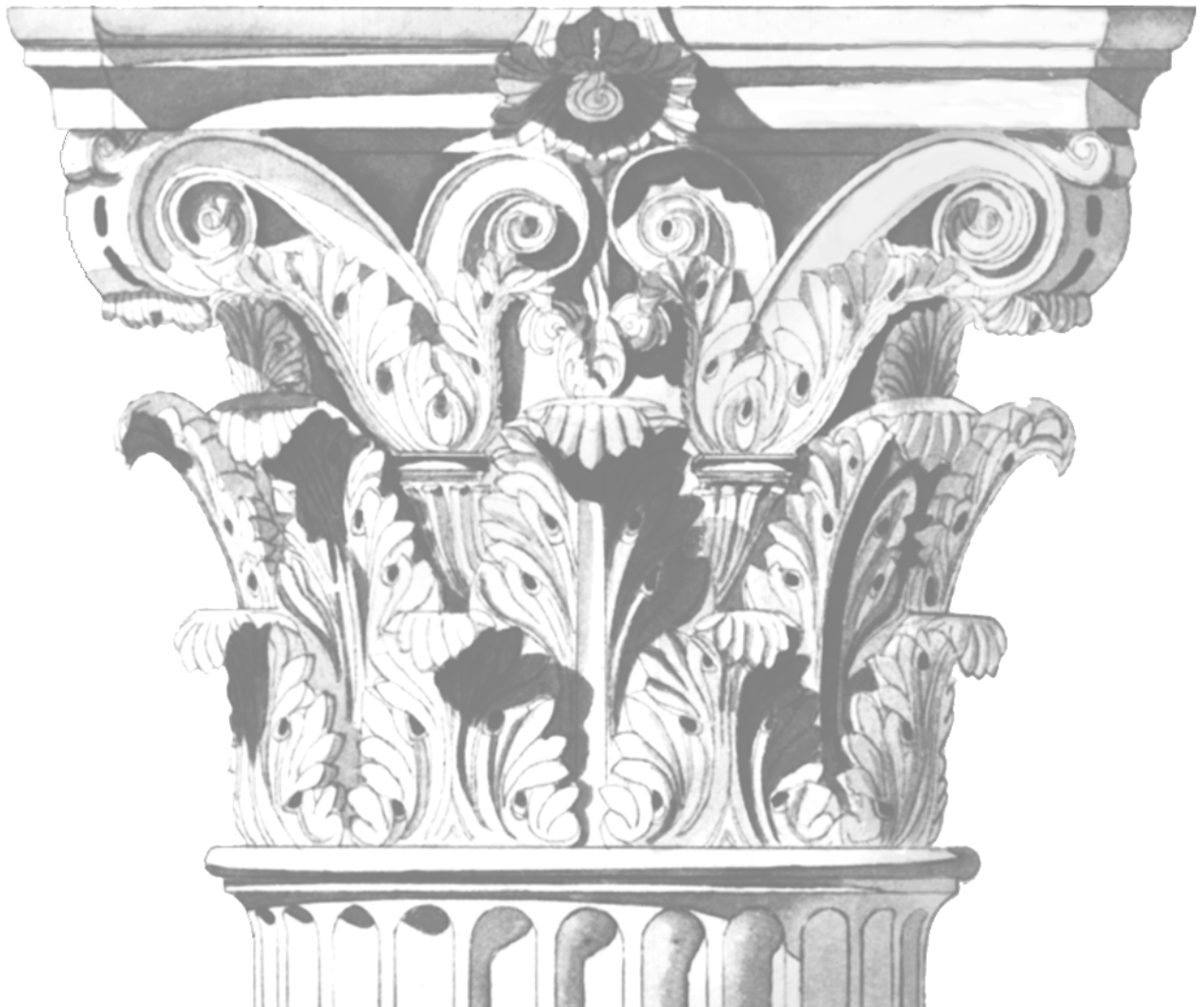


**ROLE OF IA-2 ANTIBODIES IN
CLINICAL AND PRECLINICAL
TYPE 1 DIABETES**

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Department of Paediatrics

OULU 2000



KAISA SAVOLA

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CLINICAL AND PRECLINICAL TYPE 1
DIABETES**

Academic Dissertation to be presented with the assent of the Faculty of Medicine, University of Oulu, for public discussion in Auditorium 12 of the University Hospital of Oulu, on June 16th, 2000, at 12 noon.

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Abstract

Previous scientific data suggest that beta-cell destruction in type 1 diabetes is mediated by an autoimmune process. This work was aimed at expanding existing knowledge of humoral autoimmunity by analysing antibodies against the intracellular part of the IA-2 protein (IA-2A) in 1200 patients with the disease, 750 siblings and more than 370 non-diabetic controls.

IA-2A were present at the time of diagnosis in the overwhelming majority of patients with type 1 diabetes, and were associated with human leucocyte antigen (HLA) DR4 and DQB1*0302, but not with gender. Humoral autoimmunity was more marked in patients diagnosed when younger than 20 years of age than in older ones, but no noticeable association was observed between IA-2A and age under the age of 20 years. IA-2A in combination with antibodies to GAD₆₅ (GADA) identified a higher proportion of patients younger than 15 years of age at the time of diagnosis than did islet cell antibodies (ICA) alone.

The levels of IA-2A and the proportions of antibody-positive patients decreased with increasing duration of type 1 diabetes, although more than half of the patients still tested positive for IA-2A after 10 years of clinical disease.

IA-2A, GADA, insulin autoantibodies (IAA) and ICA were detected with individual fluctuations in 8-14% of the siblings of children with type 1 diabetes monitored from the time of diagnosis of the proband, and the fluctuations were modified by HLA-defined genetic susceptibility, age of the siblings, family size and total number of detectable autoantibodies. IA-2A positivity detected at the time of diagnosis of the proband increased the risk of future disease in the siblings. The positive predictive value increased with increasing IA-2A levels, although individual risk assessment appeared to be a complex matter.

In conclusion, IA-2 appears to be an important autoantigen in type 1 diabetes, since IA-2A is associated with the HLA haplotype that most strongly predisposes subjects to the disease and have the highest positive predictive value for future disease out of the four autoantibodies used for risk assessment purposes.

Key words: humoral autoimmunity, HLA risk markers, risk assessment, seroconversion

To my sister Suvi

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Oulu, May 2000

Kaisa Savola

Abbreviations

aa	amino acids
BSA	bovine serum albumin
Cdc2	a kinase regulated during the cell division cycle (cdc)
cDNA	complementary deoxyribonucleic acid
CPP32	a cytoplasmic protease
DENIS	German Nicotinamide Diabetes Intervention Study
DiMe	Childhood Diabetes in Finland Study
DIPP	Diabetes Prevention and Prediction Study
DPT-1	Diabetes Prevention Trial of Type 1 Diabetes
ELISA	enzyme-linked immunosorbent assay
ENDIT	European Nicotinamide Diabetes Intervention Trial
Fas	a cell surface molecule linked with apoptosis
GAD	glutamic acid decarboxylase
GADA	antibodies to glutamic acid decarboxylase
HbA ₁	haemoglobin A1
HbA _{1c}	haemoglobin A1 _c
H-E staining	haematoxylin and eosin staining
HLA	human leucocyte antigen
IA-2 β	a beta cell protein belonging to the family of PTPs
IA-2, ICA512	a beta cell protein belonging to the family of PTPs
IA-2A	antibodies to the intracellular part of the IA-2 protein
IAA	insulin autoantibodies
ICA	islet cell antibodies
ICA69	69 kDa islet cell autoantigen
IFN	interferon
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible isoform of nitric oxide synthase
JDF-U	Juvenile Diabetes Foundation Unit
kDa	kilodalton
MHC	major histocompatibility complex

mRNA	messenger ribonucleic acid
NOD	non-obese diabetic mouse
PCR	polymerase chain reaction
PTP	protein tyrosine phosphatase
RIA	radioimmunoassay
ROC	receiver operating characteristics
Ser	serine
Th	T-helper lymphocyte
Thr	threonine amino acid
TNF	tumor necrosis factor
Tyr	tyrosine amino acid

List of original papers

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

- I Savola K, Bonifacio E, Sabbah E, Kulmala P, Vähäsalo P, Karjalainen J, Tuomilehto-Wolf E, Meriläinen J, Åkerblom HK, Knip M, the Childhood Diabetes in Finland Study Group. IA-2 antibodies - a sensitive marker of IDDM with clinical onset in childhood and adolescence. *Diabetologia* 1998; 41:424-429.
- II Sabbah E, Savola K, Ebeling T, Kulmala P, Vähäsalo P, Salmela PI, Ilonen J, Knip M. Genetic, autoimmune, and clinical characteristics of childhood and adult onset Type 1 diabetes. *Diabetes Care* 2000; 23, in press.
- III Savola K, Sabbah E, Kulmala P, Vähäsalo P, Ilonen J, Knip M. Autoantibodies associated with type 1 diabetes mellitus persist after diagnosis in children. *Diabetologia* 1998; 41:1293-1297
- IV Savola K, Läärä E, Vähäsalo P, Kulmala P, Åkerblom HK, Knip M, the Childhood Diabetes in Finland Study Group. Dynamic pattern of disease-associated autoantibodies in siblings of children with Type 1 diabetes. Submitted for publication.
- V Kulmala P, Savola K, Petersen JS, Vähäsalo P, Karjalainen J, Löppönen T, Dyrberg T, Åkerblom HK, Knip M, the Childhood Diabetes in Finland Study Group. Prediction of insulin-dependent diabetes mellitus in siblings of children with diabetes - a population-based study. *J Clin Invest* 1998; 101:327-336.

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1 Introduction

A group of differing metabolic diseases are characterized by similar abnormalities including hyperglycaemia, and are therefore termed diabetes mellitus (1). Diabetes mellitus is classified into four categories: type 1 diabetes, type 2 diabetes, other specific types and gestational diabetes mellitus.

Type 1 diabetes is subclassified into immune-mediated diabetes, formerly known also as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes, and idiopathic diabetes (1). In the immune-mediated type the beta cells are destroyed by a cell-mediated autoimmune process, of which ICA, IAA, GADA, IA-2A and antibodies to IA-2 β serve as markers (2-5). This disease type also has strong HLA associations (6). The destruction of beta cells leads to insulin deficiency and abnormalities in carbohydrate, fat and protein metabolism. The symptoms of hyperglycaemia caused by insulin deficiency consist of polyuria, polydipsia and weight loss, and untreated hyperglycaemia leads to life-threatening ketoacidosis or to the non-ketotic hyperosmolar syndrome. Patients need regular and life-long insulin injections for survival. In spite of diligent care, patients with type 1 diabetes carry an increased risk of long-term complications that include retinopathy, nephropathy, neuropathy and cardiovascular disease.

Type 2 diabetes, previously known also as non-insulin-dependent diabetes, or adult-onset diabetes, is characterised by impaired metabolic activity of insulin on target tissues, known as insulin resistance. Insulin deficiency is usually relative rather than absolute. Patients are often obese, and they do not usually need insulin treatment to survive, so that the risk of ketoacidosis is low. There are probably many causes, but immune-mediated beta-cell destruction does not occur in true type 2 diabetes (1).

Other specific types of diabetes include genetic defects in the beta cells, diseases of the exocrine pancreas, endocrinopathies, diabetes induced by drugs, chemicals or infections, uncommon forms of immune-mediated diabetes and other genetic syndromes that are sometimes associated with diabetes. Gestational diabetes mellitus implies hyperglycemia recognized for the first time during pregnancy, with most patients returning to normoglycaemia after delivery (1).

Although the treatment of type 1 diabetes has improved considerably over recent decades because of more reliable blood glucose monitoring and more effective insulin preparations, it is still one of the most severe chronic diseases both for the patient and for

society at large. Research into type 1 diabetes is aimed ultimately at prevention of this form of the disease.

2 Review of the literature

2.1 Pathogenesis of type 1 diabetes

2.1.1 Mechanisms of beta-cell destruction

The way in which beta cells die, by apoptosis or necrosis or a combination of both, is still controversial. A hypothetical possibility is that beta cells are killed by cytotoxic T-cells using perforin or granzymes as effector molecules (7). Perforin and granzymes enter the target cells via pores induced in their plasma membranes causing necrosis and apoptosis.

