arm, as reported at the Annual Meeting of the American Diabetes Association in 2001, show no effect of insulin treatment on progression to clinical type 1 diabetes among the high-risk relatives recruited for the trial (Skyler, personal communication).

Large-scale population screening for genetic markers of susceptibility to type 1 diabetes became possible with the introduction of methods based on polymerase chain reaction (PCR) amplification and sequence-specific oligonucleotide probe hybridization (Bugawan and Erlich 1991). The American Diabetes Autoimmunity Study in the Young is a prospective birth cohort study in which newborn babies are screened for genetic susceptibility to
type 1 diabetes. Families with babies showing an increased genetic risk are then invited for follow-up, including surveillance for the development of autoimmunity and the clinical disease (Rewers et al. 1996, Yu et al. 2000a). The Finnish Diabetes Prediction and Prevention project (DIPP) is another prospective birth cohort study targeting individuals in the general population who have a genetic risk of developing type 1 diabetes (Kupila et al. 2001). Here the appearance of signs of beta-cell autoimmunity in genetically susceptible children is recognized at an early stage and the efficacy of nasal insulin administration in delaying the onset of clinical type 1 diabetes in such cases is evaluated.

**Tertiary prevention**

Tertiary prevention covers strategies aimed at preserving beta-cell function and preventing secondary complications of the disease after the diagnosis of type 1 diabetes. Pancreatic transplantation is an invasive procedure with a substantial risk of morbidity, whereas islet transplantation has a minimal risk associated with it. The results of pancreas transplantations have regularly been more encouraging than those of islet transplantations, however, although the prospects of success with the latter have improved substantially in recent times with the introduction of the “Edmonton protocol” (Shapiro et al. 2000).
AIMS OF THE PRESENT RESEARCH

The specific objectives of the present work were:

1. To evaluate the significance of the appearance of autoantibodies for the development of type 1 diabetes in genetically susceptible children identified from the general population.

2. To define the relationship between early infant nutrition and the emergence of signs of beta-cell autoimmunity.

3. To evaluate the optimal screening strategy for identifying children at high risk of developing type 1 diabetes from among those with increased genetic susceptibility.

4. To assess the validity of diabetes-associated autoantibodies as surrogate markers of type 1 diabetes as potentials in siblings of affected children.
SUBJECTS AND METHODS

Subjects

Subjects belonging to the Type 1 Diabetes Prediction and Prevention (DIPP) project (Papers I-III)

The DIPP project was initiated to assess feasible strategies for predicting type 1 diabetes in the general population and to develop effective tools for preventing or delaying progression to clinical disease (Kupila et al. 2001). It is aimed at identifying newborn infants having an increased genetic risk of type 1 diabetes (phase I) and recognizing the appearance of signs of beta-cell autoimmunity at an early stage (phase II). Phase III is aimed at delaying the onset of type 1 diabetes in a double blind randomized intervention trial designed to evaluate the preventive efficacy of nasal insulin administration in those having genetic risk markers and signs of beta-cell specific autoimmunity.

All the infants considered in papers I-III who had increased genetic susceptibility to type 1 diabetes were carriers of the high-risk genotype DQB1*02/*0302 or the moderate risk genotype DQB1*0302/x (x = other than *02, *0301, or *0602). The first follow-up blood sample was drawn at the age of 3 months, the next at the age of 6 months and subsequent ones at intervals of 3-6 months for the first 2 years and 6 to 12 months thereafter. If an infant had autoantibodies detectable at the age of 3 or 6 months, the cord blood sample was also analyzed for ICA, IAA, GADA and IA-2A. Autoantibodies with decreasing titers in infants who had also had autoantibodies in their cord blood were excluded from the analysis, as such autoantibodies always disappeared by the age of 15 months at the latest, strongly suggesting that they had been transferred transplacentally from the mother (Hämäläinen et al. 2000).

The series reported in paper I, where ICA were used as the primary screening test for beta-cell autoimmunity, comprised 3596 genetically susceptible children (13.8%) out of 25,983 newborn infants, all of whom had been analyzed for HLA-DQB1 alleles. At the time of the analysis, at least one ICA measurement had been performed on 2448 infants followed from birth (Table 1). The median age of these index children at the end of the observation period was 1.2 years, the oldest reaching the age of 2.5 years. Altogether 1307 older sibs of the index cases were also genotyped and analyzed for ICA at least once.

The subjects in paper II comprised 65 children (32 boys) who had tested positive for ICA at least once during the follow-up (cases) and 390 children without diabetes-associated autoantibodies (controls) matched with the cases for sex, HLA-DQB1 genotype, geographical region and birth date (six controls/case). The median observation period was 2.5 years (range 0.8-3.8 years).
The series described in paper III comprised the first 1005 children (530 boys, 52.7%) to have a sample taken at the age of 2 years. All the blood samples from these children had been analyzed for ICA, IAA, GADA and IA-2A at least up to that time. In addition, 15 children from the whole DIPP cohort who had participated in the regular immunological follow-up and had developed clinical diabetes were analyzed for the appearance of autoantibodies.

Subjects belonging to the Childhood Diabetes in Finland (DiMe) Study (Paper IV)

The nationwide Childhood Diabetes in Finland (DiMe) Study is a prospective population-based survey established in 1986 to investigate the role of genetic, immunological and environmental factors in the development of type 1 diabetes. Seven hundred and fifty seven of the 801 families with a child under the age of 15 years newly diagnosed as having type 1 diabetes from September 1986 to April 1989 invited to take part agreed to do so, a participation rate of 94.5% (Tuomilehto et al. 1992), and 765 out of the 977 eligible unaffected siblings under the age of 20 years (78.3%) gave a blood sample on at least one occasion. The population considered in paper IV comprised all 180 initially unaffected siblings (92 boys) whose first blood sample was taken before the age of 6 years, their median age at sampling being 4.4 years (range 1.25-5.99 years). Blood samples were subsequently taken every 3 months during the initial 2 years after the diagnosis of type 1 diabetes in the index case and at intervals of 6-12 months thereafter. The median period elapsing from the first blood sample to the age of 6 years was 1.1 years. All the siblings were observed for the development of type 1 diabetes at least up to the age of 10 years, diagnosis being based on clinical symptoms and an increased random blood glucose concentration (> 10 mmol/l), or elevated fasting (> 6.7 mmol/l) or random blood glucose levels (>10 mmol/l) on two occasions in the absence of symptoms (Anonymous 1985).
<table>
<thead>
<tr>
<th>Paper</th>
<th>Design</th>
<th>Subjects</th>
<th>N</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Population-based prospective birth cohort study</td>
<td>Genetically susceptible index cases</td>
<td>2448</td>
<td>Autoantibodies, HLA-DQB1 genotype, Progression to type 1 diabetes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genetically susceptible siblings of index cases (DIPP study)</td>
<td>1307</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Nested case-control study within a population-based prospective birth cohort study</td>
<td>Genetically susceptible autoantibody-positive index cases</td>
<td>65</td>
<td>Autoantibodies, HLA-DQB1 genotype, Infant feeding variables, Progression to type 1 diabetes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genetically susceptible autoantibody-negative index cases (controls) (DIPP study)</td>
<td>390</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>Population-based prospective birth cohort study</td>
<td>Genetically susceptible index cases</td>
<td>1005</td>
<td>Autoantibodies, HLA-DQB1 genotype, Progression to type 1 diabetes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Genetically susceptible autoantibody-positive index cases who progressed to type 1 diabetes from the whole DIPP cohort</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>Prospective cohort study</td>
<td>Siblings of children with newly diagnosed type 1 diabetes (DiMe study)</td>
<td>180</td>
<td>Autoantibodies, HLA-DQB1 genotype, Progression to type 1 diabetes</td>
</tr>
</tbody>
</table>
**Methods**

**HLA-DQB1 genotypes**

HLA-DQB1 alleles were analyzed by a polymerase chain reaction (PCR) method as previously described (Sjöroos et al. 1995). The DQB1*02, *0301, *0302, *0602, *0603, and *0604 alleles were identified by a three-color time-resolved fluorescence method.

