SANNA HOPPU

Characterization of Autoimmune Response in Preclinical Type 1 Diabetes

ACADEMIC DISSERTATION
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To my Family

CONTENTS

LIST OF ORIGINAL COMMUNICATIONS

ABBREVIATIONS

ABSTRACT

INTRODUCTION

REVIEW OF THE LITERATURE

INNATE AND ADAPTIVE IMMUNITY

HUMORAL IMMUNITY

B CELLS AND IMMUNOGLOBULINS

HUMORAL IMMUNE RESPONSE

CELLULAR IMMUNITY

T CELLS AND T CELL RESPONSE

EPIDEMIOLOGY OF TYPE 1 DIABETES

ETIOLOGY AND PATHOGENESIS OF TYPE 1 DIABETES

GENETIC FEATURES OF TYPE 1 DIABETES

AUTOIMMUNITY IN TYPE 1 DIABETES

AUTOANTIGENS AND AUTOANTIBODIES

ENVIRONMENTAL TRIGGERS OF AUTOIMMUNITY

FUTURE DIRECTIONS OF TYPE 1 DIABETES

AIMS OF THE PRESENT RESEARCH

SUBJECTS AND METHODS

CHILDHOOD DIABETES IN FINLAND (DiMe) STUDY

THE DIABETES PREDICTION AND PREVENTION STUDY (DIPP)

STATISTICAL ANALYSES

RESULTS

DISCUSSION

SUMMARY AND CONCLUSIONS

ACKNOWLEDGEMENTS

REFERENCES

ORIGINAL COMMUNICATIONS

LIST OF ORIGINAL COMMUNICATIONS

In addition to the material included in the original papers listed below, some previously unpublished data are presented in this dissertation.

I Hoppu S, Ronkainen MS, Kulmala P, Åkerblom HK, Knip M; Childhood Diabetes in Finland Study Group (2004): GAD65 antibody isotypes and epitope recognition during the prediabetic process in siblings of children with type I diabetes. Clin Exp Immunol 136:120-128.

II Hoppu S, Härkönen T, Ronkainen MS, Åkerblom HK, Knip M; Childhood Diabetes in Finland Study Group (2004): IA-2 antibody epitopes and isotypes during the prediabetic process in siblings of children with type 1 diabetes. J Autoimmun 23:361-370.

III Hoppu S, Härkönen T, Ronkainen MS, Simell S, Hekkala A, Toivonen A, Ilonen J, Simell O, Knip M (2005): IA-2 Antibody Isotypes and Epitope Specificity during the Prediabetic Process in Children with HLA-Conferred Susceptibility to Type 1 Diabetes. Submitted

IV Hoppu S, Ronkainen MS, Kimpimäki T, Simell S, Korhonen S, Ilonen J, Simell O, Knip M (2004): Insulin autoantibody isotypes during the prediabetic process in young children with increased genetic risk of type 1 diabetes. Pediatr Res 55:236-242.

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Communication IV will be published as a part of Matti Ronkainen's dissertation at the University of Oulu.

ABBREVIATIONS

Ab antibody

AFC antibody forming cell

APC antigen presenting cell

AUC area under the curve

BB rat BioBreeding rat

B cell B lymphocyte

CD40L CD40 Ligand

C domain carboxy terminal domain

C region constant region

DASP Diabetes Autoantibody Standardization Program

DiMe the Childhood Diabetes in Finland Study

DPT-1 The American Diabetes Prevention Trial

DIPP The Diabetes Prediction and Prevention Study

ENDIT The European Nicotinamide Diabetes Intervention Trial

FPIR first-phase insulin response

GAD65 the 65 kDa isoform of glutamic acid decarboxylase

GAD67 the 67 kDa isoform of glutamic acid decarboxylase

GFAP glial fibrillary acidic protein

HLA human leukocyte antigen

IA-2 the protein tyrosine phosphatase-related IA-2 protein

IAA insulin autoantibodies

ICA islet cell antibodies

IDS The Immunology of Diabetes Society

Ig immunoglobulin

IFN interferon

IL interleukin

IPTG isopropyl-β-D-thiogalactopyranodise

JM juxtamembrane

kD kilodalton

LADA late autoimmune diabetes in the adult

MHC Major Histocompatibility Complex

NK cell natural killer cell

NOD mouse   the nonobese diabetic mouse

PCR   polymerase chain reaction

Protein A   Staphylococcal protein A

PTP   protein tyrosine phosphatase

RU   relative unites

SDS   standard deviation score

T1D   type 1 diabetes

T cell   T lymphocyte

TCR   T cell antigen receptor

Th1/Th2   T-helper 1/T-helper 2

TNF   tumor necrosis factor

TRIGR   Trial to Reduce IDDM in Genetically at Risk

V region   variable region

ABSTRACT

Type 1 diabetes (T1D), one of the most serious and common metabolic disorders in children and adolescents, is a chronic autoimmune disease. It is the result of destruction of the insulin-producing beta cells in the pancreas. This asymptomatic prediabetic period, which may last for years, finally leads to severe insulin deficiency by the time of diagnosis. The presence of circulating antibodies to various islet cell proteins is a characteristic immune phenomenon associated with T1D. These autoantibodies are well-established diagnostic markers of an ongoing immune process in the islets, and thus provide a potential tool for identifying individuals at risk for subsequent development of the disease. This thesis aimed at characterizing the humoral immune response to T1D-associated autoantigens, such as the 65 kDa isoform of glutamic acid decarboxylase (GAD65), the protein tyrosine phosphatase (PTP)-related IA-2 and IA-2β molecules, and insulin, and at assessing the utility of the epitope recognition and isotype pattern of such antibodies as surrogate markers for disease development in preclinical T1D. The study population comprised initially non-diabetic siblings of affected children derived from the Childhood Diabetes in Finland (DiMe) Study, and genetically susceptible young children, who participated in the population based Finnish Type 1 Diabetes Prediction and Prevention (DIPP) Study. The selected children expressed autoantibodies against insulin (IAA), GAD65 and/or IA-2 antigen, and almost half of them presented with clinical T1D during follow-up. Specific radiobinding assays were used to determine the epitope specifities of GAD65 and IA-2/IA-2β and the isotype specific antibodies (Abs) of GAD65, IA-2/IA-2β and insulin. Individual areas under the curve (AUC) over the observation period were calculated for autoantigen specific antibodies, and for each isotype and specific epitope responses. The humoral immune response to GAD65 was initially observed as a simultaneous response to the middle (M) and carboxy (C) terminal regions of the GAD65 molecule in most siblings of affected children, and if the response was initially restricted to the middle region, it rapidly spread to the C-terminal domain and in a few cases later to the amino (N) terminal domain. There was some heterogeneity in the GAD65 isotype response, but it was mainly composed of antibodies of immunoglobulin (Ig) G1 subclass. Responses of IgG2-, IgG4-, IgM-, and IgA-GAD65Abs were observed frequently, whereas IgE- and IgG3-GAD65Ab

responses were seen more rarely. The non-progressors tended to have initially more often IgG2- and IgG4-GAD65Abs than the progressors. The children who progressed to T1D had IA-2 juxtamembrane (JM) epitope response more frequently, but IgE-IA-2 antibodies less often than the children who did not progress to T1D. The children who did not progress to T1D had higher AUC responses of IgE-IA-2 antibodies, and the occurrence of IgE-IA-2 antibodies was protective even when combined with positivity for IA-2 JM antibodies. Accordingly IgE-IA-2 antibody reactivity may be a marker of an immune response providing protection against or delaying progression to T1D among IA-2 antibody-positive children. The children who progressed to T1D had IAA of subclass IgG3 in their first IAA positive sample more often than did the children who did not progress to disease. The progressors also had higher integrated levels of IgG1-IAA and IgG3-IAA. In summary, this study failed to identify any GAD65 epitope- or isotype-specific antibody reactivity that could be used as a marker for progression to disease, as such progression was not associated with any specific changes in reactivity over time. Our findings demonstrate that JM-reactive IA-2 antibodies are associated with an increased risk of progression to overt T1D, while an IgE response to IA-2 confers relative protection from clinical disease both among siblings of affected children and young children with Human leukocyte antigen (HLA) -conferred T1D susceptibility recruited from the general population. The data further shows that young children who progress rapidly to T1D are characterized by strong IgG1 and IgG3 responses to insulin, whereas a weak or absent IgG3 response is associated with relative protection from disease.