On the other hand, the beta cells may be destroyed via apoptosis induced by altering their microenvironment (8). Hypothetically, antigen-specific T-helper cells transcribing cytokine genes attract cytotoxic T cells, natural killer cells and macrophages into the islets and activate endothelial cells to express adhesion molecules, class I and class II HLA molecules, and to secrete mediators of inflammation (9). These include IFN- γ , which stimulates the recruited macrophages to produce IL-1 β and TNF- α , and together with these induces e.g. the inducible nitric oxide synthase (iNOS) and other putative apoptosis-activating pathways in the beta cells, leading to cell death (9). This model also implies IL-1-mediated Fas expression on beta cells, resulting in beta-cell apoptosis via interaction between these Fas molecules of the beta cells and the Fas ligands of Th1 and cytotoxic T-cells (9).

2.1.2 The Th1/Th2 paradigm

The cytokines involved in beta-cell destruction are secreted by T-helper cells, which are traditionally divided into two groups, Th1 and Th2, according to their distinct cytokine secretion, although other Th types also exist (9-11). Th1 cells produce interleukin-2 (IL-2), interferon- γ (IFN- γ) and tumor necrosis factor- β (TNF- β), while Th2 cells secrete IL-4, IL-5, IL-6, IL-10 and/or IL-13. Th1 clones activate macrophages (enhanced cellular

immunity), while Th2 clones help B cells to develop into autoantibody producing cells (11,12). Moreover, the Th1 and Th2 responses are thought to be mutually inhibitory (10). Th1 cells have been shown experimentally to induce diabetes, whereas Th2 cells do not lead to the disease (10). Mutual inhibition has not been clearly documented (13). Expression of IL-1, TNF- α , IFN- α and γ , IL-6 and IL-12 mRNAs, and partly also of the corresponding proteins, has been demonstrated in insulinitis in animal models of autoimmune diabetes (9).

The role of cytokines in human type 1 diabetes is a controversial issue. IFN- α , IFN- β and IL-6 have been detected in the pancreas of a patient with newly diagnosed type 1 diabetes (14), and IL-2, IFN- γ and the macrophage-derived cytokines TNF- α and IL-1 α were elevated in the sera of such patients as compared with control subjects or patients with long-standing type 1 diabetes, type 2 diabetes or Grave's disease (15). TNF- α and IL-1 α were elevated more frequently in prediabetic twins of patients with type 1 diabetes than in discordant twins (15), and healthy first degree relatives of patients with type 1 diabetes also exhibited signs of overproduction of TNF- α and soluble interleukin-2 receptor in their sera (16). Not all studies on this topic have provided consistent results, and assessments of cytokine secretion from stimulated peripheral blood mononuclear cells have likewise resulted in conflicting data (9).

2.1.3 Evidence for a T-cell mediated autoimmune disease

B cells synthesizing IgG were detected in the pancreas of a child who died of diabetic ketoacidosis, and the lymphocytes around the islets were primarily T lymphocytes, the predominant subpopulation being cells expressing CD8 on their surface, although cells expressing CD4 and natural killer/killer cells were also observed (17). Another patient of death from ketoacidosis at the age of 19 years had lymphocyte infiltration in 18% of the pancreatic islets, comprising T lymphocytes and macrophages but not B lymphocytes or plasma cells (14). Two patients dying as a consequence of cerebral oedema within 24 hours of the diagnosis of type 1 diabetes had a similar loss of beta cells, but showed heterogeneity in the lymphocytic infiltration in their islets: one patient had severe insulinitis in most of the islets containing both B and T lymphocytes, whereas only single islets were affected in the other one. Both patients had macrophages in their islets (18). Lymphocytic infiltration within or around the islets was hardly seen at all with H-E staining in pancreas biopsies from 7 patients aged 24 or over with newly diagnosed diabetes (19). Using various methods to define insulinitis in pancreas biopsy specimens from 18 adult patients with newly diagnosed type 1 diabetes, insulinitis was seen in eight of them (20). The mononuclear cell infiltration comprised of CD8⁺T, CD4⁺T, B lymphocytes and macrophages. More recently, nine out of 17 newly diagnosed type 1 diabetic patients aged 17 years or more were observed to have insulinitis in their islets (21). These data indicate that mononuclear cells, T lymphocytes and macrophages in particular, are associated with insulinitis at the diagnosis of type 1 diabetes and that they are involved in the pathogenesis of the disease. Heterogeneity in the intensity of insulinitis may reflect individual dynamic pattern of beta cell destruction. Evidence of delayed diabetes

manifestation in cyclosporin-treated relatives of diabetic patients with ICA ≥ 20 JDF-U, first phase insulin response $< 10^{\text{th}}$ percentile and impaired glucose tolerance (22), the observation of recurrent autoimmune diabetes in recipients of cadaveric pancreatic grafts (23) and the transfer of type 1 diabetes by bone marrow cells (24) have confirmed the immune nature of the disease and the role of T cells in the pathogenesis of type 1 diabetes. Several groups have demonstrated the presence of T cells reacting in response to human beta cells or beta-cell associated proteins in the peripheral blood of patients with newly diagnosed type 1 diabetes and in prediabetic subjects (25). The data are controversial, however, since autoreactive T cells have also been observed in healthy HLA-matched and age-matched non-diabetic control subjects, although less frequently and at a less reactive level than in patients with type 1 diabetes (25). Circulating autoreactive T cells alone do not reflect ongoing disease, as other immunological abnormalities must occur to induce a destructive process in the beta cells (25).

2.1.4 B cells and humoral immunity in relation to type 1 diabetes

Although type 1 diabetes seems to be a T-cell mediated disease, B cells play a crucial role in its pathogenesis. In the non-obese diabetic mouse (NOD), a rodent model genetically susceptible to autoimmune diabetes, the development of diabetes can be prevented by elimination of the B lymphocytes (26). Autoantibodies, although they may not have a direct cytotoxic effect on beta cells, are a powerful tool for assessing the risk of type 1 diabetes and in the search for the cause of the disease. Firstly, the target tissue, the islets of Langerhans in the pancreas, are practically inaccessible in man, whereas circulating autoantibodies are readily available in the peripheral blood. Secondly, the present T-cell methodology is imperfect, since peripheral blood lymphocytes do not reflect very well the state in the islets. Thirdly, antibody assays are relatively easy to perform.

2.2 Autoantigens in type 1 diabetes

2.2.1 IA-2/ICA512 and IA-2 β /phogrin

IA-2 (ICA512) was cloned and sequenced at the same time from a human islet cDNA library (27,28) and from a human insulinoma subtraction library (29). It is a transmembrane protein comprising of 979 amino acids with a molecular mass of approximately 106 kDa (28,29). The molecule (Fig. 1) consists of a signal peptide (amino acids 1-25), an extracellular domain (amino acids 26-576), a transmembrane region (amino acids 577-600) and an intracellular domain (amino acids 601-979). The extracellular domain contains a cysteine-rich region and two putative N-linked glycosylation sites, while the intracellular one includes several potential Ser/Thr

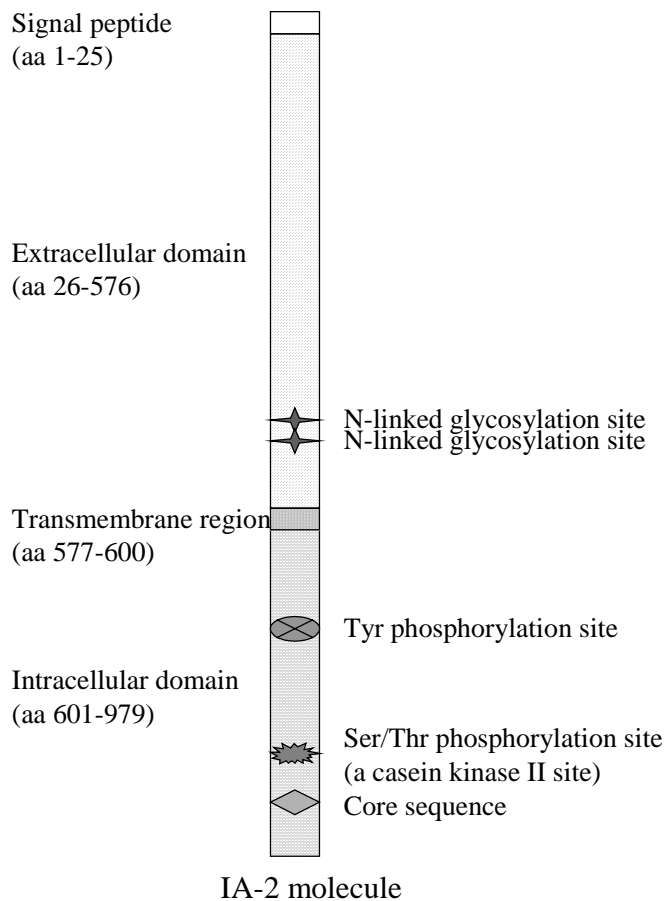


Fig. 1. Structure of the IA-2 molecule. Based on data from references 22 and 23.

phosphorylation sites and a putative Tyr-phosphorylation site. The intracellular domain of IA-2 bears some similarity to the catalytic domain of members of the PTP family, containing a core sequence of 11 amino acids at positions 907-917. Among the PTP family members, CD45 had the highest sequence similarity to the intracellular domain of IA-2 (43% identity and 58% similarity in the intracellular region spanning 180 amino acids). IA-2 has not been found to have any significant PTPase activity, however, and it is unclear whether the protein is a highly specific PTPase or an inactive one. IA-2 was found to be the precursor of the previously described 40-kDa antigen related to type 1 diabetes (30-33).

IA-2 is expressed in cells of neuroendocrine origin, including beta, alpha and delta cells in the pancreatic islets, pituitary cells and cells in the adrenal medulla (34). It is also present in autonomic nerve fibres and ganglia in the periphery and in the amygdala,

hypothalamus and infundibular tract of the central nervous system (34). Subcellularly, it is restricted to the membranes of secretory granules (34). (Fig. 2)

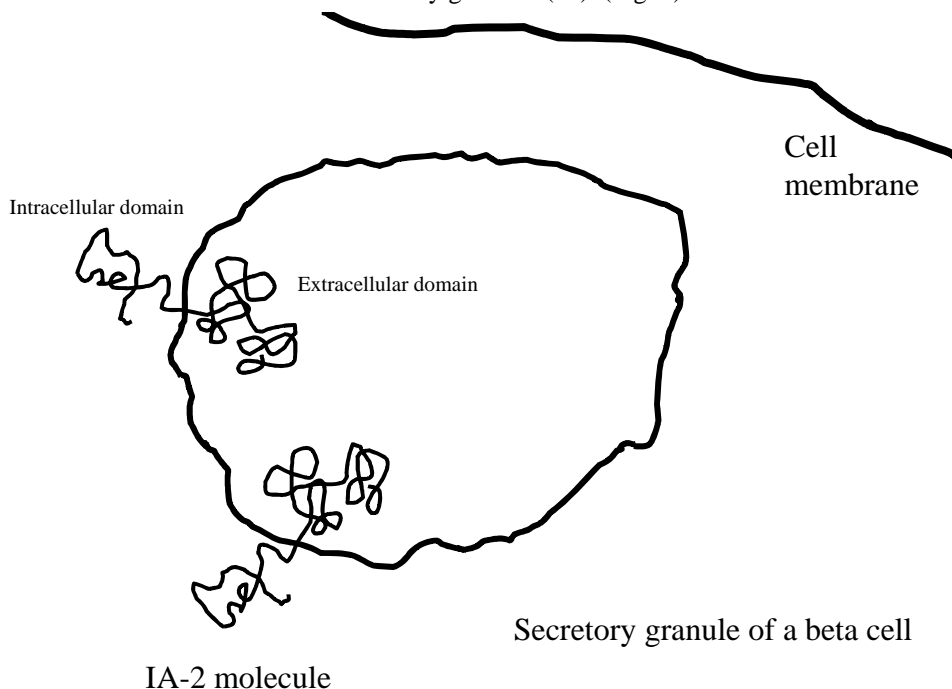


Fig. 2. Localization of I-2 in the cell. Based on data from references 22, 23 and 28.