**Autoantibody assays**

Diabetes-associated autoantibodies were analyzed at the Research Laboratory of the Department of Pediatrics, University of Oulu. All samples from the same child were analyzed in the same assay, except for ICA, to exclude the effect of interassay variation.

**Islet cell antibodies**

ICA were analyzed by a standard indirect immunofluorescence method (Bottazzo et al. 1974). The end-point dilution titers of the ICA-positive samples were recorded and the results expressed in Juvenile Diabetes Foundation Units (JDFU). The detection limit was 2.5 JDFU. All samples initially positive for ICA were retested to confirm this. The sensitivity of the ICA assay in our laboratory was 100% and the specificity 98% in the Fourth International Standardization Workshop (Lernmark et al. 1991). The interassay coefficient of variation was observed to be 22.4% for samples with low titers (10 JDFU) and 26% for samples with high titers (128 JDFU) in the ICA assay (Kulmala et al. 2000b).

**Insulin autoantibodies**

Serum concentrations of IAA were quantified in papers I-III with a microassay (Ronkainen et al. 2001) modified from radiobinding assay described by Williams et al. (1997). The IAA titers representing the specific binding were expressed in relative units (RU) based on a standard curve run on each plate using the MultiCalc™ software program (PerkinElmer Life Sciences Wallac, Turku, Finland). A subject was considered to be positive for IAA when the specific binding exceeded 1.55 RU (the 99th percentile in 371 non-diabetic Finnish subjects). The disease sensitivity of our micro-assay was 35% and the specificity 100%, based on 140 samples derived from the 1995 Multiple Autoantibody Workshop (Verge et al. 1998). The intra-assay coefficient of variation was 7% and the interassay coefficient of variation less than 9% in the IAA assay.

IAA were analyzed in paper IV using a radiobinding assay modified from the liquid phase radioimmunoassay described by Palmer et al. (1983). Endogenous insulin was removed with acid charcoal. Polyethylene glycol was used to separate the free and bound insulin fractions after incubation for 20 h with mono-\textsuperscript{125}I(Tyr\textsuperscript{A14})-human insulin (Novo Research Institute, Bagsvaerd, Denmark). The results were expressed in nU/ml, where 1 nU/ml corresponds to a specific binding of 0.01%. A subject was considered to be IAA positive if the specific insulin bind-
ing exceeded 68 nU/ml (99th percentile in 102 non-diabetic children under the age of 5 years). The sensitivity of the IAA assay was 26% and its specificity 97%, based on 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge et al. 1998). The intra-assay coefficient of variation was less than 5% and the interassay coefficient of variation less than 8% in the IAA assay.

**Autoantibodies to GAD65**

GADA were measured with a radio-binding assay as described by Savola et al. (1998b). The results were expressed in RU based on a standard curve constructed from dilutions of positive and negative samples. The cut-off limit for antibody positivity was set at 5.35 RU, the 99th percentile of 373 non-diabetic Finnish children and adolescents. The disease sensitivity of the GADA assay was 69% and its specificity 100%, based on 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge et al. 1998). The interassay coefficient of variation was 18% at a GADA level of 14.6 RU and 12% at a level exceeding 100 RU in the GADA assay (Savola 1998a).

**Assessment of early infant nutrition**

The children's height and weight were measured at clinical visits and data on breastfeeding and the introduction of cow's milk into the diet were recorded for as long as was appropriate. Exclusive breastfeeding implies that the child did not receive any other food than breast milk, and the first introduction of cow's milk formula, cow's milk or other cow's milk products was taken as the age at which cow's milk feeding was initiated. Maternal age and the duration of the mother's general education were recorded at the time of the child's birth. The duration of exclusive breastfeeding was classified into <2 months (short), 2-3.9 months (intermediate) and ≥4 months (long), that of total breastfeeding into <4 months, 4-5.9 months and ≥6 months, and ≥7 months.
months, and the age at first introduction of cow's milk into <2 months (early), 2-3.9 months (intermediate) and ≥4 months (late). Maternal age, duration of maternal education and child's relative height and weight were regarded as possible confounding factors in the analyses.

**Definitions and statistical analysis**

Persistent positivity was defined as antibody positivity in at least two consecutive samples, the latter being the last available sample. A fluctuating antibody pattern was defined as seroconversion to autoantibody positivity followed by autoantibody negativity and again conversion to autoantibody positivity. Inverse seroconversions included those antibody-positive children who seroconverted to negativity for all autoantibodies. Blood samples obtained during the randomized intervention trial were not taken into account in these definitions. Sensitivity was defined as the proportion of children with a positive outcome who had a positive test value, and specificity as the proportion of children with a negative outcome correctly identified with a negative test value. PPV was defined as the probability that a child with a positive test value will actually have a positive outcome.

When comparing age at seroconversion or seasonal variation in the appearance of the first autoantibodies, the time point for seroconversion was taken to be in the middle of the interval between the last negative sample and the first positive one. Differences in the distributions of individuals between groups were evaluated by cross-tabulation and χ² statistics with the Yates' correction, unless 20% of the cells had an expected value less than five, when the Fisher exact test was used (Swets 1988). Parametric analysis of variance, the paired t-test and the unpaired t-test were used to analyze normally distributed continuous variables and the Mann-Whitney U-test in the case of skewed distributions. The Wilcoxon signed rank test was used in paper I to compare the order of emergence of autoantibodies in those subjects who had at least two disease-associated autoantibodies. Spearman's correlation coefficients were calculated in paper II. Conditional matched logistic regression analysis and Odds ratios (OR) with 95% confidence intervals (CI) were calculated in paper II with the STATA statistical software package, version 6.0 (Stata Corporation, College Station, TX). The 95% CI in paper IV were determined by the exact method. Sensitivity, specificity and PPV were calculated as described by Swets (1988).
RESULTS

Emergence of autoantibodies (I, III)

The prevalence of children who had autoantibodies increased with age. The first IAA and GADA appeared at the age of 3 months, ICA at 6 months and IA-2A at 12 months. IAA achieved a frequency of 2.9% (29 of 1,005) by the age of 2 years, ICA 2.7% (27 of 1,005), GADA that of 1.7% (17 of 1,005) and IA-2A 1.2% (12 of 1,005). In paper I, where only 694 children where monitored to the age of 2 years, the frequency of ICA was 2.2% at the age of 2 years. ICA were detected at least once during the follow-up in thirty-eight (18 boys) out of the 2448 genetically susceptible children (1.6%), at a mean age of 1.2 years (range 0-2.5 years). In paper III, where 1005 genetically susceptible children were observed at least up to the age of 2 years, 63 children (6.3%), of whom 32 were boys, had a minimum of one autoantibody detectable. The proportion of children who had ICA detectable at least once by the age of 2 years was 3.1%, as compared with 4.8% for IAA, 2.3% for GADA and 1.3% for IA-2A, given a median observation period of 3.1 years (range 2.0-5.0 years).