LYHENNELMÄ

Lapsuusiällä alkava diabetes on tyypillisesti tyypin 1 diabetes (T1D) eli insuliininpuutosdiabetes. Suomessa diabeteksen ilmaantuvuus on maailman suurinta, ja sairastuvuus on yhä lisääntymässä erityisesti nuorimmissa ikäryhmissä. Syytä tähän ei tiedetä, mutta sekä perinnöllinen alttius että ympäristötekijät vaikuttanevat samanaikaisesti. T1D on parantumaton autoimmuunitauti, missä elimistön omat immunologiset mekanismit vähitellen tuhoavat haiman insuliinia tuottavat beetasolut. Vaikka sairauden oireet ilmaantuvat usein nopeasti, T1D kehittyy hitaasti. Insuliinipistoksia ja toistuvaa verensokeritason seurantaa vaativan sairauden diagnosointia edeltää aina vaihtelevan pituinen oireeton vaihe, jolloin seerumista on todettavissa autovasta-aineita saarekesoluja (ICA), insuliinia (IAA), glutamaattidekarboksylaasia (GAD65) ja tyrosiinifosfataasin kaltaista proteiinia (IA-2) kohtaan. Tässä työssä määritettiin T1D liittyven autovasta-aineiden alaluokkien ja isotyyppien (IgG1-4, IgA, IgE ja IgM) pitoisuuksia ja epitooppispesifisyyttä diabeteksen prekliinisessä vaiheessa. Tavoitteena oli selvittää miten humoraalinen immunivaste kypsyy T1D prekliinisessä vaiheessa, ja arvioida löytyisikö vasta-aineista uusia immunologisia mittareita, joiden avulla voidaan tulevaisuudessa tunnistaa suuressa sairastumisriskissä olevat yksilöt ja kohdistaa ehkäisevät hoitotoimet tähän ryhmään. Tutkimusaineiston muodostivat Lapsuusiän diabetes Suomessa (DiMe) –tutkimuksen sisarusaineiston vasta-ainepositiiviset lapset ja ne Diabeteksen ennustamis- ja ehkäisytutkimukseen (DIPP) osallistuvat lapset, joilla on ensinnäkin perinnöllinen alttius, mutta myös autovasta-aineita veressään. Noin puolet tähän tutkimukseen osallistuneista lapsista sairastui seurannan aikana diabetekseen. Vasta-aineiden pitoisuudet, ja niiden isotyyppi- ja epitooppispesifisyys mitattiin radioimmunologisin menetelmin. GAD65-vasta-aineiden epitooppi- ja isotyyppispesifisyyksiä mittaamalla ei kyetty tunnistamaan lapsia, joilla olisi suurentunut riski sairastua tyypin 1 diabetekseen. GAD65 vasta-aineiden epitooppi- ja isotyyppispesifiset vasta-aineet käyttäytyivät prediabeettisen seurannan aikana samalla tavalla dynaamisesti vaihdellen sekä lapsilla jotka seurannan aikana sairastuivat diabetekseen että lapsilla jotka eivät sairastuneet. IA-2 vasta-aineiden spesifisyys juxtamembraani(JM)alueen epitooppia kohtaan lisäsi lasten riskiä sairastua diabetekseen, kun taas IgE:n ilmaantuminen heijasti suojaavan vasteen kehittymistä. Niillä lapsilla, jotka eivät sairastuneet, tai joiden sairastuminen tapahtui

myöhemmin, oli havaittavissa IgE-IA-2 luokan vasta-aineita seerumissa. Insuliiniautovasta-aineiden alaluokka IgG3 havaittiin useimmiten niillä lapsilla, jotka hyvin nuorena ja nopean esivaiheen jälkeen sairastuivat tyypin 1 diabetekseen. Johtopäätöksenä voidaan todeta, että tyypin 1 diabetekseen liittyvien vasta-aineiden epitooppi- ja isotyyppispesifisyyksiä määrittämällä voidaan lapsen yksilöllistä riskiä diabekselle hieman tarkentaa, mutta immuunivaste diabetekseen esivaihessa on hyvin heterogeeninen ja dynaaminen ja ennenkaikkea antigeeni spesifinen, joten näillä analyyseillä tulee tuskin olemaan ratkaisevaa merkitystä kliinisen tyypin 1 diabeteksen ennustamisessa.

INTRODUCTION

Type 1 diabetes (T1D) is a chronic metabolic disorder that affects the host's ability to produce insulin, a hormone necessary for the conversion of food into energy. The disease presents predominantly in children, the clinical onset is unexpected and the symptoms comprise polyuria, polydipsia, weight loss and fatigue. Hyperglycemia results as a consequence of absolute insulin deficiency, and the only way to survive is permanent insulin therapy. Daily care including multiple insulin injections and glucose measurements puts a life-long burden on patients, their families and society. Good metabolic control decreases the risk of long-term complications, but T1D remains to be a threat both to quality of life and to life expectancy. The incidence of T1D in Finland is higher than anywhere else in the world (Karvonen et al. 2000), and it is still rising. The reasons for this are not known, largely because the etiology of the disease still remains to be defined. Over the past 20 years, evidence has accumulated that T1D is an immune-mediated disease, characterized by selective T cell mediated destruction of the pancreatic insulin-producing beta cells, but the exact pathogenic process or the factors affecting the disease process are so far largely unknown. It is, however, generally accepted that both genetic predisposition and diabetogenic exogenous factors are required for the initiation of the disease process leading ultimately to total beta cell destruction. This chronic prediabetic process may begin early in life, and it always starts months or even years before the presentation of clinical diabetes. Throughout this long prediabetic period, autoantibodies specific for islet antigens, such as glutamic acid decarboxylase (GAD65), the thyrosine phosphatase-like protein IA-2 (IA-2), and insulin have turned out to be useful markers for the risk of progression to overt T1D (Knip 1997). However, it is not possible to predict accurately on an individual basis whether a person will develop T1D, or when the disease will become manifest. Identification of additional parameters such as autoantibody isotypes, IgG subclasses and intramolecular spreading of the immune response to multible determinants could be helpful. An improved knowledge of the maturation of the preclinical disease process would be essential to identify those children who would most markedly benefit from preventive intervention trials. Such trials do not at present exist, but hopefully in the near future there will be methods, which delay or prevent the progression to clinical T1D.