A novel autoantigen of type 1 diabetes, phogrin/IA-2 β , was identified using rodent cDNA libraries (5,35). This is closely related to IA-2, with an overall identity of 42% and an identity of 80% in the 260-amino acid PTP domain (35). The intracellular part of IA-2 β shows 74% identity with IA-2, while the identity is only 26% in the extracellular portion (5). Phogrin was recognized as the precursor of the previously identified 37 kDa antigen associated with type 1 diabetes (30,36). Like IA-2, it is expressed in islets, pancreatic alpha and beta-cell tumor lines, brain cells and other cells of neuroendocrine lineage (35).

2.2.2 Glutamic acid decarboxylase

Sera from newly diagnosed diabetic children were reported in 1982 to immunoprecipitate a protein having a molecular weight of 64 kDa from human islets of Langerhans (37). Later the protein was identified as glutamic acid decarboxylase, the enzyme metabolizing

the inhibitory neurotransmitter γ -aminobutyric acid (GABA) (38). GAD exists in two forms: GAD65 and GAD67 (39). Human islets of Langerhans express only GAD65, and expression is not limited to the beta cells (39). GAD is also expressed in the nervous system, GAD65 primarily in the central nervous system and GAD67 in the peripheral nerves (39). Other implicated locations of GAD expression include the testis, ovary, oviduct and adrenal medulla (39).

2.2.3 Insulin and proinsulin

Insulin is a protein of the beta cells. The insulin gene is first transcribed and translated into preproinsulin, which is rapidly cleaved to proinsulin (40). During the cellular transfer of proinsulin, the protein is cleaved into insulin and a connecting peptide (C-peptide). Insulin comprises of an A and a B chain, which are connected by disulphide bonds (40). In addition to beta cells, insulin has been reported to be expressed in the human thymus (41,42).

2.2.4 Other islet cell autoantigens

The target of islet cell antibodies remains unidentified, but is presumably a heterogeneous group of antigens expressed in beta cells. ICA are partially inhibited by GAD and IA-2, suggesting that they serve as target antigens. ICA reactivity was not totally inhibited by GAD and IA-2, indicating that ICA have other target antigens. (2)

A 38 kDa autoantigen of human islet cells was precipitated using sera from patients with type 1 diabetes (37). Later this antigen was thought to be imogen 38 (43) or glima 38 (44). Imogen 38 is expressed in pancreatic beta and alpha cells and in other tissues (43), while Glima 38 has been shown to be an amphiphilic membrane glycoprotein (44).

Carboxypeptidase-H, a molecule expressed in islet secretory granules and neuroendocrine cells, was identified as being associated with type 1 diabetes (45).

ICA69 is an autoantigen identified by screening a human islet cDNA expression library with sera from relatives of patients with type 1 diabetes (46). It has two short regions of similarity to bovine serum albumin (BSA), and is transcribed in the human pancreas, brain, heart, thyroid and kidney, but not in skeletal muscle, placenta, spleen or ovary (46).

Sulphatide is a glycolipid which has been reported to be an autoantigen in human type 1 diabetes (47). It has been shown to be localized in the islets of Langerhans in the rat, more specifically in the secretory granules of both alpha and beta cells (47). It has also been detected in rodent nerve and kidney structures, but not in lung, heart, liver, adrenal, spleen, lymph node or thymus tissue.

2.3 Humoral immune responses

The quality of autoantibody assays, *ie.* their ability to distinguish between patients and control subjects, measured in terms of sensitivity and specificity, has been assessed by international workshops (48). Recent results indicate that the IA-2A radioimmunoassays (RIAs) used in different laboratories detect the majority of the patients regardless of the length of the IA-2 construct used. An ELISA assay using an extracellular construct as the antigen did not discriminate between cases and controls, while an ELISA assay using the intracellular portion resulted in lower sensitivity than the radioimmunoassays. The most common type of GADA assay, RIA with recombinant human GAD, worked well in most laboratories. The GAD ELISA assay also performed well. Based on the workshop results, the highest sensitivity of IAA was achieved with assays using large volumes of serum (600µl). Some laboratories achieved high sensitivity in the ICA assays at the expense of specificity (48). The standardization of ICA assay using JDF units has improved the assay sensitivity and specificity (49).

Type 1 diabetes-associated autoantibodies are detected at low frequencies in the general population (Tables 1-4). Most data are from cross-sectional surveys of schoolchildren. The comparability of surveys is controversial, since assays with variable performance characteristics have been used. The cut-off limit for antibody positivity is particularly critical when assessing the frequency of type 1 diabetes associated autoantibodies in the general population, since schoolchildren usually have low levels of autoantibodies, if any (50). In the general population increased antibody level may be due to assay technique in some patients, but specific autoantibody formation without clinical diabetes may also be possible.

Humoral immune responses have been extensively investigated in first-degree relatives of patients with type 1 diabetes, although the results are not fully comparable because of different study designs. Some surveys include only siblings, while others accept parents and offspring as well. Moreover, the time difference between the diagnosis in the proband and the date of sampling in the relative varies a lot both between and within surveys.

2.3.1 IA-2/ICA512 epitopes

Using sera from patients with newly diagnosed type 1 diabetes, the IA-2 autoantibody reactivity was located to the cytoplasmic portion of the molecule (amino acids 601-979). Most sera bound epitopes in the COOH terminus (within amino acids 777-937 and 687-979), while a smaller proportion reacted with epitopes in the juxtamembrane region of the intracellular domain (amino acids 605-620 and 605-682) (51). According to another study including antibody analysis of both full length IA-2 and the intracellular part, almost all patients with antibodies to the full length protein (amino acids 1-979) also had antibodies to the intracellular domain (amino acids 603-979) (52). Later a series of sera immunoprecipitating the full-length IA-2 were shown to react with the intracellular part of IA-2 (amino acids 604-979), but not with the extracellular domain (amino acids 31-

557) (53). Moreover, the major antigenic determinant of the intracellular portion of IA-2 shown to reside in the COOH terminus of the domain (amino acids 771-979) (53,54). Later the major unique epitope of ICA512 (IA-2) was localised to amino acids 762-887 (55).

2.3.2 IA-2 antibodies

The few figures for the prevalence of IA-2A in schoolchildren are presented in Table 1. The prevalence of IA-2 antibodies in schoolchildren in the Oxford region in England was 2.6% (56), while a German report mentions that the prevalence of IA-2A in schoolchildren without affected first-degree relatives was 2.35% (57).

The prevalence of antibodies to the intracytoplasmic domain of IA-2 (anti-IA-2ic) has been observed to be 4.4% among first-degree relatives (58), and that of antibodies to ICA512bcd (containing residues 256-979 of the IA-2 molecule) 2.9% in siblings (59) (Table 1).

The presence of IA-2 antibodies in newly diagnosed diabetic patients has been studied extensively over the last 5 years, having initially been detected as antibodies against 37kDa/40kDa proteolytic fragments of the 64 kDa protein (30,60,61). After the cloning of IA-2, the antigen became readily available and methods for the detection of autoantibodies were developed to facilitate the routine analysis of large numbers of serum samples. With time, the assays have spread into laboratories all over the world.

The prevalence of IA-2 antibodies at the diagnosis of type 1 diabetes has been reported to be 38%-67% (Table 1), while the frequency of IA-2A in controls was 0-1.0% (3,28,62-66). IA-2A were reported to be more frequent in patients diagnosed before 20 years of age than in those diagnosed between 20 and 40 years (3).

2.3.3 IA-2 β /Phogrin antibodies

The role of IA-2 β /phogrin antibodies has been investigated in relation to IA-2 antibodies. The former were detected in 45% of sera from patients with type 1 diabetes (54). The autoantibodies were shown to react with the intracellular domain of IA-2 β , but not with the extracellular domain, the major antigenic determinants being located in the COOH terminus of the intracellular domain (54). The major epitopes of phogrin-selective autoantibodies were located in amino acids 640-922 of phogrin (55).

A greater proportion of sera reacted exclusively with IA-2/ICA512 than exclusively with IA-2 β /phogrin (54,55,67). Moreover, the binding of phogrin autoantibodies could be totally blocked by adding ICA512 to sera positive for both ICA512 and phogrin, while the blocking of ICA512 antibodies with phogrin was not complete (55). Accordingly,

antibodies to IA-2 appear to be more important for the pathogenesis of type 1 diabetes than antibodies to IA-2 β /phogrin.

2.3.4 GAD antibodies

The major antigenic determinants of GAD have been localized to the middle and COOH-terminal regions of the molecule (4). Additional research has suggested that GAD antibodies recognise conformational epitopes (4).

The few figures for the prevalence of GADA in schoolchildren are presented in Table 2. The prevalence of GAD antibodies in schoolchildren in the Oxford region in England was 2.6% (56), while in Sweden the proportion of GAD65 antibody-positive control children was observed to be 4.1% (68). A German report mentions that the prevalences of GADA in schoolchildren without affected first-degree relatives was 2.97% (57).

Antibodies to GAD were detected in 6.3% (58) and 11% (59) of first-degree relatives and in 13% of siblings (59) (Table 2). Their frequency was lower in the offspring and parents of patients with type 1 diabetes than in their siblings (Table 2).

Various evaluations of the frequency of GAD antibodies at the diagnosis of type 1 diabetes have been published, quoting prevalences ranging from 70% to 82% (Table 2) (3,4,52,64,65,69-71). In general GAD antibodies appear to increase with age at diagnosis (4,69) and to be present more frequently in females than in males (69).

2.3.5 Insulin and proinsulin autoantibodies

The prevalences of insulin autoantibodies (IAA) reported in various studies are shown in Table 3. In southern England IAA levels exceeded the population mean by 3 SDs in 2.6% of schoolchildren (50), while the prevalence in the Oxford region was 2.49% (56). More recently, the prevalence of IAA in Germany has been reported to be 3.04% in schoolchildren without first-degree relatives with type 1 diabetes (57).

The frequency of IAA in parents has ranged from 1.3% (72) to 7.5% (59), as reported in Table 3 (59,72-74). During observation for 5.8 years (median), 6.3% of a series of siblings were reported to be IAA-positive at least once (74).

Several studies have demonstrated the presence of IAA at the diagnosis of type 1 diabetes, the prevalence ranging from 16% to 69% (75) (Table 3)(76-81). Insulin autoantibodies have been reported to be associated with young age (76,77,79-81). Antibodies to proinsulin were shown in a German study to be more prevalent at the diagnosis of type 1 diabetes than insulin autoantibodies (82).

2.3.6 Islet cell autoantibodies

The prevalence of ICA in schoolchildren is reported in Table 4. Two surveys in the Netherlands showed prevalences of ICA of 0.24% (83) and 0.29% (84) among non-diabetic children, while the proportion of ICA-positive non-diabetic children in Finland has been reported to be 4.1% (85). In Florida, ICA titres ≥ 10 JDF-U were detected in 0.59% of non-diabetic children (86), while in southern England the prevalence of ICA among schoolchildren was reported to be 2.7-2.8% at a level of ≥ 4 JDF-U (50,56) and 0.8% at a level of ≥ 20 JDF-U (50). Huge series of schoolchildren in Florida (n=9696) and Germany (n=9419) gave ICA prevalences of 0.59% (86) and 0.86% (57), respectively.

The frequency of ICA among first-degree relatives has ranged from 2.6% in parents in the United States (72) to 7.8% (74) in Finnish siblings, as shown in Table 4 (72,73,87,88). Altogether, the prevalence of ICA appears to be highest in siblings, followed by offspring and parents.

Islet cell autoantibodies were observed in 38-84% of patients with newly diagnosed type 1 diabetes (77,81,89,90) (Table 4). One study did not find any association between ICA and age (91), while another reported that ICA are associated with young age at diagnosis (81).

2.3.7 Combination of autoantibodies

Close to 80% of patients with newly diagnosed type 1 diabetes aged under 15 years were reported to test positive for multiple (≥ 2) autoantibodies when ICA, IAA and GAD65A were analysed (69). Multiple autoantibodies were more frequent among girls than among boys, and among patients younger than 5 years of age at diagnosis than among older ones (69). Elsewhere, 3.0% of first-degree relatives tested positive for anti-IA-2ic, GAD65A and ICA (58).