In paper I the first single antibody specificity to appear was IAA in eight infants, GADA in two and ICA in one. IAA were among the first antibodies to appear in 22 of the 25 children (88%) who were positive for at least one other antibody in addition to ICA during the follow-up, and they were also observed among the first antibodies in all 14 children who already had two or more antibodies in their first antibody-positive sample. In paper III, 20 of the 22 children (91%) who persistently had multiple antibodies at the age of 2 years had IAA in their initial antibody-positive sample, and IAA were among the first antibodies to appear in all 15 children who developed type 1 diabetes.

IAA emerged earlier than the other antibodies (IAA vs. ICA; p=0.002, IAA vs. GADA; p=0.019 and IAA vs. IA-2A; p<0.001), while both ICA and GADA appeared earlier than IA-2A (p=0.007 and p=0.049, respectively) in those subjects who had at least two antibodies during the observation period (25/38). In paper III, IAA also appeared at a younger age than ICA in all the cases that tested positive for both (p= 0.001: paired t-test), GADA (p= 0.016) and IA-2A (p= 0.001), whereas ICA appeared at a younger age than IA-2A (p= 0.004). The mean age at seroconversion to positivity for IAA was 1.0 years (range 0.1-1.9 years, median 0.9 years), that for GADA 1.2 years (range 0.1-1.9 years, median 1.2 years), that for ICA 1.3 years (range 0.4-1.9 years, median 1.3 years) and that for IA-2A 1.3 years (range 0.7-1.9 years, median 1.2 years). Thus no significant differences were observed between these autoantibodies in the ages at seroconversion.
The majority of the antibody-positive children (52/63; 83%) had only one autoantibody in their first antibody-positive sample (10 with ICA, 35 with IAA, 6 with GADA and 1 with IA-2A). Among the 23 children who had tested positive for at least two autoantibodies by the age of 2 years, the mean time elapsing between the appearance of the first and second autoantibodies was 0.2 years (range 0-1.1 years).

**Relation between HLA-DQB1 genotype and appearance of autoantibodies (I, III, IV)**

Almost 14% of the newborn infants and 45% of their older sibs showed increased genetic susceptibility to type 1 diabetes. Out of the 25,983 infants genotyped, 780 (3.0%) had the high risk HLA-DQB1 *02/*0302 genotype and 2816 (10.9%) the moderate risk DQB1 *0302/x genotype. About 10% of the older sibs genotyped (139/1307; 10.6%) carried the high risk genotype and more than one third (450; 34.4%) the moderate risk genotype.

The frequency of children who had ICA was significantly higher among those with the high risk genotype (15/548; 2.7%) by the age of 2 years than among those with the moderate risk genotype (23/1900, 1.2%; p=0.019). The sibs carrying the high risk genotype (6.5%; 9/139) similarly had a higher frequency of ICA, although not significantly so, at a mean age of 6.0 years (range, 1.3 to 17.1 years) than in those with the moderate risk genotype (3.3%; 15/450; p=0.17) at a mean age of 6.0 years (range, 1.2 to 22.8 years). The prevalence of autoantibody positivity was observed to increase more rapidly as a function of age in the children with a strong HLA-conferred genetic risk than in those with a moderate risk. The proportion of children who had ICA, GADA and IA-2A was significantly higher among those with the high risk genotype than among those with the moderate risk genotype (p= 0.016; p = 0.006; p= 0.029, respectively; Table 2), and similar proportions of the children tested positive for IAA at the age of 2 years. The initial positive GADA titers were also considerably higher among those who had the high risk genotype (median 26.2 RU) than among those who carried the moderate risk genotype (median 9.8 RU, P = 0.014). Among the children who tested positive for multiple antibodies, the proportion who tested positive for ICA, GADA and IA-2A at the age of 2 years was significantly higher in those with the high risk genotype than in those with the moderate risk genotype (p = 0.008; p < 0.001; p = 0.025, respectively, Fig. 1), but no significant difference was observed in the frequency of IAA. The highest frequencies of positivity for ICA (29.4%), IAA (29.4%) and GADA (35.5%) in the young unaffected siblings of the children with type 1 diabetes were observed among those carrying the high risk genotype, while the highest frequency of IA-2A was seen in the siblings with the moderate risk genotype.
FIGURE 1. Frequencies of ICA (A), IAA (B), GADA (C) and IA-2A (D) from birth to the age of 2 years in children with ≥2 autoantibodies (♦), children with the high risk genotype, HLA-DQB1*02/*0302 (■), and in children with the moderate risk genotype, HLA-DQB1*0302/x, where x = other than *02, *0301, or *0602 (▲).
TABLE 2. Frequency of autoantibodies in relation to HLA-defined genetic disease susceptibility in 1005 children at the age of 2 years

<table>
<thead>
<tr>
<th>Autoantibody</th>
<th>I *02/*0302 (N=254)</th>
<th>II *0302/x (N=751)</th>
<th>Statistics; comparison between I vs. II</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICA, no (%)</td>
<td>13 (5.1)</td>
<td>14 (1.9)</td>
<td>p=0.016</td>
</tr>
<tr>
<td>IAA, no (%)</td>
<td>10 (3.9)</td>
<td>19 (2.5)</td>
<td>p=0.344</td>
</tr>
<tr>
<td>GADA, no (%)</td>
<td>10 (3.9)</td>
<td>7 (0.9)</td>
<td>p=0.006</td>
</tr>
<tr>
<td>IA-2A, no (%)</td>
<td>7 (2.8)</td>
<td>5 (0.7)</td>
<td>p=0.029</td>
</tr>
</tbody>
</table>

The frequency of positivity for multiple antibodies (≥ 2) was closely associated with the degree of genetic susceptibility to type 1 diabetes in the young unaffected siblings of children with type 1 diabetes (Figure 2), and the young children from the general population with the high risk genotype also had persistent multiple antibodies more frequently than did those with the moderate risk genotypes (11 of 254, 4.3%, vs. 11 of 751, 1.5%; p=0.02).

FIGURE 2. Frequencies of siblings testing positive for one autoantibody (open bars) and multiple (≥2) autoantibodies (closed bars) in relation to HLA-defined genetic risk in 180 siblings of children with type 1 diabetes.
Relation between environmental factors and appearance of autoantibodies (I, II)

Seasonal variation in the appearance of the first antibodies

A significantly higher proportion of the antibodies appeared in the fall and winter, from September to February [30 seroconversions (79%) in 4129 samples obtained during that period], than in the spring and summer, from March to August [eight seroconversions (21%) in 5159 samples; p<0.001].