REVIEW OF THE LITERATURE

INNATE AND ADAPTIVE IMMUNITY

The immune system defends the host against infections while maintaining homeostasis in the body and thereby avoiding chronic inflammatory processes and autoimmune diseases. The immune system comprises two arms, innate reactions that are ready to function without any previous contact with an antigen (innate immunity) and specific immunological reactions (adaptive immunity), which have high antigenic specificity and immunological memory (Janeway 2001, Beyan et al. 2003). The nature of the antigen influences the type of immune response that occurs. Polysaccharide capsule antigens of bacteria generally induce innate immune responses, whereas proteins mainly induce the adaptive system, including both humoral and cell-mediated immune responses. Neutrophils, eosinophils, basophiles, mast cells, natural killer (NK) cells, and professional antigen presenting cells (APCs), such as dendritic cells and macrophages are principal effector cells of the innate immune system. Adaptive immune responses are mediated by lymphocytes with antigen-specific receptors. (Delves and Roitt 2000, Beyan et al. 2003)

Lymphocytes may be divided into two main groups: T cells, which are responsible for cell-mediated reactions, and B cells, which are responsible for antibody production. On contact with an antigen, the lymphocytes start to divide, giving rise to a large clone of cells with the capacity to recognize and respond to the antigen. (Delves and Roitt 2000, Janeway 2001, Beyan et al. 2003)

HUMORAL IMMUNITY

B CELLS AND IMMUNOGLOBULINS

The function of B lymphocytes is to produce antibodies, the central effector molecules of the humoral immune response. Antibodies (immunoglobulins) are a group of soluble glycoproteins present in serum. Direct contact with the antigen and the help of T cells is needed to induce the B cell to develop into antibody forming cells (AFCs), called plasma cells, which secrete large amounts of Abs. After primary antigen stimulation the B cell can produce IgM; after stimulation by an antigen, and with the help of T cells, the cell can switch classes to produce IgG, IgA or IgE. Class switching and somatic hypermutation of the variable region are important in the

maturation of the immune response. Five distinct classes of immunoglobulin molecules mentioned above have been identified in humans. They differ in size, charge, amino acid composition and carbohydrate content. In addition to the difference between classes, the immunoglobulins within each class are also relatively heterogeneous, which is related to their functions. (Porter 1967, Huber 1980, Taussig 1988)

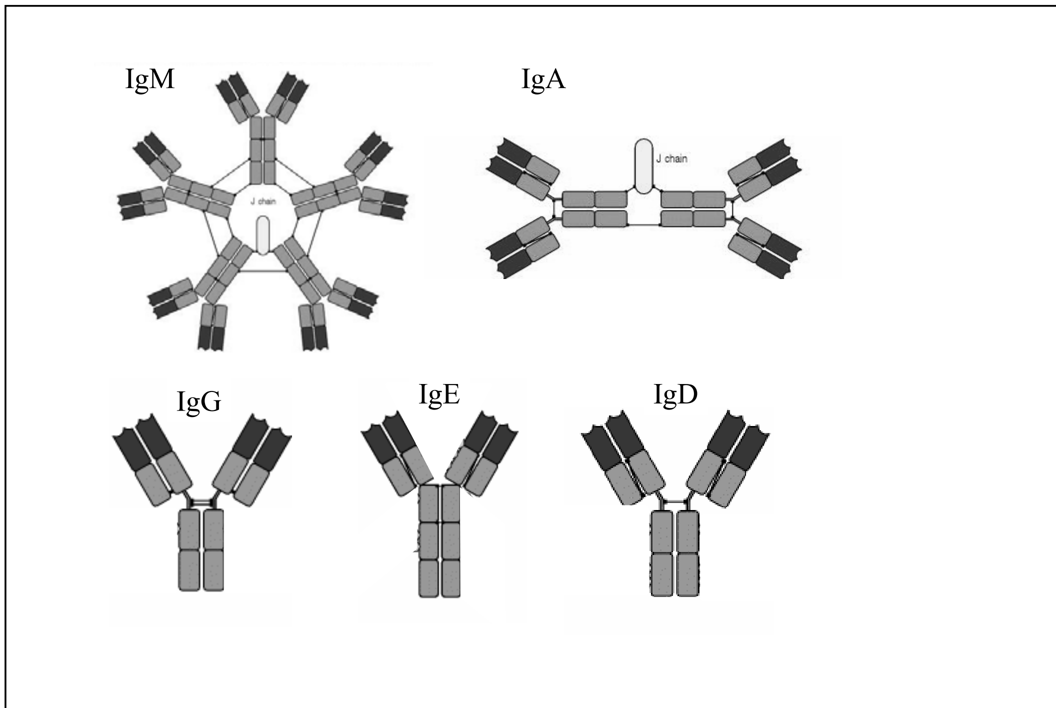

Fig 1. Schematic drawing of the basic structure of the five human immunoglobulin molecules. IgM is presented as a pentamer and IgA as a dimer. The amino-terminal end, meaning the variable (V) region of both light and heavy chains, is marked in black in each molecule, and the constant (C) parts in gray. The hinge region is a segment of the heavy chain, located between first and second constant portions in IgA, IgG and IgD. IgM and IgE molecules lack a hinge region, but each contains an extra heavy-chain domain. Dimers and pentamers are held together by the J chain. Figure modified from Meulenbroek 2000.

The basic structure of all immunoglobulin molecules is a Y-shaped unit consisting of two identical light polypeptide chains and two identical heavy polypeptide chains, which are linked together by disulfide bonds. Its heavy chain type determines the class and subclass of an immunoglobulin molecule. A typical antibody forms two

arms, which apexs (the amino-terminal regions) vary between different antibody molecules, and are called the variable (V) regions. These V regions are involved in antigen binding, whereas the stem of the Y, or the constant (C) region, is far less variable, and is the part of the immunoglobulin that interacts with effector cells. A molecule has two intrachain disulfide bonds in the light chain, one in the variable region and one in the constant region. There are four or five such bonds in the heavy chain, which is twice the length of the light chain. The peptides of the molecule make loops that are enclosed by these disulfide bonds and form a tertiary structure called domain. In both the heavy and light chains the first Ig domain is in the variable region. The constant regions of the IgG, IgA and IgD have three and those of IgM and IgE chains four additional Ig domains (Fig1). (Huber 1980, Taussig 1988)

IgG. IgG is the major immunoglobulin in normal human serum, accounting for 70-75% of the total immunoglobulin pool. It comprises a single 4-chain molecule with a molecular weight of 146 000 kilodalton (kD). There are four IgG subclasses (IgG1, IgG2, IgG3 and IgG4) having heavy chains called γ1, γ2, γ3 and γ4 respectively. The four subclasses of human IgG (IgG1-IgG4) differ only slightly (Table 1) and occur in approximate proportions of 66%, 23%, 7%, and 4% respectively. The most striking structural difference within molecules is the hinge region, which is the part of the molecule containing disulfide bonds between the γ-heavy chains and Fab arms. IgG3 has an elongated hinge region which accounts for its higher molecular weight (170 000 kD) and possibly for some of its enhanced biological activity, and susceptibility to proteolytic enzymes. These IgG subclasses have arisen relatively late in evolution, and therefore differ greatly from those identified in the mouse. (Papadea and Check 1989, Herrod 1992)

Table 1. Some biological properties of human IgG subclass and reference ranges (g/l) of IgG subclasses in healthy Caucasian children. Table modified from Meulenbroek 2000