2.3.8 Age at diagnosis and humoral immune responses

Humoral immune responses have been reported to be weaker in patients with type 1 diabetes diagnosed at an older age than in young patients. Antibodies to the intracytoplasmic domain of IA-2, full-length GAD65 and cytoplasmic ICA were all more common among patients diagnosed before the age of 40 than in older patients (66). The results are controversial, however, since in another population GADA levels tended to be higher in newly diagnosed patients with type 1 diabetes aged 20-39 years than in younger patients (92). Moreover, the prevalence of GADA was observed to be of the same magnitude in different age groups at the time of diagnosis (92). In that population, however, the younger patients were more often positive for IAA or ICA than the older

ones (92). In general, combinations of ICA, IAA and/or GAD65A appeared to be more frequent in younger patients than in older ones (92). Antibodies to the COOH-terminal portion of IA-2 (amino acids 256-979) did not improve the sensitivity of antibodies to GAD65 for type 1 diabetes in older patients with slowly progressive disease as it did among younger patients with an acute onset (93). In the case of diabetic patients aged 40 years or more, 9.4% were classified as having type 1 diabetes, 8% had ICA and 8% tested positive for GADA (94).

2.3.9 Studies of patients with long-term type 1 diabetes

Although a humoral immune response to islet cells is a well-known characteristic of type 1 diabetes before and at diagnosis, little is known about this phenomenon after diagnosis. Most data are based on cross-sectional studies including patients with different durations of disease. The prevalence of ICA appeared to be higher in patients with a short duration of diabetes than in those with a longer duration (89), and the ICA titre of patients with a short duration decreased in 48% of cases when ICA were reanalysed after a time interval of 5 weeks to 3 years (89). In a subpopulation of patients with type 1 diabetes, GADA were shown to persist after diagnosis but ICA disappeared (95). The prevalence and levels of ICA and GADA appeared to be higher in patients with a short duration of type 1 diabetes (<1 year) than in ones with a long-standing disease (3-22 years) (96). GADA were detected in 38% of patients with type 1 diabetes with an average disease duration of more than 20 years: some patients became positive and in others GADA disappeared during the prospective follow-up (97). A gradual decline in GADA levels was reported in another longitudinal study (98), and in a third follow-up study the GADA titres were shown to vary substantially in patients after diagnosis, the prevalence being 59% after a mean duration of 6 years of diabetes (99). The frequencies of IA-2A and GADA were reported to be 45% and 50%, respectively, 5 years after the onset of type 1 diabetes in a Japanese study (65).

Table 1. Frequencies of IA-2A in various populations detected with different autoantibody assays.

Reference	Frequency, %	Population			Cut-off limit
		N	Age, y	Characteristics	
General population					
Bingley et al. 1997 (56)	2.63	2855	9.0-13.8	Schoolchildren	97.5th percentile
Strebelow et al. 1999 (57)	2.35	9419	6-17	Schoolchildren without first-degree relatives with type 1 diabetes	98th percentile of the control group
First-degree relatives					
Seissler et al. 1996 (58)	4.4	1238	0.1-57	First-degree relatives	mean + 4 SD in 100 control sera
Verge et al. 1996 (59)	2.9	683		Parents, siblings, offspring	Indistinctly defined
Verge et al. 1996 (59)	3.3	303		Siblings	Indistinctly defined
Verge et al. 1996 (59)	2.8	287		Offspring	Indistinctly defined
Verge et al. 1996 (59)	2.2	93		Parents	Indistinctly defined
Patients with newly diagnosed type 1 diabetes					
Rabin et al. 1994 (28)	48	80	Not reported		mean + 2 SD of the normal sera signals
Gianini et al. 1995 (62)	38	42	2.2-33		99th percentile of 205 controls
Lan et al. 1996 (63)	66	50	Not reported		Visible band on SDS/PAGE gel
Vandewalle et al. 1997 (64)	56	608	0-39		Based on ROC analysis ($\geq 0.4\%$ tracer bound)
Gorus et al. 1997 (3)	58	474	0-39		Based on ROC curve analysis
Gorus et al. 1997 (3)	71	97	0-9		Based on ROC curve analysis
Gorus et al. 1997 (3)	69	158	10-19		Based on ROC curve analysis
Gorus et al. 1997 (3)	45	219	20-39		Based on ROC curve analysis
Yokota et al. 1998 (65)	58	40	1.7-20		+ 3 SD of 65 control subjects
Lohmann et al. 1997 (66)	39	23	12-38		mean +/- 4 SD of 100 controls
Lohmann et al. 1997 (66)	0	24	>40		mean +/- 4 SD of 100 controls
Patients with long-term type 1 diabetes					
Yokota et al. 1998 (65)	45	20	1.7-20 at onset	Duration of diabetes 5 years	+ 3 SD of 65 control subjects

Table 2. Frequencies of GADA in various populations detected with different autoantibody assays.

Reference	Frequency		Population		Cut-off limit
	%	N	Age, y	Characteristics	
General population					
Bingley et al. 1997 (56)	2.63	2855	9.0-13.8	Schoolchildren	97.5th percentile
Hagopian et al. 1995 (68)	4.1	412	<15	Control children	
Strebelow et al. 1999 (57)	2.97	9419	6-17	Schoolchildren without first-degree relatives with type 1 diabetes	98th percentile of the control group
First-degree relatives					
Seissler et al. 1996 (58)	6.3	1238	0.1-57	First-degree relatives	mean + 4 SD in 100 control sera
Verge et al. 1996 (59)	11	683		First-degree relatives	99th percentile in 205 controls
Verge et al. 1996 (59)	13	303		Siblings	99th percentile in 205 controls
Verge et al. 1996 (59)	9.1	287		Offspring	99th percentile in 205 controls
Verge et al. 1996 (59)	9.7	93		Parents	99th percentile in 205 controls
Patients with newly diagnosed type 1 diabetes					
Kaufman et al. 1992 (70)	78	23	Not reported		SDS-PAGE analysis
Velloso et al. 1993 (71)	76	50	5-44		Not reported
Sabbah et al. 1996 (69)	73	747	0.8-14.9		99th percentile in 372 controls
Sabbah et al. 1996 (69)	71	165	<5		99th percentile in 372 controls
Sabbah et al. 1996 (69)	69	291	5-9.9		99th percentile in 372 controls
Sabbah et al. 1996 (69)	79	291	10-14.9		99th percentile in 372 controls
Vandewalle et al. 1997 (64)	79	608	0-39		Based on ROC analysis ($\geq 2.6\%$ tracer bound)
Gorus et al. 1997 (3)	82	474	0-39		$\geq 1.7\%$
Gorus et al. 1997 (3)	69	97	0-9		$\geq 1.7\%$
Gorus et al. 1997 (3)	88	158	10-19		$\geq 1.7\%$
Gorus et al. 1997 (3)	85	219	20-39		$\geq 1.7\%$
Hawa et al. 1997 (52)	77	60	5-31		>3 SD above the controls
Yokota et al. 1998 (65)	70	40	1.7-20		+ 3 SD (8 U/ml)

Continued. Frequencies of GADA in various populations detected with different autoantibody assays.

Reference	Frequency		Population		Cut-off limit
	%	N	Age, y	Characteristics	
Patients with long-term type 1 diabetes					
Christie et al. 1990 (95)	80	15	6-17 at onset	Duration of diabetes max 3 years	Not reported
Sundkvist et al. 1994 (97)	38	95	18-77	Duration of diabetes 2-62 years	mean + 2 SD in controls
Batstra et al. 1997 (99)	59	28	1.7-16.3 at dg	Duration of diabetes 6 years (mean)	A GAD index of 0.16
Yokota et al. 1998 (65)	50	20	1.7-20 at onset	Duration of diabetes 5 years	+ 3 SD (8 U/ml)

Table 3. Frequencies of IAA in various populations detected with different autoantibody assays.

Reference	Frequency %	Population			Cut-off limit
		N	Age, y	Characteristics	
General population					
Bingley et al. 1993 (50)	2.6	78		Schoolchildren	mean + 3 SD
Bingley et al. 1997 (56)	2.49	2855	9.0-13.8	Schoolchildren	97.5th centile
Strebelow et al. 1999 (57)	3.04	9419	6-17	Schoolchildren without first-degree relatives with type 1 diabetes	98th percentile of the control group
First-degree relatives					
Krischer et al. 1993 (72)	2.5	4060		Parents, siblings, offspring	mean + 3 SD
Krischer et al. 1993 (72)	1.3	1992		Parents	mean + 3 SD
Krischer et al. 1993 (72)	4.0	1390		Siblings	mean + 3 SD
Krischer et al. 1993 (72)	3.1	678		Offspring	mean + 3 SD
Gorus et al. 1994 (73)	6	310	0-39	Siblings	mean + 3 SD
Karjalainen et al. 1996 (74)	1.4	765	<20	Siblings	mean + 3 SD in 100 controls
Verge et al. 1996 (59)	6.7	683		Parents, siblings, offspring	99th percentile in 205 healthy controls
Verge et al. 1996 (59)	6.9	303		Siblings	99th percentile in 205 healthy controls
Verge et al. 1996 (59)	7.5	93		Parents	99th percentile in 205 healthy controls
Verge et al. 1996 (59)	6.3	287		Offspring	99th percentile in 205 healthy controls
Patients with newly diagnosed type 1 diabetes					
Gorus et al. 1997 (3)	42	474	0-39		≥0.7%
Palmer et al. 1983 (75)	16	112	5-20		mean + 5 SD in controls
Palmer et al. 1983 (75)	32	112	5-20		95th percentile of controls
Arslanian et al. 1985 (76)	36	124	1-17		95th percentile of controls
Atkinson et al. 1986 (78)	37	38	15.3, mean		>3.2% (mean + 2 SD)
Vardi et al 1988 (79)	69	39	1-32		39 nU/ml (mean + 3 SD in controls)
Karjalainen et al. 1986 (77)	28	60	1.0-15.8		Upper range in the controls
Karjalainen et al. 1988 (80)	35	46	1.3-15.8		Upper range in 68 controls
Vähäsalo et al. 1996 (81)	47	781	0.8-14.9		mean + 3 SD in 105 controls

Table 4. Frequencies of ICA in various populations detected with different autoantibody assays.

Reference	Frequency %	Population			Cut-off limit
		N	Age, y	Characteristics	
General population					
Bruining et al. 1989 (83)	0.24	3383	5-19	Schoolchildren	≥2 JDF-U
Bingley et al. 1993 (50)	2.8	2925	9-13	Schoolchildren	≥4 JDF-U
Bingley et al. 1993 (50)	0.8	2925	9-13	Schoolchildren	≥20 JDF-U
Bingley et al. 1997 (56)	2.73	2855	9.0- 13.8	Schoolchildren	≥97.5th percentile
Karjalainen 1990 (85)	4.1	1212	3-18	Schoolchildren without type 1 diabetes	Evidently 3 JDF-U
Aanstoot et al. 1994 (84)	0.29	2805	5-19	Schoolchildren	CF-ICA
Schatz et al. 1994 (86)	0.59	9696	5-17	Schoolchildren without type 1 diabetes	10 JDF-U
Strebelow et al. 1999 (57)	0.86	9419	6-17	Schoolchildren without first-degree relatives with type 1 diabetes	10 JDF-U
First-degree relatives					
Bonifacio et al. 1990 (149)	3.6	719		Parents and siblings	4 JDF-U
Bonifacio et al. 1990 (149)	2.7	376		Parents	4 JDF-U
Bonifacio et al. 1990 (149)	4.7	343		Siblings	4 JDF-U
Krischer et al. 1993 (72)	3.6	4060		Parents, siblings, offspring	10 JDF-U
Krischer et al. 1993 (72)	2.6	1992		Parents	10 JDF-U
Krischer et al. 1993 (72)	4.7	1390		Siblings	10 JDF-U
Krischer et al. 1993 (72)	4.4	678		Offspring	10 JDF-U
Riley et al. 1990 (87)	3.3	3413		Parents, siblings, offspring	10 JDF-U
Riley et al. 1990 (87)	2.5	1711		Parents	10 JDF-U
Riley et al. 1990 (87)	4.2	1190		Siblings	10 JDF-U
Riley et al. 1990 (87)	3.7	512		Offspring	10 JDF-U
Deschamps et al. 1992 (88)	5.5	401	2-29	Siblings	4 JDF-U
Gorus et al. 1994 (73)	7	310	0-39	Siblings	12 JDF-U
Gorus et al. 1994 (73)	5	310	0-39	Siblings	20 JDF-U
Gorus et al. 1994 (73)	2	310	0-39	Siblings	80 JDF-U
Karjalainen et al. 1996 (74)	7.8	765	<20	Siblings, non-affected	2.5 JDF-U

Continued. Frequencies of ICA in various populations detected with different autoantibody assays.