Early infant feeding

There were differences in the duration of exclusive breastfeeding and age at the introduction of cow's milk between the children who had diabetes-associated autoantibodies and their controls. A long duration of exclusive breastfeeding was protective against the appearance of IA-2A (OR 0.24; CI 0.06-0.94; p=0.04) and the development of all four autoantibodies (OR 0.17; CI 0.03-0.86; p=0.03) as compared with a short duration, while an intermediate duration failed to influence the development of positivity in either of these respects. Similarly, the risk of the appearance of IA-2A or positivity for all four autoantibodies was higher in those who received cow's milk early (OR 4.37; CI 1.33-14.42; p=0.02 and OR 5.02; CI 1.27-19.89; p=0.02, respectively) or at an intermediate age (OR 5.50; CI 1.21-25.04; p=0.03 and OR 6.19 (CI 1.10-34.84; p=0.04, respectively) than in those first exposed to it at an older age. These associations remained significant after adjustment for confounding factors (maternal age, duration of mother’s general education and relative height and weight of the child at 12 months) (Table 3). Positivity for ICA, IAA and GADA showed no significant association with duration of breastfeeding or age at the introduction of cow's milk, nor was positivity for IA-2A or all four autoantibodies associated with the total duration of breastfeeding.

The longer the duration of exclusive breastfeeding, the lower the risk of testing positive for IA-2A tended to be when analyzed as a continuous variable (Table 4). This association was significant after adjustment for confounding factors (adjusted OR 0.70, CI 0.49-0.99), and the same risk also tended to be lower the older the infant was when cow's milk-based supplementary feeding was first introduced. There was no significant association between positivity for ICA, IAA and GADA and either the duration of breastfeeding or the introduction of supplementary milk feeding. Consistent with the above observations, the crude ORs showed a protective effect against positivity for all four antibodies (crude OR 0.72, CI 0.52-0.98) the longer the duration of exclusive breastfeeding was when analyzed as a continuous variable (Table 5), and the risk of developing positivity for all four autoantibodies also tended to be lower, the older the infant was when cow's milk-based supplementary feeding was first introduced. There was no significant association between positivity for
one, two or three antibodies and either the duration of breastfeeding or the introduction of supplementary milk feeding.

**Transient and fluctuating antibody patterns (I, III, IV)**

*Inverse seroconversions*

Eight out of the 38 antibody-positive children discussed in paper I (21%) reverted to antibody negativity during the first 2 years of follow-up, and all of them tested positive for ICA only (maximum titer 18 JDFU). Four of them were positive for ICA only once, their highest ICA titer being 10 JDFU. In paper III, where all four autoantibodies were analyzed in all samples at least to the age of 2 years, close to half of the antibody-positive children (29 of 63, 46%) reverted to antibody negativity during the median observation period of 3.1 years (range 2.0-5.0 years) after one (19 of 29) to six positive samples. All of these children tested positive for only one autoantibody by the age of 2 years, and three of them had ICA (maximum titers 5 JDFU, 5 JDFU, 8 JDFU), 22 had IAA (median 4.34 RU, maximum titer 1.93-63.7 RU), three had GADA (maximum titers 10.6 RU, 13.3 RU, 13.9 RU), and one had IA-2A (maximum titer 0.61 RU). IAA disappeared more often than the other autoantibodies (IAA vs. ICA; $P = 0.001$, IAA vs. GADA; $P = 0.012$ and IAA vs. IA-2A $P = 0.021$). Autoantibodies disappeared as frequently in the high-risk genotype group as in those with the moderate-risk genotype. Autoantibodies also disappeared and fluctuated among the children who developed type 1 diabetes, as one child reverted to GADA negativity and another to IA-2A negativity, both presenting later with type 1 diabetes (Figure 3). These two subjects remained positive for the other diabetes-associated autoantibodies, however.

The frequency of inverse seroconversions among the unaffected siblings of the children with type 1 diabetes was 25%, as eight of 33 siblings reverted to antibody negativity by the age of 6 years. Seven of these had had only one autoantibody detectable (three with ICA, two with IAA, one with GADA and one with IA-2A). In one sibling who at one point tested positive for three autoantibodies (ICA, IAA and IA-2A) all of them had disappeared before the age of 6 years.
TABLE 3. ORs (CI) for early infant feeding variables in relation to positivity for autoantibodies, adjusted for maternal age, duration of mother’s general education and relative height and weight at the age of 12 months

<table>
<thead>
<tr>
<th>Variable</th>
<th>ICA (N=65) OR (95% CI)</th>
<th>IAA (N=41) OR (95% CI)</th>
<th>GADA (N=32) OR (95% CI)</th>
<th>IA-2A (N=24) OR (95% CI)</th>
<th>One antibody (N=22) OR (95% CI)</th>
<th>Two to three antibodies (N=24) OR (95% CI)</th>
<th>Four antibodies (N=19) OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration of exclusive breastfeeding</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 - 3.9 months vs. &lt;2 months</td>
<td>1.25 (0.60-2.57)</td>
<td>1.14 (0.44-2.95)</td>
<td>1.13 (0.40-3.21)</td>
<td>0.97 (0.30-3.18)</td>
<td>1.82 (0.50-6.56)</td>
<td>0.95 (0.26-3.51)</td>
<td>0.87 (0.20-3.79)</td>
</tr>
<tr>
<td>≥4 months vs. &lt;2 months</td>
<td>0.89 (0.43-1.83)</td>
<td>0.60 (0.22-1.61)</td>
<td>0.62 (0.21-1.82)</td>
<td>0.10 (0.01-0.82)</td>
<td>1.82 (0.55-6.00)</td>
<td>1.19 (0.34-4.12)</td>
<td>0.07 (0.01-0.79)</td>
</tr>
<tr>
<td><strong>Age at introduction of supplementary milk</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;2 months vs. ≥4 months</td>
<td>1.02 (0.53-1.97)</td>
<td>1.50 (0.62-3.60)</td>
<td>1.56 (0.61-3.97)</td>
<td>7.29 (1.48-36.04)</td>
<td>0.41 (0.13-1.31)</td>
<td>0.77 (0.24-2.53)</td>
<td>9.46 (1.50-59.79)</td>
</tr>
<tr>
<td>2 - 3.9 months vs. ≥4 months</td>
<td>1.61 (0.66-3.96)</td>
<td>2.77 (0.84-9.15)</td>
<td>2.02 (0.54-7.62)</td>
<td>11.98 (1.76-81.68)</td>
<td>0.67 (0.15-3.01)</td>
<td>1.14 (0.23-5.77)</td>
<td>17.91 (1.87-171.52)</td>
</tr>
</tbody>
</table>
TABLE 4. ORs for continuous early infant feeding variables in relation to positivity for ICA, IAA, GADA and IA-2A