	IgG1	**IgG2**	**IgG3**	**IgG4**
Serum level range (g/l) and proportion of total IgG (%)				
Age in months				
1-6	1.8-7.0 (60-69%)	0.4-2.1(15-19%)	0.2-0.8 (5-7%)	<0.1-0.3 (1-2%)
6-12	2.0-7.7 (60-77%)	0.3-2.3 (15-23%)	0.2-1.0 (6%)	<0.1-0.4 (1-3%)
Age in years				
<2	2.9-8.5 (60-100%)	0.5-2.6 (16-18%)	0.2-1.1 (6-8%)	<0.1-0.8 (1-6%)
<4	3.5-9.4 (60-70%)	0.6-3.0 (13-22%)	0.1-1.3 (3-9%)	<0.1-1.3 (1-9%)
<6	3.7-10.0 (70-75%)	0.7-3.4 (14-25%)	0.1-1.3 (3-10%)	<0.1-1.6 (1-12%)
<9	4.0-10.8 (76-77%)	0.9-4.1 (16-27%)	0.1-1.4 (2-9%)	<0.1-1.9 (1-13%)
<12	3.7-12.8 (52-80%)	1.1-6.1 (17-38%)	0.2-1.6 (3-10%)	0.1-2.3 (1-15%)
Adults	4.9-11.4 (43-75%)	1.5-6.4 (16-48%)	0.2-1.1 (2-8%)	0.1-1.4 (1-8%)
Half-life (days)	21	21	7	21
Antibody response to:				
proteins	++	+/-	++	+/-
polysaccharides	+	++	(-)	(-)
allergens	+	(-)	(-)	++
Binding to protein A	++	++	-	++

In the circulation these IgG class antibodies are distributed evenly between the intravascular and extravascular pools, and easily cross the placenta (Pitcher-Wilmott et al. 1980). After the first 6 months of life, during which maternal IgG levels decrease, the individual's own IgG production sets in, eventually reaching adult levels. Each IgG subclass has an individual pattern of development, IgG1 and IgG3 attaining adult levels at an earlier age than IgG2 and IgG4. The serum levels of IgG1 and IgG3 reach about 50% of the adult serum levels by the age of one year and about

75% by the age of five, all with large inter-individual variations. IgG2 and IgG4 rise much slower. By the age of five, 50% of the adult level has been reached. IgG subclasses differ from one another functionally. An isolated absence of a certain specific subclass may be associated with disease. For example, a low concentration of serum IgG4 may be associated with severe recurrent pyogenic infections in the respiratory tract. The properties of IgG1 are similar to those of IgG3 and those of IgG2 appear to resemble those of IgG4 in certain respects. In general, IgG1 and IgG3 are the major component of the T cell dependent response to protein antigens, and IgG3 antibodies are first to appear after anti-viral stimulation. IgG2 (and IgG4) are produced in T cell independent response to polysaccharide antigens. Repeated, long-term antigenic stimulation with T cell dependent antigens may lead to marked IgG4 response. (Heiner 1984)

IgM. IgM accounts for approximately 10% of the immunoglobulin pool. It is synthesized before birth, and by the age of 12 months the infant produces 75% of its adult level. The molecule is a pentamer of the basic four-chain structure. The μ chains of IgM differ from γ chains in amino acid sequence and have an extra constant region domain. The individual heavy chains have a molecular weight of approximately 65 000 kD and the whole molecule has a molecular weight of 970 000 kD. IgM is largely confined to the intravascular pool and is the predominant "early" antibody with a low affinity to antigens. IgM may also activate the classical pathway of complement. (Spiegelberg 1989)

IgA. IgA represents 15-20% of the human serum immunoglobulin pool. In human serum more than 80% of IgA occurs as a monomer of the 4-chain unit, but in most mammals the IgA in serum is mainly polymeric, present mostly as a dimer. IgA, which may be either subclass IgA1 (90%) or IgA2 (10%), is produced at the mucous membranes and is the predominant immunoglobulin in seromucous secretions such as saliva, colostrums, milk, and tracheobronchial and genitourinary secretions. A feature shared with IgM is an additional C-terminal 18-residue peptide, which is able to bind covalently to a J chain to form dimers. Secretory IgA is made up of two units of IgA, and is secreted by submucosal plasma cells. It appears around birth, but reaches adult concentrations by the age of 2 years. (Spiegelberg 1989)

IgE. IgE comprises 1-2% of the circulating immunoglobulin pool. IgE is bound to the high-affinity Fc receptors of basophiles and mast-cells, which induces histamine and leukotriene release. This immunoglobulin class may play a role in the immune response to helminthes parasites, but in developed countries is more commonly associated with allergic diseases such as asthma and hay fever. The IgE is formed from five domains. (Spiegelberg 1989)

IgD. IgD accounts for less than 1% of the total plasma immunoglobulin but is present in large quantities on the membrane of immature B cells. The precise biological function is unknown, but it may play a role in antigen-triggered B lymphocyte differentiation. This protein is more susceptible to proteolysis than other immunoglobulin isotypes and has also a tendency to undergo spontaneous proteolysis. (Spiegelberg 1989)

THE HUMORAL IMMUNE RESPONSE

The antibody response is the consequence of a series of cellular and molecular interactions occurring in orderly sequence, but the primary function of an antibody is to bind an antigen with its specific antigen binding receptor. In a few cases this has a direct defensive effect, for example by neutralizing bacterial toxin (IgG), or by preventing viral penetration of cells. In general, however, the interaction between the antibody and the antigen is without significance unless secondary effector functions are initiated. Activation of the complement system is one of the most important effector mechanisms of IgG1, IgG3 and IgM molecules. The complement system also mediates inflammatory reactions in nonorgan-specific (involves IgG1 and IgG3) and organ-specific autoimmune disease. IgG2 appears to be less effective in activating complement, while IgG4, IgA, IgD and IgE are ineffective. (Raff 1973, Papadea and Check 1989, Spiegelberg 1989)

Following the primary antigenic response, there is an initial lag phase, followed by phases in which first the IgM and later IgG antibody titers increase logarithmically to a plateau and then decline again. The decline occurs because the antibodies are either naturally catabolized or bind to the antigen and are cleared from the circulation. The secondary antigenic challenge differs from the primary one in four major respects. Firstly the secondary response has a shorter lag phase and an extended plateau and

decline. The plateau of the antibody levels is substantially higher in the secondary response, and comprises mainly IgG and very little IgM, which is the major component of the primary response (Fig 2). The affinity of the antibodies is also usually higher in the secondary phase than those produced during the primary response, as there is no affinity maturation of the IgM response. The degree of affinity is inversely related to the dose of antigen administered. It has been suggested that, in the presence of low antigen concentrations, only B cells with high-affinity receptors bind sufficient antigen and are triggered to divide and differentiate. (Raff 1973, Delves and Roitt 2000)