Reference	Frequency %	Population			Cut-off limit
		N	Age, y	Characteristics	
Patients with newly diagnosed type 1 diabetes					
Lendrum et al. 1976 (89)	38	829	≤83		Not reported
Mustonen et al. 1984 (90)	84	44	0.7-16.7		Not reported
Karjalainen et al. 1986 (77)	75	60	1.0-15.8		Not reported
Vähäsalo et al. 1996 (81)	84	781	0.8-14.9		2.5 JDF-U
Gorus et al. 1997 (3)	73	474	0-39		>12 JDF-U, based on ROC plot
Patients with long-term type 1 diabetes					
Sundkvist et al. 1994 (97)	31	95	18-77	Duration of diabetes 2-62 years	3 JDF-U

2.4 Genetics of type 1 diabetes

2.4.1 Family studies

About one tenth of patients with type 1 diabetes have an affected first-degree relative at diagnosis (100,101), and the proportion of familial diabetes increases with increasing disease duration, so that a Danish study reported a proportion as high as 25% after more than 30 years (102). On the other hand, a monozygotic co-twin has an estimated 44% risk of developing diabetes if the proband is diagnosed before the age of 15 years (103). Elsewhere, the probability of progression to diabetes was 23% after 20 years of discordance in monozygotic twins, who have a higher risk of developing diabetes than dizygotic twins (104). These observations point to a genetic background for type 1 diabetes mellitus.

2.4.2 The gene search

A genome-wide search for disease susceptibility genes revealed that type 1 diabetes is associated most strikingly with IDDM1 (the major histocompatibility complex on chromosome 6p21), but also to some extent with IDDM2 (the insulin gene region on chromosome 11p15) and at least three other genes (6,105). The genes of the major histocompatibility complex (MHC) are classified into class I, II and III regions, and it is

the class II region that contains the genes most closely associated with type 1 diabetes, ie. the HLA DP, DQ and DR genes (106). It has been suggested that HLA genes are associated with type 1 diabetes through their impact on the maturation, selection and activation of T-cells (107).

2.4.3 HLA genes in relation to humoral immune responses

HLA genes modify humoral immune responses in type 1 diabetes. The earliest studies from the 1980's failed to show any association between ICA or IAA and HLA DR antigens (76,77,91), but contrasting results have been reported since then. (Table 5) In a population of first-degree relatives, IAA appeared to be more frequent in subjects who were heterozygous for HLA DR3/DR4 than in ones with HLA non-DR3/non-DR4 (78). According to findings in another series of first-degree relatives, the presence of ICA was associated with a 57-Asp negative DQ-B chain at a single or double dose (108). IAA and ICA are both associated with the HLA DQA1*0301-DQB1*0302 haplotype at the clinical onset of type 1 diabetes (109). In a series of relatives of patients an increase in the frequency of HLA DR3/4 heterozygotes was observed among subjects who were positive for ICA or IAA as opposed to those who were negative for both antibodies (72). Among 310 Caucasian siblings, cases carrying at least one copy of the DQA1*0301-DQB1*0302 haplotype tested positive for IAA and ICA more frequently than relatives lacking this haplotype. Moreover, IAA and ICA positivity were associated with the heterozygous DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 high risk genotype, and ICA positivity with homozygosity for DQA1*0301-DQB1*0302 as well (73).

A series of Finnish patients with HLA DR3/DR4 appeared positive for at least two antibodies out of ICA, IAA and GADA more often than did the other patients. In addition, higher GADA levels were associated with HLA DR3/non-DR4 (69). Another Finnish study reported that ICA and IAA positivity was more frequent in siblings with HLA DR3/4 than in the others (74). In a series of more than 100 relatives of patients with type 1 diabetes, cases testing positive for ICA512bdcAA (antibodies against a protein containing amino acid residues 256-979 of IA-2) more often carried the high risk haplotype DR4-DQ8 (DQA1*0301-DQB1*0302) than cases testing negative for ICA512AA, and the same also held true for IAA positivity (59). Among cases positive for GADA neither DR4-DQ8 nor DR3-DQ2 (DQA1*0501-DQB1*0201) were increased as compared with GADA-negative relatives (59). GADA were shown to be associated with DQA1*0501/B1*0201 (DQ2) in patients with newly diagnosed type 1 diabetes, but not with DQA1*0301/B1*0302 (DQ8), while IAA were associated with DQA1*0301/B1*0302 (DQ8) but not with DQA1*0501/B1*0201 (DQ2) (68). The frequency and level of IA-2A were higher at diagnosis in patients with HLA DR4 than in other patients, whereas the prevalence and level of GADA were associated with the HLA DR3 allele (110). Somewhat contradictory data do exist, however, since the frequencies of both GADA and IA-2A have been reported to be associated with DQA1*0301-DQB1*0302 in patients with newly diagnosed type 1 diabetes and their siblings, although an association with GADA was found only in patients aged 20-39 years (64). Supporting

the previous observations, an association was observed between GADA and DQA1*0501-DQB1*0201 together with insulin gene risk marker I/I in patients negative for DQA1*0301-DQB1*0302 (64). In a Belgian study of patients with type 1 diabetes, the level of IA-2A was positively associated with HLA DQA1*0301-DQB1*0302 but not with HLA DQA1*0501-DQB1*0201 (3).

Among discordant monozygotic twins carrying HLA DQ8/DQ2, the probability of developing IAA, GADA, IA-2A and/or ICA positivity was higher than among twins without this genotype (104). The frequencies of HLA genotypes DRB1*03/04(DQB1*57non-Asp) and DRB1*04/04(DQB1*57non-Asp) were increased in the offspring of parents with type 1 diabetes developing diabetes-associated autoantibodies (IAA, GADA, IA2A and/or ICA) during the first 2 years of life (111).

The results regarding humoral immunity and HLA associations are heterogeneous, but logical trends can be detected. Taken together, DR3/DR4 and DQ8/DQ2 are associated with ICA and IAA, whereas the single antigens DR4 and DQ8 are linked with IA-2A. The most contradictory results concern GADA, but DR3 and DQ2 appear to play an essential role in GADA immunity.

Table 5. Associations between humoral autoimmunity and HLA genes.

Reference	Study population	N	IA-2A	GADA	IAA	ICA
Relatives						
Atkinson et al. 1986 (78)	First-degree relatives	245			DR3 and/or DR4	
Thivolet et al. 1991 (108)	First-degree relatives	658				57-Asp negative DQ-B chain
Krischer et al. 1993 (72)	Relatives	915			DR3/4	DR3/4
Gorus et al. 1994 (73)	Siblings	310			DQ8	DQ8
Gorus et al. 1994 (73)	Siblings	310			DQ8/ DQ2	DQ8/ DQ2
Gorus et al. 1994 (73)	Siblings	310				DQ8/ DQ8
Verge et al. 1996 (59)	First-degree relatives	118	DR4-DQ8		DR4-DQ8	
Karjalainen et al. 1996 (74)	Siblings	765			DR3/4	DR3/4
Vandewalle et al. 1997 (64)	Siblings	480	DQ8	DQ8		
Patients with type 1 diabetes						
Vandewalle et al. 1993 (109)	Newly diagnosed	279			DQ8	DQ8
Sabbah et al. 1996 (69)	Newly diagnosed	719		DR3/non-DR4		
Hagopian et al. 1995 (68)	Newly diagnosed	425		DQ2	DQ8	DQA1*0301
Genovese et al. 1996 (110)	Newly diagnosed	160	DR4	DR3		
Vandewalle et al. 1997 (64)	Newly diagnosed	608	DQ8	DQ8 and DQ2 together with INS I/I		
Gorus et al. 1997 (3)	Newly diagnosed	474	DQ8			

DQ8 = DQA1*0301-DQB1*0302, DQ2 = DQA1*0501-DQB1*0201, INS I/I = insulin gene risk marker I/I

2.5 Cell-mediated autoimmunity

Cellular immune responses associated with type 1 diabetes are technically difficult to measure. This is attributable to several factors: the quality of the recombinant autoantigen preparations used, the heterogeneity of control populations, and the lack of standardization for the definition of 'positivity' (112). Although several solutions have been proposed for improving the quality of T-cell assays, only a few laboratories are able to discriminate between patients with type 1 diabetes and non-diabetic control subjects (112). Furthermore, the patient populations in studies on T-cell responses are often small. Since the human pancreas has largely remained inaccessible to immunological investigations, T-cell responses are usually measured using peripheral blood mononuclear cells. Such an approach has often been criticized, however, as these cells are likely to reflect poorly the situation in the islets of Langerhans.

2.5.1 Cellular immunity against IA-2

T-cell responses to ICA512 were detected in the majority of patients with newly diagnosed type 1 diabetes and in first-degree relatives with no association between humoral and cellular autoimmunity (113,114). Similarly, patients with newly diagnosed type 1 diabetes and their autoantibody-positive relatives were reported to have an increased frequency of peripheral blood mononuclear cell proliferation against IA-2 as compared with that in autoantibody-negative relatives and healthy control subjects (115). The mean stimulation index was also higher in the former subjects. Later, 13 T-cell epitopes of IA-2 were characterised and shown to have sequence identity and similarity to human rotavirus, some other micro-organisms and certain dietary proteins (116).

2.5.2 Other autoantigens

T-cell reactivity against GAD and an inverse relation between the humoral and cellular immune responses to GAD have been reported (117). The T-cell response to GAD were confirmed later by other groups (113), but the inverse relation between humoral and cellular immunity is a more controversial issue (114).

T-cell responses to some other antigens have been reported to be associated with type 1 diabetes, including beta-cell membrane preparations (25,113,118) and human pancreas

(113). According to one report, there is no evidence for enhanced cellular reactivity to proinsulin in type 1 diabetes (119).

2.6 Assessment of the risk of future type 1 diabetes

2.6.1 Risk assessment in the general population

A half (n=4) of the ICA positive subjects in a series of non-diabetic schoolchildren became insulin-dependent over a period of 10 years, whereas a negative ICA result gave a 99.9% probability of remaining non-diabetic (83). Another study of schoolchildren found that 6% (n=3) of the ICA-positive subjects developed type 1 diabetes during an 8-year follow-up while none of the ICA-negative children did so (85). A survey of a selected, and possibly biased, population of 111 schoolchildren suggested that a single GADA test may be more sensitive for assessing the risk of type 1 diabetes than a single ICA test (84). In an extensive series of schoolchildren, the estimated risk of developing type 1 diabetes was observed to be 45% for ICA-positive subjects over 7 years of observation, although the weakness of this study was the great variability in follow-up periods (86). The estimated risks of developing type 1 diabetes in the population of the Oxford region between the ages of 10-20 years were 6.7% for ICA, 6.6% for GADA, 5.6% for IA-2A and 4.8% for IAA, whereas the estimated risk for two antibodies was 9%, that for three antibodies 59% and that for four antibodies 61% (56). For risk assessment in the general population, the authors recommend a strategy of measuring IA-2A and GADA primarily, followed by ICA. This model is based on questionable assumptions, however. Because of a lack of information on the natural history of humoral islet autoimmunity, the authors have assumed that antibodies appear in most patients very early in life and are stable until diagnosis, with only individual exceptions (56). Follow-up studies of first-degree relatives have nevertheless demonstrated that seroconversions from autoantibody positivity to negativity do occur (108,120-123).

The sensitivity of a positive GADA test among Finnish prediabetic mothers was more than 80%, while the specificity was 100% (124). The authors concluded that GADA are associated with a high risk of subsequent type 1 diabetes.