<table>
<thead>
<tr>
<th></th>
<th>ICA (N=65)</th>
<th>IAA (N=41)</th>
<th>GADA (N=32)</th>
<th>IA-2A (N=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of exclusive breastfeeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude OR</td>
<td>0.93 (0.80-1.08)</td>
<td>0.93 (0.77-1.14)</td>
<td>0.91 (0.73-1.14)</td>
<td>0.76 (0.58-1.00)</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>0.93 (0.79-1.10)</td>
<td>0.89 (0.71-1.11)</td>
<td>0.88 (0.69-1.13)</td>
<td>0.70 (0.49-0.99)</td>
</tr>
<tr>
<td>Duration of total breastfeeding</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude OR</td>
<td>0.99 (0.93-1.05)</td>
<td>0.97 (0.89-1.06)</td>
<td>0.99 (0.90-1.09)</td>
<td>0.99 (0.89-1.11)</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>1.00 (0.93-1.08)</td>
<td>0.97 (0.87-1.07)</td>
<td>0.99 (0.89-1.11)</td>
<td>1.04 (0.90-1.19)</td>
</tr>
<tr>
<td>Age at introduction of supplementary milk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude OR</td>
<td>0.97 (0.88-1.06)</td>
<td>0.96 (0.84-1.09)</td>
<td>0.94 (0.81-1.08)</td>
<td>0.83 (0.69-1.01)</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>0.96 (0.86-1.07)</td>
<td>0.93 (0.80-1.07)</td>
<td>0.91 (0.77-1.07)</td>
<td>0.78 (0.61-1.00)</td>
</tr>
</tbody>
</table>

*Adjusted for maternal age, duration of mother’s general education and relative height and weight at the age of 12 months
### TABLE 5. ORs for continuous early infant feeding variables in relation to positivity for diabetes-associated autoantibodies

<table>
<thead>
<tr>
<th>Duration of exclusive breastfeeding</th>
<th>One antibody (N=22)</th>
<th>Two to three antibodies (N=24)</th>
<th>Four antibodies (N=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude OR</td>
<td>0.97 (0.76-1.24)</td>
<td>1.10 (0.85-1.41)</td>
<td>0.72 (0.52-0.98)</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>1.07 (0.81-1.42)</td>
<td>0.97 (0.73-1.29)</td>
<td>0.68 (0.46-1.01)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Duration of total breastfeeding</th>
<th>One antibody (N=22)</th>
<th>Two to three antibodies (N=24)</th>
<th>Four antibodies (N=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude OR</td>
<td>1.01 (0.91-1.11)</td>
<td>0.98 (0.88-1.10)</td>
<td>0.97 (0.85-1.10)</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>1.05 (0.94-1.17)</td>
<td>0.93 (0.82-1.05)</td>
<td>1.04 (0.88-1.22)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Age at introduction of supplementary milk</th>
<th>One antibody (N=22)</th>
<th>Two to three antibodies (N=24)</th>
<th>Four antibodies (N=19)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude OR</td>
<td>1.01 (0.87-1.18)</td>
<td>1.02 (0.88-1.19)</td>
<td>0.81 (0.65-1.01)</td>
</tr>
<tr>
<td>Adjusted OR*</td>
<td>1.07 (0.89-1.27)</td>
<td>0.97 (0.82-1.16)</td>
<td>0.77 (0.59-1.02)</td>
</tr>
</tbody>
</table>

*Adjusted for maternal age, duration of mother’s general education and relative height and weight at the age of 12 months
FIGURE 3. Autoantibody status during the observation period in two children who developed type 1 diabetes. The upper panel shows the autoantibodies in a boy carrying the DQB1*0302/x genotype who reverted to negativity for GADA before the presentation of symptoms of type 1 diabetes at the age of 2.5 yr. The lower panel illustrates the autoantibody profile of a boy with the DQB1*0302/x genotype who had low, fluctuating levels of IA-2A and who manifested clinical type 1 diabetes at the age of 2.2 yr. The open symbols represent antibody-negative samples and the closed symbols positive samples.
Genetic disease susceptibility and autoantibodies as predictive markers of type 1 diabetes in young children (I, III, IV)

Genetic screening identified seven out of the 11 young children in the general population (63.6%) who eventually developed type 1 diabetes during the observation period. All the siblings who developed type 1 diabetes showed increased genetic susceptibility to the disease, and the siblings with increased susceptibility had a 18.3% of risk of clinical manifestation before the age of 10 years (15/82). The rate of progression to clinical type 1 diabetes was related to the degree of genetic disease susceptibility among the siblings, with close to 30% of those who had the high risk genotype (5/17) developing clinical diabetes before the age of 10 years, while none of those carrying protective alleles had done so by that age.

Positivity for only one autoantibody was not associated with an increased risk of progression to type 1 diabetes among the unaffected siblings of the affected children, while all six of the 38 ICA-positive children in the general population who progressed to type 1 diabetes had at least two autoantibodies before diagnosis. Positivity for at least two autoantibodies among genetically susceptible children in the general population entailed a 24% (6/25 CI 0.9-45%) risk of developing clinical diabetes during the mean observation period of 2.2 years. Similarly all five of the 1005 children who developed type 1 diabetes tested positive for at least two autoantibodies before diagnosis.

Of the 15 children in the whole DIPP cohort who developed type 1 diabetes, ten (67%) persistently tested positive for at least one autoantibody before diagnosis and 9 (60%) for multiple antibodies. ICA screening identified 93% of those with persistent positivity for at least one autoantibody (27 of 29), IAA 79% (23 of 29), GADA 66% (19 of 29) and IA-2A 41% (12 of 29). All these children were identified by combined screening for ICA and IAA. This antibody combination identified 95.5% of those who persistently tested positive for multiple antibodies (21 out of 22), while GADA identified 86.4% (19 of 22) and IA-2A 54.5% (12 of 22). Among the single antibodies, positivity for IA-2A had the highest specificity (99.9%) and PPV (92.3%) for persistent multiple antibody positivity by the age of 2 years, while combined positivity for ICA and IAA had a sensitivity of 90.9%, a specificity of 99.9% and a PPV of 95.2% in this respect. All 22 children (100%) with persistent positivity for multiple antibodies by the age of 2 years were identified with combined screening for ICA and IAA.

Those young siblings of children with type 1 diabetes who showed increased genetic disease susceptibility and tested positive for at least two autoantibodies by the age of 6 years were found to have a risk of 76.5% (13/17; CI 50-93%) of developing clinical diabetes before the age of 10 years. Positivity for at least two disease-associated autoantibodies as a surrogate marker of the
disease identified 86.7% (13/15; CI 60-98%) of the initially unaffected siblings who reached clinical manifestation before the age of 10 years.

All six children in paper I who developed type 1 diabetes had IAA as their first autoantibody, and three of them also tested positive for ICA in that sample. Primary screening for ICA identified all six children who developed the disease and who had participated in the immunological surveillance. The five children in the cohort of 1005 in paper III who progressed to type 1 diabetes had IAA as their first autoantibody, and one of them also had ICA in that sample, while one other tested positive for ICA, IAA and GADA. Of the 15 children in the DIPP cohort who developed type 1 diabetes, 14 (93%) tested positive for IAA before diagnosis. The one child in whom no autoantibodies were detected in the preclinical period tested positive for ICA and IAA at the time of diagnosis, 8 months after the last antibody-negative sample had been obtained. Twelve of the children (80%) had ICA before diagnosis and 14 had ICA at the time of diagnosis. One of these 15 children tested negative for ICA beforehand and at the time of diagnosis but had had these autoantibodies 0.5 years previously and had GADA at the time of diagnosis. Nine of the 15 children (60%) tested positive for GADA and six for IA-2A (40%) before diagnosis.
DISCUSSION

The establishment of preventive strategies for type 1 diabetes is hampered by our lack of knowledge of the natural history of the pathogenetic process involved in the disease and the fact that environmental factors play a major role in its etiology. The identification of environmental triggers, the timing of the initial event, the progressiveness and persistence of beta-cell autoimmunity once initiated, and the role of factors promoting or preventing progression to clinical disease are some of the open issues, and there has also been intense interest in the identification of the primary autoantigen, in order to develop specific therapies to prevent or delay the onset of type 1 diabetes.