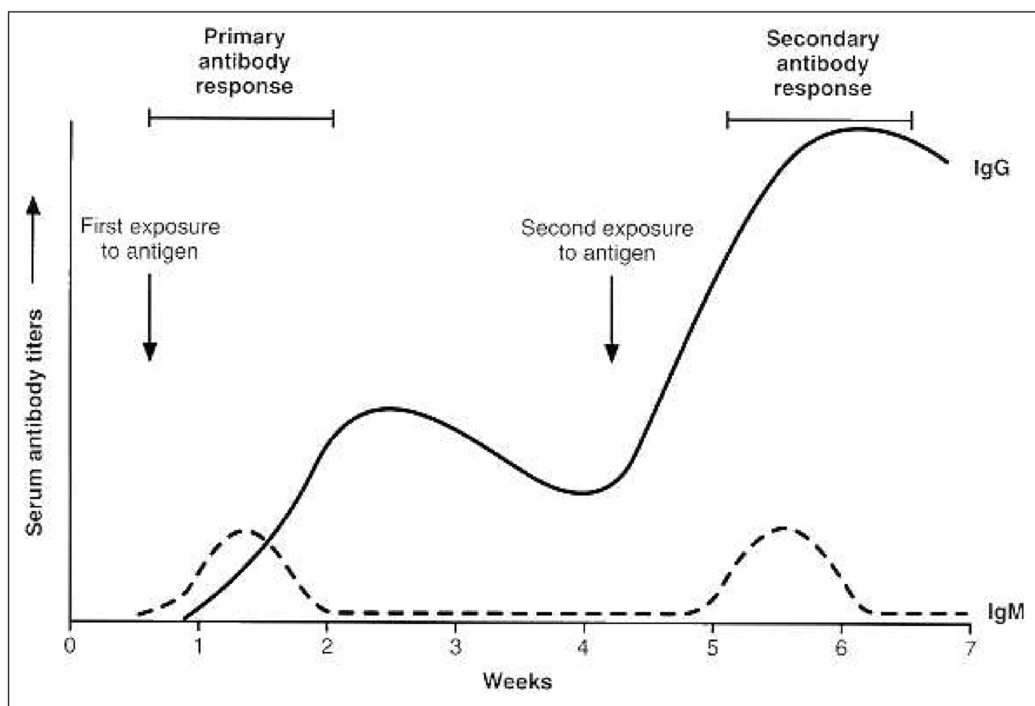

Fig 2. Schematic drawing of primary and secondary antibody responses. Due to the activation of adaptive immunity, the IgG antibody levels are higher, and the affinity of antibodies more mature in the secondary phase than those produced during the primary response. Figure based to Meulenbroek 2000.

During the immune response, there is a progressive change in the predominant immunoglobulin class of the specific antibody produced, usually to IgG. The IgG subclasses produced by the plasma cells vary depending on the stimulus. Isotype switching is based on DNA rearrangements in the immunoglobulin gene in the

nucleus of the B cells, which further depend on signals from T-helper cells and cytokines that determine the new isotype. (Seide and Kehoe 1983, Bich-Thuy and Revillard 1984, Taussig 1988)

Cytokines have a central role in the positive and negative regulation of immune reactions. It is now clear that CD4-positive T-helper (Th) lymphocytes have two different profiles. Cytokines from Th 1 cells inhibit the actions of Th2 cells and vice versa. The cytokines produced, and the balance of different cytokines, depends on antigen, the dose of antigen and genetic backround of host, but little is known about which cytokines are associated with pathogenesis of human T1D. At present, only IFN-α and and IFN-γ have been associated with beta cell destructive insulitis in human T1D (Table 2). (Rabinovitch and Suarez-Pinzon 2003)

Table 2. Properties of few selected cytokines. Each cytokine interacts with one or more receptors, and affects one or more types of cell. Table modified from Rabinovitch and Suarez-Pinzon 2003.

Cytokine		**Producer cells**	**Actions**	**Effect of cytokine or receptor knockout**	**Correlation with bening or destructive insulitis**
Pro inflammatory	IL-1	macrophages epithelial cells	T cell activation, macro-phage activation, fever	Toxic effect to beta cells in vitro, decreased IL-6 production	NOD MOUSE +, BB RAT +, HUMANS 0
	TNF-α	T cells, macrophages		Toxic effect to beta cells in vitro	NOD MOUSE +, BB RAT +, HUMANS nd
	IFN-α	Leukocytes	Antiviral	Impaired antiviral defences, no effect to beta cells in vitro	NOD MOUSE 0, BB RAT +, HUMANS +

Type 1 cytokines	IL-12	B cells, macrophages	Activates NK cells, induces $CD4^+$ T cell differentiation to Th1 –like cells	Impaired in IFN-γ production and in Th1 responses	NOD MOUSE 0, BB RAT +, HUMANS ?
	IFN-γ	T cells, NK cells	Macrophage and cytotoxic T cell activation	Impaired Th1 responses, Toxic effect to beta cells in vitro	NOD MOUSE +, BB RAT +, HUMANS +
	TNF-β	T cells, B cells	Endothelial activation	Decreased antibody, increased IgM, Toxic effect to beta cells in vitro	NOD MOUSE +, BB RAT +, HUMANS ?
	IL-2 (T-cell growth factor)	T cells	T cell proliferation and activation	May increase T cell autoimmunity, no effect to beta cells in vitro	NOD MOUSE +, BB RAT +, HUMANS nd
Type 2 cytokines	IL-4	T cells, mast cells	B cell activation, IgE switch, suppresses Th1 cells	Decreased IgE synthesis, no effect to beta cells in vitro	NOD MOUSE -, BB RAT 0, HUMANS 0
	IL-6	T cells, macrophages	Acute phase protein production, fever	Decreased acute phase reaction, reduced IgA production, no effect to beta cells in vitro	NOD MOUSE +, BB RAT ?, HUMANS 0
	IL-10			No effect to beta cells in vitro	NOD MOUSE -, BB RAT -, HUMANS ?
Type 3 cytokine	TGF-β	Chondrocytes, monocytes, T cells	Inhibits cell growth, anti-inflammatory, induces IgA secretion	Lethal inflammation	NOD MOUSE -, BB RAT -, HUMANS ?

Other	IL-5	T cells, mast cells	Easinophil growth, class switching, suppress Th2	Decreased IgE	
	IL-9	T-cells	B cell growth, inhibits macrophage cytokine production and Th1 cells	Defective regulation of isotype-specific responses	
	CD40 ligand	T cells, mast cells	B cell activation, class switching	Poor antibody response, no class switching, dimished T cell priming (hyper IgM syndrome)	

+, cytokine presence correlates with beta cell destructive insulitis; -, cytokine presence correlates with bening insulitis; 0, cytokine presence does not correlate with either destructive or bening insulitis; nd, not detected; ?,not reported.