2.6.2 Risk assessment in first-degree relatives

Considerable resources have been put into the development of risk assessment strategies for use with first-degree relatives of patients with type 1 diabetes. The strategies proposed have been based on genetic and humoral autoimmune markers. The earliest research on combining genetic and humoral markers in risk assessment dates back to the 1980's.

The future risk for type 1 diabetes in relatives varied between 3 and 100% (3,52,59,72,74,87,88,123,125-128). The figures of the risk are not fully comparable, for numerous reasons. Firstly, the population in most cases includes first-degree relatives (siblings, parents and/or offspring) (59,123,125-128), but sometimes it comprises only siblings (3,74,88). Moreover, some investigators have accepted second- and/or third-degree relatives (72,87). In the survey giving the highest predictive characteristics for type 1 diabetes the population comprised of the initially non-diabetic counterparts of monozygotic twin pairs (52). Secondly, autoantibodies have been analysed in different laboratories and there are still some variations in performance characteristics (48). Thirdly, the follow-up times and the intervals between diagnosis of the proband and sampling of the relative may vary substantially. Fourthly, different statistical methods have been used, the risk being assessed by the cumulative risk method, life-table analysis, relative risk, actuarial risk and/or positive predictive value. Fifthly, some studies are based on relatively small numbers of subjects (3,52,123), and some populations are biased because of initial selection of autoantibody-positive relatives (123,127). Furthermore, risk assessment based on autoantibodies may be inaccurate, because autoantibodies may both appear and disappear with time (73). However, these studies show that the risk of developing clinical type 1 diabetes in first-degree relatives is increased in the presence of genetic susceptibility and disease-associated autoantibodies. The combining of various autoantibodies tends to be a useful tool for assessing the risk of type 1 diabetes. In any case, as specificity (the predicted individuals do develop diabetes) increases when several risk markers are combined, the sensitivity (prediction of all future type 1 diabetes cases in the population) decreases (129).

2.7 Prevention of impending type 1 diabetes

The ultimate goal of research into the pathogenesis and development of type 1 diabetes is to prevent the disease. The destructive immune process should be stopped before the majority of the insulin-producing beta cells have perished. There are several trials in progress aimed at the prevention of type 1 diabetes (130,131).

One idea is to protect the remaining beta cells by treatment with nicotinamide, a well-tolerated inexpensive compound belonging to the family of B vitamins (132). The evidence that nicotinamide can provide protection from type 1 diabetes comes from both chemically induced and immune-mediated diabetes (132). Nicotinamide is assumed to improve beta-cell regeneration, to protect the cells from lysis and to affect cytokine expression. A meta-analysis of studies on patients with newly diagnosed type 1 diabetes demonstrated a therapeutic effect of nicotinamide, with higher baseline C-peptide concentrations for the treated patients (133). The effect of nicotinamide in high-risk relatives of patients with type 1 diabetes has been the topic of two large clinical trials: the German Nicotinamide Diabetes Intervention Study (DENIS) and the European Nicotinamide Diabetes Intervention Trial (ENDIT) (10). The DENIS study was terminated in 1997 after it turned out that nicotinamide was not capable of reducing the

incidence of type 1 diabetes by 80% in high-risk siblings (134), while the final results of ENDIT will be available in year 2003.

According to many clinical trials, early aggressive insulin treatment may result in the preservation of residual beta-cell function (10). A small pilot study in the USA achieved promising results in delaying or preventing type 1 diabetes in high-risk relatives with insulin prophylaxis (135), and similar results were reported from another pilot trial with parenteral insulin administration in seven relatives with high risk of type 1 diabetes as compared to seven in the control group (136,137). Two major clinical trials are evaluating the role of insulin as a preventive drug for type 1 diabetes. Firstly, the Diabetes Prevention trial of Type 1 Diabetes (DPT-1) is attempting to determine whether parenteral insulin will delay the expected development of type 1 diabetes in relatives with a $\geq 50\%$ risk of developing the disease over 5 years, and whether oral insulin will induce immunological tolerance, delaying the onset of disease in relatives with a 25-50% risk of overt disease within 5 years (10). Secondly, the Diabetes Prevention and Prediction (DIPP) study is aimed at delaying type 1 diabetes by nasal insulin administration in subjects from the general population with a high risk of the disease as detected by genetic and autoimmune markers.

It is thought that the gut immune system may play a role in the induction of the pathological process leading to type 1 diabetes, and that cow milk proteins may be associated with the process (138). Thus the TRIGR project is determining the effect of the avoidance of nutritional cow milk proteins on the prevention of type 1 diabetes. Interim data from the second pilot study revealed that disease-associated autoantibodies appeared more frequently in subjects who received cow milk proteins during the first months of life than in those who received a hydrolyzed casein formula (139).

3 Aims of the research

This work was aimed at gaining new insights into the immunopathogenesis of type 1 diabetes in order to understand the pathomechanism better and to improve assessment of the risk of developing the disease. Humoral autoimmunity was of special interest.

The specific aims of the present work were

1. to investigate the occurrence of IA-2A in newly diagnosed type 1 diabetes;
2. to study the effect of age at diagnosis on the humoral immune responses to beta-cell antigens, particularly IA-2;
3. to study humoral immune responses to beta-cell antigens after clinical diagnosis;
4. to investigate the associations between HLA genes and humoral autoimmunity, particularly IA-2A, in patients and their siblings
5. to investigate the dynamics of humoral beta cell autoimmunity in non-diabetic siblings of affected children;
6. to improve the assessment of the risk of type 1 diabetes in siblings of children with the disease.

4 Subjects and methods

4.1 Subjects

4.1.1 Patients with type 1 diabetes (Papers I-III)

The population of the first report includes 758 children aged under 15 years (range 0.78-14.97) with newly diagnosed diabetes, 340 of whom (44.9%) were girls. The subjects represent index cases in the nationwide "Childhood Diabetes in Finland" (DiMe) study, invited to participate in the project between September 1986 and April 1989 (100).

The population in paper II consists of 352 patients with newly diagnosed type 1 diabetes. One hundred and thirty-seven of them (38.9%) were females. The patients were divided into two age groups, less than 20 years (range 0.6-19.9) and 20 years or older (range 20-61.9). The mean age of the younger group (n=252) was 9.5 years and that of the older group 35.3 years (n=100). The patients were recruited between April 1, 1988 and September 30, 1995 from the Department of Pediatrics and Department of Internal Medicine, Oulu University Hospital, which serves approximately 300,000 inhabitants, including about 90,000 under 20 years of age. Thus a small proportion of the younger patients in report II (n=35) are also included in paper I.

In paper III the subjects comprise of 90 children and adolescents with type 1 diabetes recruited from Department of Pediatrics, Oulu University Hospital, between January 1, 1983 and December 31, 1986 and followed up for 10 years. Blood samples were obtained at diagnosis and approximately 2, 5 and 10 years later. There were 60 patients (67%) whose follow-up lasted 10 years. All four samples were available from 52 patients (58%), three samples from 27 patients (30%) and two samples from 11 patients (12%). The mean age of the patients at diagnosis was 8.2 years (range 0.9-15.6), and 36 of the subjects were girls (40%).

4.1.2 Siblings of children with type 1 diabetes (Papers IV-V)

The population in paper IV includes 710 siblings of children with type 1 diabetes from the DiMe study. One requirement for inclusion was that there should be at least two blood samples available for the sibling. More than half of the siblings were girls (n=386; 54.4%) and their mean age at the beginning of the study was 9.8 (range -0.3 - 19.7) years. The samples were scheduled to be taken at or close to the time of diagnosis in the proband, and subsequently at 3 month intervals during the first 2 years of follow-up and annually thereafter up to 4 years. Extra samples were obtained between or after the time points mentioned from siblings of special interest, mainly those who were positive for ICA or IAA during the early follow-up. Five hundred and sixty-nine of the siblings (80.1%) gave their first sample within the first month after diagnosis of the proband, and the siblings were observed for autoantibodies for a median period of 3 years 7 months (range 0.1 months - 9 years 10 months).

Paper V is based on a population comprising of 755 siblings of children with newly diagnosed type 1 diabetes from the DiMe Study (100). The subjects are mainly the same as in paper IV. The majority of the siblings were girls (n=406;53.8%), and the mean age of the population was 9.9 years (range 0.8-19.7) at the time of initial sampling. The siblings were followed for subsequent type 1 diabetes for 9.1 years (median, range 7.7-10.7).

Altogether 801 families were invited to take part in the DiMe Study. The probands of these families had 1064 siblings, of whom 819 were eligible for the study (alive, non-diabetic and between 3-19 years at enrolment) (100). Thus the vast majority of eligible siblings were included in the autoantibody analyses (Paper IV: n=710, 87% and paper V: n=755, 92%).

4.1.3 Control subjects

A total of 374 non-diabetic children (age 0-19 years) comprised the control population when assessing the cut-off limits for positivity for IA-2A, GADA and IAA. The samples were taken at child welfare clinics and schools in Oulu or at prearranged visits to the outpatient clinic at the Department of Pediatrics, University of Oulu.

4.2 Laboratory methods

4.2.1 IA-2 autoantibody assay

The recombinant plasmid encoding the intracellular portion of the IA-2 protein (aa 605-979) was multiplied using *E. Coli* cells and then purified by standard techniques (140) (Fig. 3). The TNT Coupled Reticulocyte Lysate System (Promega, Madison, Wi., USA)

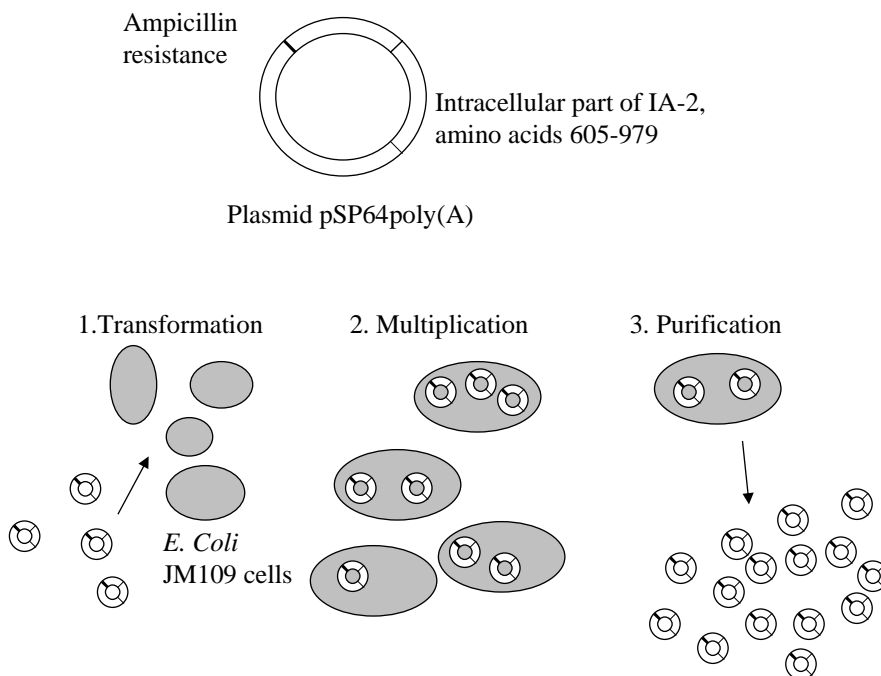


Fig. 3. Multiplication of cDNA encoding the IA-2 antigen using *E. Coli* JM109 cells.

was used to transcribe and translate the IA-2 protein, which was labelled with ^{35}S -methionine (Amersham, Little Chalfont, Bucks, UK). Serum samples were incubated overnight with labelled protein, and Protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden) was used to isolate the immune complexes (Fig. 4). After washing the bound activity was measured in a liquid scintillation counter (1450 Microbeta Trilux, PerkinElmer Wallac, Turku, Finland). Relative units were calculated with a software program (MultiCalc, PerkinElmer Wallac) using a standard curve for a dilution series run on each plate. The cut-off limit was defined as the 99th percentile in 374 non-diabetic Finnish children and adolescents (0.43 RU). The interassay coefficient of variation was 12% at an IA-2A level of 0.63 RU, 10% at a level of 21.3 RU and 8% at a level of 82.6

RU. Based on 140 samples included in the 1995 Multiple Autoantibody Workshop, the disease sensitivity of the assay was 62% and the specificity 97% (48).