**Induction of beta-cell autoimmunity**

Genetic factors may control the early activation of autoimmunity, as a strong HLA-conferred disease susceptibility has been associated with young age at diagnosis. Our observation that the first autoantibodies often appear in infancy supports such a hypothesis. It has been suggested previously that diabetes-associated autoantibodies appear before the age of 5-6 years in the majority of subjects who later develop type 1 diabetes (reviewed by Leslie and Elliott 1994, Roll et al. 1996), and that the initial events in the development of the disease could occur early in life. There may be a critical period when environmental triggers are deleterious, whereas these putative environmental factors may also induce immunological tolerance and thereby protect the individual from the development of the disease other than during a limited critical period in early childhood. Infants may be most susceptible to environmental agents during the first months of life, when their intestine is relatively permeable (Jakobsson et al. 1986). Furthermore, breastfeeding protects them against infections during the first months of life via maternally transmitted antibodies. We found a closer association between short or intermediate duration of exclusive breastfeeding (<4 months) and the appearance of IA-2A and of all four autoantibodies than in a corresponding analysis performed with continuous variables, indicating that the first few months of life may be a critical period for susceptibility to various environmental factors capable of triggering the destructive process that leads to clinical type 1 diabetes. Previous prospective studies of first-degree relatives of subjects with type 1 diabetes in the United States (Norris et al. 1996), Australia (Couper et al. 1999) and Germany (Hummel et al. 2000) failed to point to any association between early infant nutrition and the appearance of diabetes-associated autoantibodies. The discrepancy between these results and the above observations may be due...
to differences in study design, for the relation between the appearance of IA-2A or of all four diabetes-associated autoantibodies and early infant nutrition has not been analyzed previously. In addition, the controls in our prospective population-based DIPP project were matched for the HLA-DQB1 genotype. Further evidence for the role of environmental factors in the pathogenesis of type 1 diabetes was obtained, as there was an apparent seasonal variation in the appearance of the first autoantibodies, suggesting that infectious agents could be involved in the induction of beta-cell autoimmunity. The role of putative environmental triggers in the pathogenesis of type 1 diabetes may be complex, since our knowledge of their interactions and relations with genetic factors is still limited. It is also unclear whether single or multiple exposure to environmental triggers is needed to initiate and promote the disease process.

It has been suggested that insulin may be a central autoantigen in the development of type 1 diabetes (Eisenbarth 1994), and further evidence for a single target autoantigen was provided by our observation that insulin may be the primary antigen. A recent study employing NOD mice demonstrated that the age of appearance of IAA correlated negatively with development of autoimmune diabetes (Abiru et al. 2001). The present results revealed that IAA were the most common autoantibody specificity among infants and young children, that they appeared earlier than ICA, GADA or IA-2A, and that they were also among the first autoantibodies to appear in those children who developed type 1 diabetes or had signs of biologically significant beta-cell autoimmunity and tested positive for at least two autoantibodies. It was concluded in a German report that no specific order prevailed in the emergence of autoantibodies, regardless of the findings of these authors and others that IAA are common in young children and are among the first autoantibodies to appear (Roll et al. 1996). The role of insulin as a primary antigen in the pathogenesis of type 1 diabetes is challenged by the finding that GAD expression is essential for the induction of diabetogenic T cells in NOD mice and that this cannot take place in its absence GAD (Yoon et al. 1999). GAD has also been observed to provoke the earliest T-cell proliferative response in NOD mice (Kaufman et al. 1993). It is likely, however, that insulin-specific T cells may play an important role in the pathogenesis of type 1 diabetes, as it has been shown that autoimmune diabetes can be transferred experimentally by insulin-reactive T cells (Daniel et al. 1995). The development of IAA may be controlled by specific HLA genes, as young patients with type 1 diabetes are more likely to have HLA susceptibility genes than patients with onset of the disease later in childhood. Thus although IAA emerged at a similar rate in the high and moderate risk genotype groups in our series, all the infants in these two groups carried at least one DQB1*0302 allele. Interestingly, IAA may be related to the early introduction of cow's milk, as cow's milk feeding has recently been
reported to be an environmental trigger of an immune response to bovine insulin in infancy (Vaarala et al. 1999). In that series a few infants developed signs of beta-cell autoimmunity and failed to develop the normal tolerance of bovine insulin, raising the possibility that the initial immune response to bovine insulin may have been switched for an autoimmune response to human insulin in these individuals. Insulin could still play a role in the induction of beta-cell autoimmunity in older children although IAA may disappear from the peripheral circulation in early childhood (Ziegler et al. 1999). Autoimmunity in early childhood may be a more common phenomenon than has been suggested earlier, in view of our finding that transient IAA positivity was relatively frequent in young children. The possibility that the conspicuously high frequency of transient IAA observed here compared with earlier reports may be attributable to methodological differences is hardly relevant, since the microassay used in our analysis is more specific than the conventional radioimmunological method more commonly used previously. Although T cells are thought to play a central role in the pathogenesis of type 1 diabetes, the relation of IAA and T-cell autoreactivity to insulin in young children has not been defined.

The development of beta-cell autoimmunity

Beta-cell destruction may be a cumulative process, each stage being initiated by an environmental trigger and multiple episodes succeeding in destroying the beta cells, eventually resulting in the development of clinical type 1 diabetes (Gorsuch et al. 1981, reviewed by Palmer and McCulloch 1991). The rate of progression to type 1 diabetes varies widely. Rapid progression was observed in some young children in the DIPP project (paper III), whereas slow progression was found in some of the DiMe children (paper IV). The young children who experienced rapid progression to type 1 diabetes may have been exposed to an exceptionally strong environmental trigger early in life, or alternatively they might have been more susceptible to the putative trigger than those who developed the disease more slowly or not at all. The rate of the progression of beta-cell autoimmunity may also be affected by genetic differences, as supported by the finding that the prevalence of autoantibodies increased as a function of age more rapidly in children carrying a high genetic risk of type 1 diabetes than in those with a moderate risk genotype. Also the rate of progression to clinical disease in the young initially unaffected siblings of children with type 1 diabetes was related to the degree of genetic disease susceptibility. It is generally assumed that once diabetes-associated autoantibodies have appeared, they will usually be detectable at least until diabetes is diagnosed and in some cases for
years afterwards. Our finding of a high prevalence of transient autoantibodies supports the idea that the process of beta-cell destruction need not always lead to presentation with the clinical disease. The mechanisms that regulate the progression of the autoimmune process remain open, although our results suggest that early introduction of cow's milk may act as a promoter of progressive beta-cell destruction rather than as a trigger of the process, at least in some children who develop signs of autoimmunity during the first years of life.