CELLULAR IMMUNITY

T CELLS AND T CELL RESPONSE

T lymphocytes are mediators of the cell-mediated immune responses. Once they have completed their development in the thymus, naive T cells enter the bloodstream and are activated. They circulate through specific lymphoid tissues returning to blood until they encounter their specific antigen. Naive T cells are activated when they meet their specific antigen bound to the major histocompatibility complex (MHC) on the surface of an APC. Antigen recognition is mediated through a specific receptor, the T cell antigen receptor (TCR). TCR consists of α and β polypeptide chains (95% of T cells) or structurally similar γδ polypeptides (Bentley and Mariuzza 1996). αβ-T cells can be divided into three functional classes that detect peptide antigens bound to MHC I or II molecules. (Delves and Roitt 2000)

There are two major classes of T lymphocytes: CD8-positive cytotoxic T lymphocytes, which recognize processed antigens (i.e. peptides) bound to MHC class

I molecules on the surface of various cells (e.g. beta cells), and CD4-positive T-helper lymphocytes, which recognize processed antigens bound to MHC class II molecules on the surface of antigen-presenting cells (APCs), macrophages and dendritic cells or B cells. CD8-positive T cells differentiate into cytotoxic T cells that kill infected target cells, while the CD4-positive subset of T cells mainly help and initiate immune responses. CD4-positive T cells can further be divided into T helper class 1 and 2 cells (Th1 and Th2) according to differential cytokine secretion profiles. Pathogens that accumulate in large numbers inside macrophage and dendritic cell vesicles tend to stimulate the differentiation of Th1 cells, whereas extracellular antigens tend to stimulate the production of Th2 cells. Th1 cells activate the macrophages, and induce B cells to make IgG1 antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. Th2 cells initiate the humoral immune response by activating naive antigen-specific B cells to produce antibodies. These Th2 cells can subsequently stimulate the production of different isotypes, including IgA and IgE, as well as subtypes of IgG (specially IgG4 and IgG2). (Mosmann and Sad 1996, Germain 1994, Delves and Roitt 2000)

The regulatory T cells are important in controlling autoreactivity. Some of these regulatory T cells mature in the thymus, while others require activation by autoantigens in the periphery. (Germain 1994)

EPIDEMIOLOGY OF TYPE 1 DIABETES

T1D may develop at any age, but the majorities (over 50%) of patients are diagnosed within the first two decades of life (Reunanen 2002), however, an increasing number of cases are being recognized in older individuals as well. During the first year of life the disease is rare, but thereafter the incidence increases. The highest incidence has been reported in early puberty in the 10-14 year-old group (Green et al. 1992, Chueca et al. 1997), but over the past decades the clinical manifestation of T1D has moved into younger age groups, among whom the increase of incidence has been most conspicuous in relative terms, reflecting an acceleration of the disease process (Charkaluk et al. 2002). According to the EURODIAB registry the greatest rate of increase is seen among children under the age of 5 years (EURODIAB ACE study group 2000). The risk of disease is now approximately the same in all age groups in Finnish children. (Tuomilehto et al. 1999, Karvonen et al. 1999 and 2000)

Finland is known to have the highest incidence of T1D in the world among children younger than 15 years of age. The first nation-wide study in 1953 showed an annual rate of 12/100 000 (Somersalo 1955). Since then the incidence has gradually increased, being 45.4 per 100 000 children in 2003 (Reunanen, personal communication). The average increase has been 3.4% per year (Tuomilehto et al. 1999). Approximately 500 new children in Finland are affected annually by T1D.

Over the last decades the incidence of T1D has increased significantly worldwide (3.0% per year) (Onkamo et al. 1999), but it has remained principally a disease of Caucasians. The worldwide incidence rates of T1D vary greatly among different racial groups and populations, with Finland and Sardinia showing the highest incidence rates, while Chinese and populations in Southern America show the lowest incidence rates (Karvonen et al. 2000). Incidence varies considerably from one country to another in the same geographical region. Estonia, for example, has an incidence that is less than one fourth of that in Finland (Podar et al. 2001), whereas the incidence rate in Sardinia is at least five times higher than in continental Italy (7.2/100 000 children ≤14 years of age) (Karvonen et al. 2000). Large interethnic differences in the incidence rates between Jewish (18.5/100 000) and Arab (2.9/100 000) populations have been reported from Israel (Shamis et al. 1997). A proportion of the international variation in the incidence rates is explained by differences between populations in the distribution of particular DQ genotypes, which confer increased risk of T1D (Ronningen et al. 2001), but the rapidly increasing incidence within the same population cannot be explained by an increase in the pool of genetically susceptible individuals (Pitkäniemi et al. 2004).

In addition to the worldwide differences in incidence rates, a higher rate of diabetes has been reported in offspring of diabetic fathers vs. mothers (Warram et al. 1984), and male excess among newly diagnosed patient’s children especially in high-incidence countries (Tuomilehto et al. 1992, Lévy-Marchal et al. 1995, Chueca et al. 1997, Padaiga et al. 1997, Charkaluk et al. 2002) and among those diagnosed after puberty (Gale EA 2002).

ETIOLOGY AND PATHOGENESIS OF TYPE 1 DIABETES

T1D results from an immune-mediated loss of insulin-secreting beta cells. It is the clinical end point of a sequential cascade of chronic destructive processes which involves both cellular and humoral autoimmune response detectable in peripheral blood, months or even years, before the clinical presentation. Throughout this long prediabetic period, metabolic changes, including reduced insulin secretion with eventual glucose intolerance, develop at a variable rate toward full-blown T1D in genetically susceptible individuals.

GENETIC FEATURES IN TYPE 1 DIABETES

T1D is not an inherited disorder, but family studies provide evidence that genetic factors are important (Salvetti et al. 2000). Most cases of T1D are sporadic, and anyone can develop the disease, but some individuals are at greater risk than others, because they are genetically susceptible. Alleles or genetic variants associated with T1D provide either susceptibility or protection against the disease (Todd et al. 1987, Devendra et al. 2004). The genetic background of T1D is complex and the genetic susceptibility is polygenic (Davies et al. 1994, Cordell and Todd 1995, Field and Tobias 1997, Pitkäniemi et al. 2004). More than 20 years ago, the HLA region on chromosome 6 was found to contain a major locus that influences predisposition to T1D and a decade ago a locus with a smaller effect was identified in the insulin gene region (Field and Tobias 1997). In T1D, certain alleles of the MHC class II locus show the strongest association with the development of diabetes. The contribution of HLA genes to the familial clustering of T1D has been estimated to be in the range of 30-60% (Redondo and Eisenbart 2002). The linkage of HLA class II D locus (alleles DR3 and DR4) with T1D was originally demonstrated in the early 1980's (Platz et al. 1981), and when the HLA genotyping became more exact through the sequencing of HLA alleles the disease associations could be defined more precisely, which shows that a subregion of the DQ locus rather than a subregion of the DR locus has the strongest impact on disease susceptibility. Later it has become evident that e.g. DR4 subtypes may modify the genetic risk conferred by the DQ8 allele. (Redondo and Eisenbart 2002)

Linkage disequilibrium means that some genes co-occur more often than would be expected by chance in the general population, for example the DR4 allele is usually

present together with the DQ8 allele and the DR3 allele together with the DQ2 allele (Redondo and Eisenbart 2002). The genes in the class II region of the MHC complex encode molecules, which are expressed on the surface of APCs (i.e. macrophages, dendritic cells and B lymphocytes) and present antigens to antigen-specific receptors of $CD4^+$ T lymphocytes. To make the nomenclature of frequent genes more understandable the World Health Organizations made recommendations for the use of a coding system. Every letter and number in the HLA gene has a specific meaning (Schreuder et al. 2001). The class I molecules, expressed on all nucleated cells, are encoded by genes within the HLA-A, -B, and -C loci, whereas class II molecules are encoded by genes within the HLA-DP, -DQ, and -DR loci. The first letter D means that the gene is located in the class II region, and a letter for the subregion (M, N, O, P, Q, R) follows (Todd et al. 1988). Further each subregion contains A and B genes, which encode the α and β chains of the protein, and the number code seen after the letters refers to different genes from the same subregion.