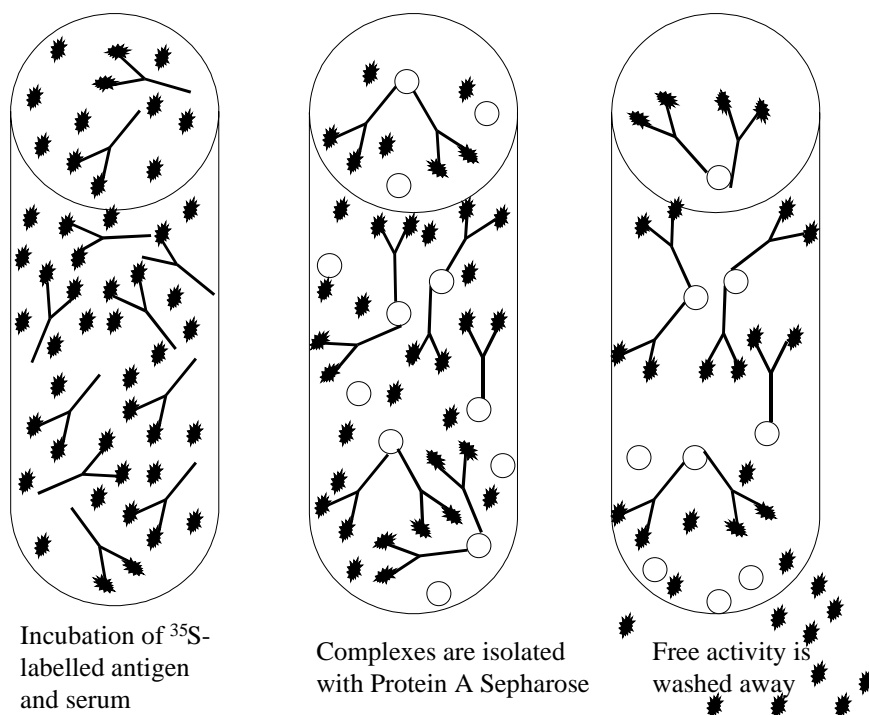


Fig. 4. Protocol for measuring IA-2 antibodies. * = ^{35}S -methionine-labelled IA-2, —< = circulating autoantibody against IA-2, O = particle of Protein A Sepharose. After washing, the IA-2A activity was measured in a liquid scintillation counter.

4.2.2 GAD autoantibody assay

Two technically different modifications were used to analyse GAD antibodies. The older method was that described by Sabbah et al. (69) (Papers I, IV and V), but a technically simpler method corresponding to that used for IA-2A was established later (Papers II-IV). The recombinant plasmid encoding the whole glutamic acid decarboxylase molecule (GAD_{65}) was multiplied using *E. Coli* cells and then purified by standard techniques (140). The TNT Coupled Reticulocyte Lysate System (Promega) was used to transcribe and translate the GAD_{65} protein, which was labelled with ^{35}S -methionine (Amersham).

Serum samples were incubated overnight with labelled protein, and Protein A Sepharose (Pharmacia Biotech) was used to isolate the immune complexes. After washing, the bound activity was measured in a liquid scintillation counter (1450 Microbeta Trilux, PerkinElmer Wallac). Relative units were calculated with a software program (MultiCalc, PerkinElmer Wallac) using a standard curve for a dilution series run on each plate. The cut-off limit was defined as 99th percentile in 373 unaffected Finnish subjects (5.35 RU). The receiver operating characteristic (ROC) curve demonstrates the performance characteristics of the GADA assay in distinguishing the cases of type 1 diabetes (n=757) from the controls (n=373). (Fig. 5) Based on 140 samples included in the 1995 Multiple Autoantibody Workshop, the disease sensitivity of the assay was 69% and the specificity 100% (48).

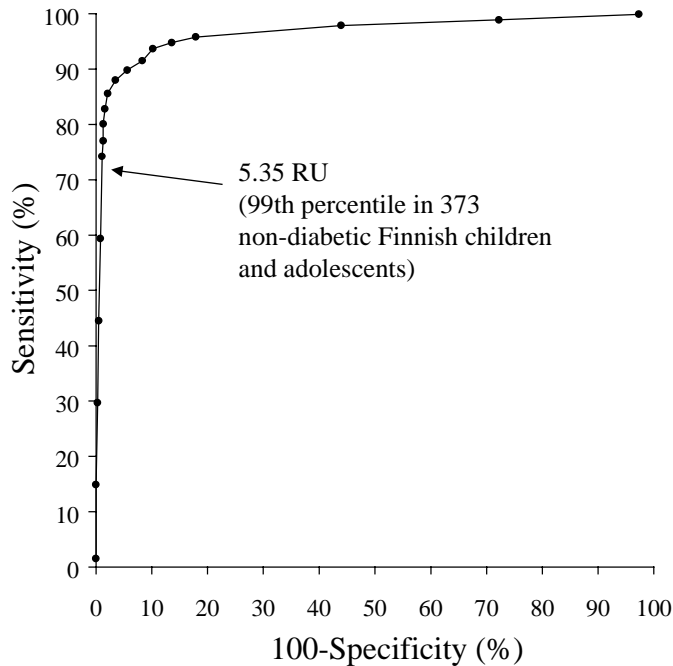


Fig. 5. ROC curve for the GADA assay. Results for 757 children with newly diagnosed type 1 diabetes and 373 control children were used to construct the curve.

4.2.3 Insulin autoantibody assay

Insulin autoantibodies were measured with a radiobinding assay modified from that described by Palmer et al. (75) After removal of endogenous insulin, the sera were incubated with mono-¹²⁵I(Tyr A 14) human insulin [Novo Research Institute (NRI), Bagsvaerd, Denmark or Amersham] in the presence or absence of an excess of unlabelled insulin. A specific binding of 0.01% of labelled protein corresponded to an antibody level of 1 nU/ml. The 99th percentile in a population of 105 non-diabetic children and adolescents was regarded as the cut-off limit for positivity, corresponding to 54 nU/ml with the NRI label (Papers I-V) and 68 nU/ml with that from Amersham (Paper IV). The disease sensitivity of the assay was 26% and the specificity 97%, based on 140 samples included in the 1995 Multiple Autoantibody Workshop (48).

4.2.4 Islet cell autoantibody assay

Islet cell antibodies were analysed by a standard immunofluorescence assay (141), the detection limit being 2,5 JDF-U. In the fourth international workshop on standardization of the ICA assay the sensitivity of our technique was 100%, specificity 98%, validity 98% and consistency 98% (49).

4.2.5 HLA typing

HLA DR alleles were typed using a standard serological test as described earlier (142) (Papers I, III and IV). HLA DQB1 alleles *02, *0302, *0301 and *0602 or *0603 were detected by a method based on PCR amplification, triple-label hybridization and time resolved fluorescence (143), and subjects positive for *02 were further analysed for the possibility of carrying either DQA1*05 or DQA1*0201 (144) (Paper II).

4.2.6 Other laboratory methods

Serum C-peptide concentrations were measured using a commercial kit (NRI) (Paper II, III), and HbA_{1c} (Paper II) and HbA₁ (Paper III) were determined electrophoretically, with reference ranges of 4-6% and 5.5-8.4% in non-diabetic subjects, respectively. A hexokinase method was used to determine blood glucose concentrations, and capillary blood gases, bicarbonate and base excess were analysed by routine laboratory methods (Paper II). Clinical data were collected from the case records (Paper II).

4.3 Statistical analysis

The data were analysed statistically using cross-tabulation and the chi-square test, Fisher's exact test, the Mann-Whitney U test, Kruskal-Wallis one-way ANOVA, Spearman's non-parametric correlation analysis, the *t*-test for unpaired and paired analyses, one-way analysis of variance, one-way analysis of variance for repeated measures, the Kaplan-Meier method and the log-rank test.

4.4 Ethics

Informed consent was obtained from subjects and/or their parents included in these studies. The study protocols were approved by the local ethical committees.

5 Results

5.1 Humoral immune responses to beta-cell antigens at the diagnosis of type 1 diabetes (Papers I and II)

IA-2A. An overwhelming majority of the patients with type 1 diabetes tested positive for IA-2A at the time of diagnosis, with highly variable levels. The results were unrelated to gender. These antibodies were more common in the patients under 20 years of age than in the older ones (II/Table 2) and appeared to decrease with increasing age up to 40 years (II/Fig. 1), although they showed no association with age among the patients younger than 15 years (I/Table 1)

Combinations of autoantibodies. Approximately 90% of the patients under 20 years tested positive for at least two autoantibodies, whereas less than 50% of the older ones did so. Only less than 5 percent of the patients under 20 years appeared to have no detectable autoantibodies at the time of diagnosis, as opposed to nearly one third of the older ones (II/Fig. 2). IA-2A levels correlated with ICA, and weakly with IAA, whereas IA-2A and GADA did not have positive correlation. A higher proportion of the patients were identified as having IA-2A and/or GADA than ICA alone, especially in the age group from 10 to 14.9 years (I/Fig. 2).

5.2 Humoral immune responses to beta-cell antigens in long-term type 1 diabetes (Paper III)

In general the levels of IA-2A, GADA and ICA decreased during the first 10 years of clinical type 1 diabetes (III/Fig. 1), as also did the proportions of antibody positive patients. Although the proportion of patients with three antibodies decreased, two thirds of the patients still had at least one antibody detectable after 10 years of clinical disease

(III/Fig. 2). An increase over the initial level of antibody was more commonly observed for GADA than for IA-2A, while no such a change was seen in ICA.

5.3 Humoral immune responses to beta-cell antigens are modulated by HLA genes (Papers I-IV)

Patients with type 1 diabetes. IA-2A levels and positivity appeared to be associated with DR4/non-DR3 in children and adolescents with newly diagnosed type 1 diabetes, but not with DR3/non-DR4 (I/Table 2 and Fig 3). A similar association appeared between the frequency of IA-2A and DQB1*0302/non-*02 but not with DQB1*02/non-*0302 in patients under or over the age of 20 years (II/Table 3). Moreover, the prevalence of IA-2A was remarkably high among patients younger than 20 years with the DQB1*02/0302 genotype. Similar associations were seen between the levels of IA-2A and the DQB1 genotypes. IA-2A were less frequent in subjects with the DQB1*0602 or *0603 allele than in other subjects, while no association was observed between IA-2A and the DR2 allele among children with newly diagnosed type 1 diabetes.

No relation was seen between IA-2A levels and HLA DR phenotypes over the first 10 years after the clinical presentation of type 1 diabetes.

Siblings of children with type 1 diabetes. The overall frequency of IA-2A positivity was increased in the HLA-identical siblings and siblings carrying the HLA DR3/4 or DR4/non-DR3 phenotype, and the same held true for GADA, IAA and ICA (IV/Table 2). The HLA phenotype appeared to be a stronger risk factor for antibody positivity than HLA identity (IV/Table 3), so that seroconversions to IA-2A positivity, and also to GADA, IAA and ICA positivity, were consistently associated with HLA DR3/DR4 and DR4/non-DR3 (IV/Table 4), while seroconversions to IA-2A, GADA and ICA negativity were associated with other phenotypes than those mentioned (IV/Table 5).

5.4 Natural course of disease-associated humoral immune responses in siblings of affected children (Paper IV)

After the clinical manifestation of diabetes in the proband, single autoantibodies (IA-2A, GADA, IAA and ICA) were detected in 8-14% of siblings under 20 years of age (IV/Fig. 2). Autoantibody positivity appeared to be associated with HLA identity with the proband, the DR3/DR4 and DR4/non-DR3 phenotypes and the presence of several autoantibodies in the initial sample (IV/Table 2). The incidence rates of seroconversions to antibody positivity varied between 6 and 23 per 1000 person-years. Positive seroconversions occurred in all age groups, but they seemed to be associated with young

age, a family size of four or more children and a high initial number of detectable autoantibodies (IV/Table 4). The incidence rates for inverse seroconversions were higher than those for positive ones, varying between 64 and 580 per 1000 person-years. Inverse seroconversions with respect to each antibody were associated with a small family size and a low maximal number of autoantibodies, and those of IA-2A, IAA and ICA also with greater age (IV/Table 5).