**Identification of subjects with a high risk of type 1 diabetes**

The identification of genetically susceptible infants within the general population at birth would substantially reduce the number of subjects to be observed for the appearance of signs of beta-cell autoimmunity in order to recognize individuals with a high risk of progression to type 1 diabetes. This would also increase the predictive characteristics of diabetes-associated autoantibodies. In order to achieve the optimal predictive value, the autoantibodies should have certain characteristics. Ideally every subject who is progressing to clinical disease should have the autoantibody (100% sensitivity), while everyone without the autoantibody would remain healthy (100% specificity), and every healthy autoantibody-positive individual should eventually present with the clinical disease (100% PPV). Although the maximal PPV for type 1 diabetes will be reached in genetically susceptible subjects testing positive for autoantibodies, the sensitivity will be reduced when autoantibodies and genetic susceptibility are combined, since a proportion of patients with type 1 diabetes carry neutral or even protective HLA genotypes.

Although markers characterized by high sensitivity and high specificity for type 1 diabetes have been identified, the rate of progression to the disease is difficult to predict. Also, only sparse data are available on the risk associated with the early appearance of diabetes-associated autoantibodies, although prospective studies have shown that the prevalences of various autoantibodies at diagnosis are similar to the frequencies seen in the preclinical period, this does not necessarily reflect their predictive characteristics during the early phases of the autoimmune process. As the initial event of the pathogenetic process may occur early in life, prevention studies should mainly target young children. The younger the children are, the more frequent the monitoring should be, in order to identify the signs of beta-cell autoimmunity as early as possible. Non-diabetic young siblings of affected children comprise another special group of subjects at high risk. Although only about 10% of new cases with type 1 diabetes are diagnosed in individuals with affected first-degree relatives, the diagnosis of the disease in one child in the family will most likely arouse parental concern regarding its possible development in other chil-
dren. Increased knowledge of the risk of an unaffected sibling developing the disease may relieve the feelings of uncertainty and concern in many parents, even when a child has a high risk of progression. On the other hand, the knowledge of an increased risk may cause some parents great anxiety, since there are no effective, clinically applicable means available for preventing or delaying the disease at the moment. As the American Diabetes Association has stated, screening for the risk of type 1 diabetes and intervention for its prevention should at present be attempted only in the context of well-defined research efforts (Anonymous 1999a).

Our genetic screening identified most of those children in the general population who developed type 1 diabetes, in line with a previous estimate that our genetic criteria would recognize 60 to 80% of future cases (Ilonen et al. 1996). The strategy based on two-phase screening aimed at identifying subjects with a high risk of progression within the general population following initial genetic screening, turned out to be feasible and to provide a cost-saving alternative to a pure immunological screening strategy (Hahl et al. 1998).

Primary screening for ICA identified the majority of the children who developed type 1 diabetes among those who participated in the immunological surveillance. This suggests that the present screening strategy will identify most of the young children who will eventually develop autoimmune diabetes. The results also showed that IAA provide a sensitive marker of beta-cell autoimmunity, particularly in young children, although transient IAA positivity was common. IA-2A were characterized by high specificity, but they were among the last antibodies to appear during the preclinical process. The combination of ICA and IAA yields probably the highest predictive characteristics for the identification of young children with an increased risk of developing type 1 diabetes. The present sample was relatively small in size, however, and additional studies on the predictive characteristics of ICA, IAA, GADA and IA-2A are needed to establish optimal type 1 diabetes screening strategies for use with young children.

Positivity for a single autoantibody was not associated with an increased risk of type 1 diabetes among unaffected siblings of children with the disease. As a conspicuous proportion of the autoantibodies observed in young children in the general population disappear spontaneously, the risk of progression to type 1 diabetes was low among such children when they tested positive for a single autoantibody during the first years of life. This high prevalence of transient autoantibodies in fact supports the idea that there is a critical period early in childhood when type 1 diabetes might be induced. Findings in recent animal experiments nevertheless suggest that the emergence of IAA does not always predict the development of type 1 diabetes (Abiru et al. 2001). The significance of transient autoantibody positivity is unclear, although it might still be associated with a risk of progression to
type 1 diabetes later in childhood. Conversely, positivity for multiple autoantibodies was associated with high risk of developing type 1 diabetes, and was closely linked to the degree of genetic susceptibility among young siblings of affected children. Risk assessment may be further improved by gathering further information on the insulin response to intravenous glucose in carriers of two or more autoantibodies.

**Future prospects**

This work was focused on evaluating the natural history of beta-cell autoimmunity. When the genetically susceptible children derived from the general population in the DIPP project have been monitored for some time to come it may prove possible to define more precisely the complex pathogenetic process involved in this multifactorial disease. The impact of early infant nutrition can definitely be assessed by intervention trials such as Trial to Reduce IDDM in the Genetically at Risk (TRIGR), but large prospective cohort studies starting from birth are needed in addition in order to assess the role of the diet throughout childhood in the etiology of type 1 diabetes, including several other proposed food diabetogens. Possible interactions between nutritional factors and infections, e.g. enterovirus infections, pose intriguing challenges for future research. Increased knowledge of the putative environmental factors that may initiate the pathogenetic process could facilitate the elimination of such factors in high-risk individuals, and more extensive knowledge of the natural history of beta-cell autoimmunity, more accurate means of risk assessment, and more effective screening techniques would make it possible to establish effective preventive measures for type 1 diabetes. Such intervention could replace or postpone insulin therapy and thereby reduce the long-term complications of type 1 diabetes. Even a delay in clinical presentation during early childhood could have a profound effect on the quality of life of both the child and the family. A critical factor in achieving success in preventing type 1 diabetes may be the initiation of therapy early in the pathogenetic process, for which purpose the identification of subjects having a high risk of developing the disease is of vital importance.
The purpose of this research was to evaluate the natural history of beta-cell autoimmunity and the impact of the appearance of autoantibodies on the development of type 1 diabetes among young children. The population comprised genetically susceptible children identified from the general population and observed from birth, together with young unaffected siblings of children with type 1 diabetes.

Although clinical type 1 diabetes is still rare in infants, the autoimmune process may be initiated early in life. Evidence for a single target autoantigen is presented here, indicating that insulin may be the primary antigen in most cases of human type 1 diabetes. IAA were the most frequently detected autoantibody type in the young children studied, appearing earlier than ICA, GADA or IA-2A. IAA was also among the first antibodies to emerge in those children who developed type 1 diabetes or showed signs of biologically significant beta-cell autoimmunity by testing positive for at least two autoantibodies. There is substantial individual variation in the prediabetic process, however, and transient beta-cell autoimmunity seems to be a more common phenomenon than had previously been assumed, supporting the suggestion that beta-cell autoimmunity does not necessarily lead to the development of clinical disease. The present results provide evidence that multiple autoantibodies (≥ 2) can be used as a marker of biologically significant beta-cell autoimmunity in young siblings of affected children that will result in the manifestation of clinical type 1 diabetes within the next few years in an overwhelming majority of the cases. In fact, the vast majority of subjects with a high risk of developing type 1 diabetes during early childhood could be identified by the emergence of multiple autoantibodies.