Most patients affected by T1D carry the DR4, DQ8 (DR4-DQA1*0301-DQB1*0302) haplotype and/or the DR3, DQ2 (DR3-DQA1*0501-DQB1*0201) haplotype. DR4-DQ8/DR3-DQ2 heterozygous individuals have a particular high risk (Redondo and Eisenbart 2002). According to Finnish data 44% of all patients with T1D carry that combination, compared with a Finnish population prevalence of 4% (Reijonen et al. 1991, Ilonen et al. 1996, Veijola et al. 1996). Heterozygosity is even more frequent among children who progress to clinical diabetes before age 5. Some HLA alleles, for example HLA-DR2 or HLA-DQB1*0602, are protective, and subjects carrying protective alleles rarely develop T1D even when combined with a susceptibility allele (Ilonen et al. 1996, Nejentsev et al. 1999). The mechanism of this protective effect is not very well defined, although various hypotheses have been proposed. It has been suggested that it may arise from individual affinities in the interaction between the diabetogenic peptide and the HLA molecule and subsequent T cell recognition of the bound peptide (Nepom 1990), either during T cell maturation in the thymus or as a failure of T cell tolerance to different autoantigens (Nepom and Kwok 1998). Regardless of the mechanism, HLA typing has proved useful in population screening for identification and follow-up of individuals at increased risk for disease (Knip 2002).

Other genes within the HLA complex, particularly class I genes, have also been linked to T1D, but the strongest linkage by far is with the DQ and DR class II genes. According to Redondo and Eisenbarth (2002) 17 other genetic loci have been associated with T1D. However, only two of these associations have been confirmed, i.e. the polymorphism in the insulin gene region on chromosome 11p15 (IDDM2) and the polymorphism in the gene region encoding the cytotoxic T lymphocyte antigen 4 (CTLA4) on the long arm of chromosome 2 (IDDM7) (Ueda et al. 2003). The former may explain a maximum of 10% of the familial aggregation of T1D (Devendra et al. 2004), while the latter is associated with increased risk of common autoimmune disorders such as Grave's disease, autoimmune thyroiditis and T1D.

AUTOIMMUNITY IN TYPE 1 DIABETES

T1D is characterized by infiltration of mononuclear cells into pancreatic islets, a phenomenon called insulitis (Gepts 1965, Gepts and De Mey 1978). Evidence for the presence of insulitis first came from the histology of pancreas in patients with newly diagnosed T1D (Gepts 1965, Bottazzo et al. 1985). The knowledge of the immunopathogenesis has increased considerably over the last two decades, as studies on animal models such as the BioBreeding (BB) rat and the nonobese diabetic (NOD) mouse, both of which spontaneously develop autoimmune T1D, have shown without doubt that T cells are present in the inflammatory lesion, and play an important role in the disease process. The cause of the loss of tolerance to islet autoantigens and which autoantigens are critically involved in the initiation or progression of the disease are, however, still open, and more direct information on the etiology and pathogenesis at the human level is definitely needed. (Roep 2003)

What is known about the pathogenesis of human T1D comes largely from random autopsy specimens obtained from patients who died either close to the time of diagnosis from acute metabolic diabetic ketoacidosis or later in the course of the disease, and both of these time points represent a late stage of the disease process, as its has been estimated that 80-90% of the beta cells are put out of function at the time of the clinical presentation of T1D (Knip 2002). The autopsy samples show a decrease in the number of beta cells and a chronic autoimmune inflammation and lymphocytes surrounding and infiltrating the islets (Bottazzo et al. 1985). The degree of beta cell destruction varies substantially from almost intact islets to islets totally devoid of beta

cells. The nature, function and significance of the T cells seen in the infiltrates, are poorly defined, since in most countries pancreas biopsies are not allowed for ethical reasons, and the detection of autoreactive antigen-specific T cells from peripheral blood is technically difficult. (Roep et al. 1999, Peakman et al. 2001)

Once beta cell autoimmunity has been initiated, the progression of the process is quite variable, with some subjects progressing rapidly to clinical diabetes, while others remain in a nonprogressive state. The presentation of beta cell specific autoantigens by APCs to $CD4^+$ Th cells in association with MHC class II molecules is considered to be the first step in the initiation of the disease process. Denritic cells and macrophages play an essential role as APC in the development and activation of beta cell cytotoxic T cells by secreting interleukin (IL)-12, which stimulates $CD4^+$ Th1 cells to secrete IFN-γ and IL-2. These cytokines favor the destructive process by stimulating other still resting macrophages to release, in turn, cytokines such as IL-1ß, IFN-γ, tumor necrosis factor (TNF)-α and free radicals, which are toxic to the beta cells (Mauricio and Mandrup-Poulsen 1998). During this cytokine storm, the migration of beta cell autoantigen-specific $CD8^+$ cytotoxic T cells is induced, on recognizing specific autoantigen on beta cells in association with class I molecules, these $CD8^+$ T cells induce beta cell damage and apoptosis. Th2 cells try to suppress and regulate the disease process by secreting cytokines such as IL-4, IL-5, IL-6, IL-10, and IL-13, which stimulates the production of autoantibodies and antagonizes the Th1 responses. (Roep et al. 1999, Rabinovitch and Suarez-Pinzon 2003)

The cytokine profile of the infiltrating inflammatory cells plays a crucial role in the destructive process. A series of T cell assays have been established to characterize autoreactive T cells in the hope of learning more about the disease process leading to overt T1D. It has, however, turned out to be complicated to develop T cell assays based on the use of mononuclear cells from peripheral blood, which would differentiate between subjects with preclinical or clinical T1D and non-diabetic subjects (Meierhoff et al. 2002). It has been hypothesized alternatively that immunoglobulin subclasses and isotypes may reflect cytokine responses and that studies on the isotypes of diabetes-associated autoantibodies might provide information on the Th1/Th2 balance (Hawa et al. 2000).

THE Th1/Th2 PARADIGM

The cross-regulatory, polarized roles of Th1 and Th2 cells in the induction and regulation of T cell-mediated immune responses is now well established, and they are differentiable by the cytokines they secrete. Typical human Th1 cytokines include IL-2 and TNF-α and in particular IFN-γ, and they are involved in cell-mediated inflammatory reactions. By contrast, Th2 cells are characterized by the production of IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13. Th2 cells stimulate the production of antibodies, especially IgE involved in allergic responses. (Bach and Chatenoud 2001, Rapinovitsh and Suarez-Pinzon 2003)

Several factors influence the development of Th1 and Th2 cells, including the type of pathogen, the avidity of the TCR interaction with the antigen, and cytokines involved (Mosmann and Sad 1996). Pathogens that are inside APCs tend to stimulate the differentiation of Th1 cells, whereas extracellular antigens tend to stimulate the production of Th2 cells (Mosmann and Sad 1996). Th1 cells and their cytokines activate macrophages as mentioned earlier, and also induce B cells to produce IgG antibodies that are very effective at opsonizing extracellular pathogens for uptake by phagocytic cells. If the microbe/antigen persists despite such a response, the inflammatory reaction proceeds to result in tissue injury. Th2 cells initiate the humoral immune response stimulate the production of different isotypes, including IgE, as well as neutralizing and/or weakly opsonizing subtypes of IgG. (Abbas et al. 1996, Kolb 1997, Romagnani 2000 and 2004).