5.5 Humoral immune responses to beta-cell antigens in the assessment of the risk of future type 1 diabetes (Paper V)

IA-2A. The siblings of children with type 1 diabetes who later progressed to the disease usually tested positive for IA-2A at the time of diagnosis in the proband, and these progressors had higher antibody levels than the corresponding non-progressors (V/Fig. 2). IA-2A positivity close to the diagnosis of type 1 diabetes in the proband resulted in a positive predictive value of 55% over the subsequent 7.7 years, whereas the figures for ICA, IAA and GADA were lower (V/Fig. 1). When the cut-off limit of IA-2A was raised, the positive predictive value increased, but at the same time the sensitivity decreased (V/Table 2).

Multiple antibodies. Initial positivity for four, three, two, one and no antibodies gave estimated risks of 40%, 70%, 25%, 2% and 0.8%, respectively, for progression to type 1 diabetes in siblings over a period of 7.7 years (V/Table IV). Siblings with at least two antibodies initially had an estimated risk of 55%, whereas those with one or no antibodies had a risk of only 0.8%. The positive predictive value, sensitivity and specificity of IA-2A and/or GADA positivity were similar to those achieved with ICA positivity.

6 Discussion

The goal of this work was to gain new insights into the immunopathogenesis of type 1 diabetes and to make assessment of the risk of developing the disease more precise. Practical considerations made us to approach the issue from the viewpoint of the humoral immune responses of patients with newly diagnosed or long-standing type 1 diabetes and their siblings.

6.1 Study design

IA-2 and GAD, as autoantigens in type 1 diabetes, are expressed not only in the beta cells, but also in the alpha and delta cells of pancreatic islets, in the central nervous system, in peripheral nerves and in the adrenal medulla (34,39). The clinical disease is a consequence of specific beta cell destruction, however (145). The cellular and humoral autoimmune responses to beta cell antigens cannot alone explain the specific beta cell destruction, but the special characteristics of beta cells must account for the pathogenetic process leading to the cell destruction and the disease. One possible explanation for beta cell selective destruction may be their expression of a cytokine-inducible nitric oxide synthase (9). This work does not focus specifically on the characteristics of beta cells that lead to cell destruction.

It was set out here to investigate humoral immunity in type 1 diabetes, even though it is more evident that cellular rather than humoral autoimmunity mediates the beta cell destruction in type 1 diabetes (10). In fact, there is no consensus on the pathomechanism of the disease. T-cell mediated beta-cell destruction and destruction via apoptosis together with microenvironmental alterations have been proposed as possible pathogenetic models (8), but both hypotheses are difficult to prove. The T cells in the islets of Langerhans are virtually inaccessible and there are many technical problems concerning the methodology of T-cell responses in peripheral blood. Accumulating data on apoptosis in beta cell destruction in rodent models of autoimmune diabetes (8) cannot be applied directly to human type 1 diabetes. Human pancreas material is available only from patients who

have died soon after the diagnosis and from pancreas biopsies, which entail severe risks and are therefore impossible to perform without clinical indications, particularly in the case of small children. On the other hand, circulating autoantibodies are easily available in peripheral blood and relatively easy to analyse in extensive populations. In addition, previous studies have suggested that humoral autoimmunity is undisputedly related to the pathogenesis of type 1 diabetes (26,52,59,87). New molecular cloning technology has revealed hitherto unknown proteins which potentially act as autoantigens in type 1 diabetes - one of which is IA-2 (27-29). Because all of these advantages, relatively easy performance of the assay, and well characterized populations, it was decided to embark on an assessment of the role of humoral autoimmune responses to IA-2 in the pathogenesis of type 1 diabetes.

Accurate assessment of the risk of developing type 1 diabetes was of special interest, since if the disease can be predicted before clinical manifestation, the patients will not be exposed to ketoacidosis at diagnosis and all intervention studies and potential prevention trials could target subjects with preclinical type 1 diabetes. An optimal risk assessment strategy would predict the disease in the general population and at an early age. For such a strategy one would need a marker related to the pathogenesis of the disease, which would be rare in the general population. In the present work the risk of future diabetes was assessed in siblings of children with the disease at the time of diagnosis in the proband. This is not a rational approach, in the sense that 90 % of new cases with type 1 diabetes are of sporadic origin, whereas only about one tenth of them are familial (100,101). However, when developing risk assessment strategies one must begin by investigating the role of the hypothetic risk marker in manifested disease, advancing to populations with a known increased risk and then to the general population, as we have done with IA-2A. Siblings and subjects with specific genetic risk markers carry an increased risk of type 1 diabetes, and for us the population of siblings was more easily accessible. Accordingly, the role of IA-2A in assessment of the risk of type 1 diabetes in siblings of affected children was chosen as one focus of the present study.

As the incidence of type 1 diabetes in Finland is the highest in the world, so the absolute number of subjects is high in all the papers I-V. The subjects in the papers I, IV and V were enrolled between 1 September 1986 and 30 April 1989 in the population-based, nation-wide DiMe Study. Participation in the study was very active, and the probands in paper I represent the vast majority of patients with type 1 diabetes diagnosed in Finland during that time period. The participation rate of siblings was high as well. Moreover, the research on relatives was limited to siblings, and their follow-up was scheduled to begin at or close to the diagnosis in the proband, which lessens the possibility of biases. The extensive populations in all of the papers increase the informativeness and reliability of the results.

Since the controls were collected in Oulu, they represent a very small geographical area in Finland. Hypothetically, autoantibody levels could have geographical variation, so that the cut-off limits based on results gained from the controls might be biased. There is no variation in the incidence of type 1 diabetes between the provinces of Finland (100), however, and therefore no significant geographical variation in autoantibody levels is probable.

6.2 The IA-2A assay

A poor autoantibody assay may introduce bias into the reported levels and frequency of IA-2A. The assay used here was carefully developed, modifying a widely accepted method (31). A standard curve based on a dilution series was constructed on each plate, and the results were expressed in relative units based on this standard curve. The cut-off limit of positivity was set at the 99th percentile in 374 non-diabetic controls. The receiver operating curve (ROC) that was constructed using the results of 758 newly diagnosed patients and 374 controls was close to an optimal curve. The quality characteristics of our assay based on international standard samples were good, and the inter-assay variation was acceptable. Most of the results reported in these papers have been analyzed within a relatively short period of time, which lessens the risk of biases resulting from assay drift. Later the assay stability was assessed using internal control samples on each plate. The assay is quite simple to perform, and a huge number of samples can be analysed in a short time at relatively low cost. Accordingly, special efforts were made to optimize the assay and to avoid methodological biases.

Most of the samples were stored for years before the autoantibody assessment and the samples may have been melted and refrozen even many times before autoantibody assay. Hypothetically, this might interfere with the autoantibody levels of the sample. However, there are no data suggesting any rapid disappearance of autoantiantibodies during the storage time or after refreezing, so presumably these processes have only a slight effect on autoantibody levels, if any.

6.3 IA-2A at diagnosis

IA-2A were measured in patients with type 1 diabetes at the time of diagnosis in order to assess whether they play any role in the pathogenesis of the disease. The majority of the patients younger than 20 years of age tested positive at the time of diagnosis, indicating that IA-2A have a relevant association with the disease process.

Both the population studied and the assay method can affect the results. It has been reported previously that both age under 20 years (3) and the HLA DR4 allele (110), which is especially common among Finnish patients with type 1 diabetes (146), are associated with IA-2A. Obviously the methods used for analysing IA-2A have become more sensitive over time, so that the high frequency of IA-2A in the patients and the low frequency among the controls may point to a sophisticated and well-developed autoantibody assay rather than a poor one. These factors are likely to contribute to the observation of an exceptionally high prevalence of IA-2A in patients with newly diagnosed type 1 diabetes. The low levels of autoantibodies in control children may represent non-specific binding or low amount of real autoantibodies that are without clinical relevance.

6.4 Age at diagnosis of type 1 diabetes

Patients younger than 20 years of age appeared to have more usually multiple autoantibodies to beta-cell antigens than older patients. It is possible that the older patients had been autoantibody positive earlier and had become negative before presentation with the clinical disease. Some patients testing negative for autoantibodies have been reported to have antigen-specific T-cell responses (66). The lack of any signs of an autoantibody response in nearly one third of the adult patients points to the possibility of a non-immune aetiology for their diabetes (1). Although the adult patients in paper II were selected to represent type 1 diabetes on the basis of clinical examinations, it is possible that those without autoimmunity suffer from other specific types of diabetes, e.g. genetic defects or diseases of the exocrine pancreas.

6.5 Humoral autoimmunity after clinical manifestation of the disease

The autoimmune process went on in the majority of the child and adolescent patients for years after diagnosis, but became more silent as measured by autoantibody levels and frequency of positivity. It is difficult to assess why the process should persist for such a long time when practically all the beta cells have already been destroyed. It is unlikely that other cells in the body would release enough antigen to maintain the autoimmune response. Exogenous proteins or a continuous small-scale regeneration of beta cells (147) could account for the persistence of autoantibodies. This remaining autoimmunity may make the long-term curing of type 1 diabetes by means of beta-cell transplantation complicated.

6.6 Risk assessment

Humoral autoimmunity against beta-cell antigens has been used successfully to assess the risk of future type 1 diabetes (56,59,127). Single autoantibody positivities in siblings of children with diabetes already represent an increased risk, but positivity for two or more autoantibodies enhances the risk considerably. Risk assessment on an individual basis is still complicated, however, partly due to the dynamic pattern of autoantibodies in siblings of children with type 1 diabetes. The use of the appearance and disappearance of autoantibodies as an inclusion criterion and an outcome measure, respectively, in intervention trials may be questioned on the basis of the observation of positive and inverse seroconversions. On the other hand, the spontaneous disappearance of autoantibodies suggests that their production may become silent.

The results regarding the dynamics of type 1 diabetes-associated autoantibodies should be considered critically. Although the study design described was better in many respects

than earlier ones, some limitations still existed. The follow-up began too late, the samples were not always obtained at the optimal frequency, some seroconversions may result from the assay methodology, the statistics used could not analyze multiple seroconversions and the low total number of seroconversions did not reach optimal statistical evidence.

The dynamics of the autoantibodies appeared to be associated with age, HLA-conferred genetic disease susceptibility and family size. The clustering of positive seroconversions in large families could be explained by environmental factors such as increased exposure to infections.

6.7 HLA genes and type 1 diabetes

An association between HLA genes and humoral autoimmunity was observed in both newly diagnosed patients and their siblings. These findings confirmed the earlier reported relationship of IA-2A to HLA DR4 (110) and DQB1*0302 (148).

The siblings of children with diabetes appeared to have a stronger propensity for autoantibody positivity if they carried the HLA DR3/DR4 or DR/4non-DR3 phenotype, whereas seroconversion to IA-2A negativity was associated with phenotypes other than DR3/DR4 or DR4/non-DR3. These findings indicate that persistent beta-cell autoimmunity is likely to develop in siblings with strong HLA-defined genetic disease susceptibility.

7 Conclusions

Autoantibodies against the intracellular part of a membrane-bound, protein tyrosine phosphatase-like molecule, IA-2, are rarely detected in non-diabetic children and adolescents. On the other hand, although most siblings of children with type 1 diabetes remain without any signs of humoral beta-cell autoimmunity, the frequency of IA-2A is increased in siblings as compared with a control population. When detected, this autoantibody positivity appears to be a dynamic process modified by HLA-conferred genetic susceptibility, the age of the sibling and family size. Analysis of IA-2A facilitates the assessment of the risk of future diabetes in siblings, giving the highest positive predictive value out of the four autoantibody markers detected here. A combined analysis of autoantibodies seems to be a useful way of assessing the future risk of developing the disease, but accurate risk assessment on an individual basis is an intricate matter.

IA-2A are detected in the overwhelming majority of children and adolescents at clinical presentation with type 1 diabetes, and these antibodies have an association with the HLA DR4 and DQB1*0302 alleles. Humoral immune responses are stronger in patients diagnosed under the age of 20 years than in older ones, as reflected by higher autoantibody frequencies in younger patients. IA-2A levels decrease after the diagnosis of the disease, but more than half of the patients still remain positive for IA-2A after a disease duration of 10 years.

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