Further evidence for the role of environmental factors in the pathogenesis of type 1 diabetes was obtained, in that a long duration of exclusive breastfeeding and late introduction of cow's milk may protect genetically susceptible children from progressive beta-cell destruction during the first years of life. The seasonal variation observed in the appearance of the first autoantibodies suggests that infectious agents could be implicated in the induction of beta-cell autoimmunity.

The age of the target population should be taken into account when planning screening for type 1 diabetes, since there seem to be age-related differences in the predictive characteristics of the autoantibodies. Early screening for signs of beta-cell autoimmunity is essential in order to identify children with a high risk of developing type 1 diabetes, so as to facilitate intervention before the disease process is too advanced. In conclusion, the autoimmune process involved in type 1 diabetes may already be initiated during the first
year of life, and both genetic and environmental factors are involved in the emergence of beta-cell autoimmunity.
ACKNOWLEDGEMENTS

The present work was carried out at the Department of Pediatrics, Medical School, University of Tampere and at the Department of Pediatrics, Tampere University Hospital in collaboration with Departments of Pediatrics and Virology, University of Turku and Department of Pediatrics, University of Oulu.

I wish to express my deepest gratitude to Professor Mikael Knip, M.D., previous Head of the Department of Pediatrics, Medical School, University of Tampere, and one of the Principal Investigators of the DIPP project, who as a supervisor of this thesis introduced me to scientific work. His skillful guidance, expertise and encouragement have been indispensable to this work.

I am grateful to Professor Markku Mäki, M. D., and Docent Matti Salo, M.D., for providing me with the research facilities of the Department of Pediatrics.

My sincere thanks are due to the official referees of this study, Professor Timo Palosuo, M.D. and Docent Ilkka Sipilä, M.D. for their careful review and constructive comments to the manuscript.

I am grateful to the Principal Investigators of the DIPP project, Professor Olli Simell, M.D., and Docent Jorma Ilonen, M.D., for their kind collaboration and their contribution to the original articles presented in this thesis. I wish to express my warm thanks to Emeritus Professor Hans K. Åkerblom, M.D., Principal Investigator of the Childhood Diabetes in Finland (DiMe) Study, for his encouraging comments and for the possibility to use data from the DiMe Study. I wish to thank Docent Suvi Virtanen, M.D., M.Sc., for her valuable advice during various parts of this work.

I wish to express my warm thanks to my co-authors Maijaliisa Erkkola, M.Sc., Anu-Maaria Hämäläinen, M.D., Päivi Keskinen, M.D., Sari Korhonen, M.D., Marika Kukko, M.B., Petri Kulmala, M.D., Antti Kupila, M.D., Helena Reijonen, Ph.D., Kaisa Savola, M.D., Tuula Simell, Ph.D., and Paula Vähäsalo, M.D., with whom it has been a pleasure to work.

Malcolm Hicks, M.A., whom I sincerely thank, revised the English language of the manuscript and the original papers.

I wish to thank all the other members of the DIPP project in Tampere, Turku and Oulu.

I am grateful to the members of the Tampere Diabetes Research Center for creating a pleasant and stimulating research atmosphere; special thanks are due to Docent Heikki Hyöty, M.D. and Maria Lönnrot, M.D.

I wish to thank the staff in the Tampere DIPP project and the personnel of the Department of Pediatrics for their friendly attitude and cooperation during these years.

All children and their parents participating in the DiMe Study and the DIPP project are warmly acknowledged.
I sincerely thank the personnel of the Medical Libraries of the Medical School and the University Hospital in Tampere.

I wish to thank warmly all my friends for their supporting attitude during these years. I am especially grateful to Taina Arvola, M.D., my dear friend and colleague for encouragement and for sharing various joys and sorrows of research work and life during these years.

I am also deeply grateful to my parents for their support over these years.

Last but not least I wish to thank my beloved husband Harri for his generous and skillful IT-support, encouragement and loving care. In addition, his sympathetic care of our dear daughter Anni has made the final preparation of this work possible. I am grateful to Anni for filling our lives with joy and for reminding me of the basic values of life.

Permission from the copyright owners of the original articles to reproduce the original contributions of this thesis is gratefully acknowledged. This work was financially supported by grants from the Foundation for Pediatric Research in Finland, the Medical Research Fund of Tampere University Hospital, the Finnish Medical Foundation, and the University of Tampere. The DIPP project also received financial support from the Juvenile Diabetes Research Foundation International, the Medical Research Funds; Turku and Oulu University Hospitals, the Medical Research Council, Academy of Finland and Novo Nordisk Foundation and EU Biomed 2.

Nummela, February 2002
REFERENCES


of patients with IDDM. J Clin Endocrinol Metab 80:3739-3743.


Dahlquist G, Blom L, Tuvemo T, Nyström L, Sandström A and
Wall S (1989): The Swedish childhood diabetes study - Results from a nine year case register and a one year case-referent study indicating that type 1 (insulin-dependent) diabetes mellitus is associated with both type 2 (non-insulin-dependent) diabetes mellitus and autoimmune disorders. Diabetologia 32:2-6.


GAD$_{65}$-autoantibodies in new-onset IDDM patients and help predict impending diabetes in their siblings. Diabetologia 40:95-99.


cohort of twins in Finland. Diabetologia 35:1060-1067.
Komulainen J, Knip M, Sabbah E, Vähäsalo P, Lounamaa R, Åker-


Myers MA, Rabin DU and Rowley MJ (1995): Pancreatic islet cell cytoplasmic antibody in diabetes is
represented by antibodies to islet cell antigen 512 and glutamic acid decarboxylase. Diabetes 44:1290-1295.


Platz P, Jakobsen BK, Morling N, Ryder LP, Svejgaard A, Thomsen M, Christy M, Kromann H, Benn J,


Rewers M, Bugawan TL, Norris JM, Blair A, Beaty B, Hoffman M, McDuffie Jr. RS, Hamman RF,


Rubinstein P, Walker ME, Fedun B, Witt ME, Cooper LZ and Gins-


Vandewalle CL, Decraene T, Schuit FC, De Leeuw IH, Pipeleers DG, Gorus FK and the Belgian Diabetes Registry (1993): Insulin autoantibodies and high titre islet cell antibodies are preferentially associated with the HLA DQA1*0301-DQB1*0302 haplotype at clinical onset of type 1 (insulin dependent) diabetes mellitus.
before age 10 years, but not at onset between age 10 and 40 years. Diabetologia 36:1155-1162.


Virtanen SM, Räsänen L, Aro A, Ylönen K, Lounamaa R, Åkerblom HK, Tuomilehto J and the 'Childhood Diabetes in Finland' Study Group (1994b): Is children's or parents' coffee or tea consumption associated with the risk for type 1 diabetes mellitus in


Vreugdenhil GR, Geluk A, Ottenhoff THM, Melchers WJG, Roep BO and Galama JMD (1998): Molecular mimicry in diabetes mellitus: the homologous domain in coxsackie B virus protein 2C and islet autoantigen GAD65 is highly conserved in the coxsackie B-like entroviruses and binds to the diabetes associated HLA-DR3 molecule. Diabetologia 41:40-46.