Th1 and Th2 cells also regulate each others function through their respective cytokines (Romagnani 2000). A variety of approaches, mostly in NOD mice and BB rats, have been used to study the roles of cytokines in the pathogenesis of T1D for the past 20 years. These studies largely support the concept that pernicious insulitis is associated with increased expression of proinflammatory Th1-type cytokines, whereas nondestructive (benign) insulitis is associated with increased expression of Th2 cytokines, that are beneficial or at least benign, in combating beta cell destruction (Rabinovitch and Suarez-Pinzon 2003). However, IL-4 producing Th2 cells are capable of transferring insulitis, and accelerating beta cell destruction or even diabetes in non-diabetic NOD mice (Katz et al. 1995, Daniel et al. 1995), and incapable of preventing disease (Katz et al. 1995), which suggests that the role of the Th1/Th2

balance is far more complicated in the pathogenesis of immune-mediated diabetes than initially perceived.

One possible mechanism that leads to disease development is a loss of immunological tolerance to self. T cell tolerance is established centrally in the thymus and further strengthened and maintained through multible mechanisms of peripheral tolerance (Bach and Chatenoud 2001, Walker and Abbas 2002). Intrest has focused on a feature of tolerance that seems to bridge the central and peripheral processes, namely $CD4^+CD25^+$ regulatory T cells. These cells are able to suppress both $CD4^+$ and $CD8^+$ T cells, and a very recent data has demonstrate that deficiency in the function of the $CD4^+CD25^+$ regulatory T cell population may influence the pathogenesis of human T1D (Lindley et al. 2005).

EPITOPE SPREADING

Epitope spreading within any given autoantigen in addition to the spreading from one autoantigen to another occurs during the maturation of immune response. As an autoimmune disease progresses from initial activation to a chronic state there is often an increase in the number of autoantigens targeted by T cells and antibodies ("epitope spreading") and, in some cases, also changes in participating cells, cytokines, and other inflammatory mediators. Both autoreactive T cells and B cells contribute to epitope spreading. Activated autoreactive B cells present novel cryptic peptides of autoantigens and express co-stimulatory molecules. They thereby generate peptides that have not previously been presented to T cells, and accordingly T cells have not become tolerant of such cryptic peptides. Over time, multiple novel peptides within a molecule can activate T cells. (Katz et al. 1995, Roep 2003).

AUTOANTIGENS AND AUTOANTIBODIES

Although the beta cell destruction is T cell-mediated, autoantibodies specific to islet antigens, such as insulin (IAA), glutamic acid decarboxylase (GAD65Ab), the thyrosine phosphatase-like protein IA-2 (IA-2Ab), and islet cells (ICA) have turned out to be useful markers of increased risk for T1D (Bingley et al. 1994, Verge et al. 1998, Ziegler et al. 1999). The possible role of a recently reported autoantigen, i.e. the glial fibrillary acidic protein (GFAP) in peri-islet Schwann cells remains open (Winer et al. 2003). This antigen is present at high concentration in the central nervous

system similar to GAD and IA-2. The nature, intensity, and antigenic spreading of the classical autoantibodies (IAA, GAD65Ab, IA-2Ab) identify individuals at increased risk for T1D, but it is still challenging to predict accurately whether a person will progress to clinical T1D, or not. Neither it is possible to transfer immune-mediated T1D by antibodies, strongly indicating that the autoantibodies are a passive bystander in beta cell destruction, but they do show that the disease process has been initiated (Schloot et al. 1999).

Despite increasing knowledge of specific pathogenetic factors in T1D, there is a need to identify additional markers facilitating disease prediction on an individual basis among subjects at increased risk of progression to clinical diabetes. Epitope spreading and isotype and IgG subclass distribution might reflect the maturation of the immune response and impending progression to T1D.

GLUTAMIC ACID DECARBOXYLASE (GAD)

The initial evidence for autoantigens in patients with T1D came from immunofluorescence studies, which showed that a high proportion of sera from patients with newly diagnosed T1D reacted with pancreatic islets. This led to an intensive search for the actual autoantigens with which the islet cell autoantibodies (ICA) reacted. The 64 kD antigen initially described by Baekkeskov et al. in 1982 was identified as the 65 kD isoform of glutamic acid decarboxylase (GAD65) by the same group 8 years later (Baekkeskov et al. 1990). GAD65 is a protein comprising 585 amino acids with a molecular weight of 65 kD encoded by a gene on chromosome 10p11. There are two isoforms of GAD, GAD65 being the smaller one and GAD67 the larger one. GAD65 is the principal autoantigen in T1D (Kulmala et al. 1998).

GAD is expressed in the nervous system and in neuroendocrine cells, including pancreatic islets, and it is located within neuron-like small vesicles. GAD67 is the dominant form in the central nervous system, while GAD65 is the dominant form in the islet cells. In the central nervous system GAD is involved in the synthesis of the neurotransmitter GABA, but in the beta cells its function is not clear. The presence of GABA receptors in islet beta cells has led to the suggestion that GABA is involved in paracrine signaling in the islet (Baekkeskov et al. 1990, Ellis and Atkinson 1996).

The two human GAD isoforms are approximately 65% identical at the amino acid level, with much of the variance in primary structure located within their first 100 amino acids (Karlsen et al. 1991, Bu et al. 1992), and they both have enzymatic activity (Ellis and Atkinson 1996). Both GAD isoforms are synthesized as hydrophilic and soluble molecules, but later the N-terminal domain of GAD65 is modified so that it anchors the molecule within the membranes of islet beta cells (Fig 3) (Bu et al. 1992).

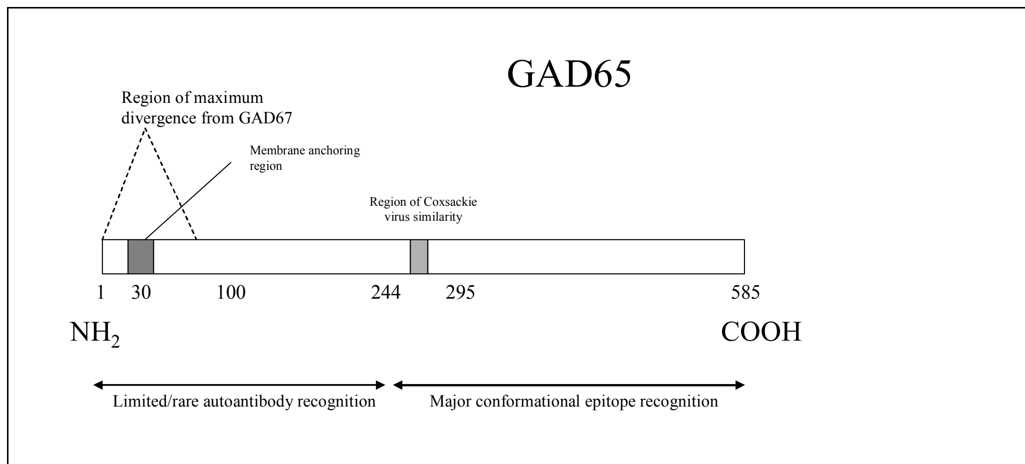

Fig 3. Diagrammatic representation of GAD65 protein. Regions labeled include membrane anchoring region, region with maximum divergence from GAD67 and region similarity with the Coxsackie virus. Based to Leslie et al. 1999.

AUTOANTIBODIES TO GAD65

Antibodies against glutamic acid decarboxylase 65 (GAD65) are one of the major antibodies detected in patients with T1D (Baekkeskov et al. 1982). The antigenic region of GAD65 with which autoantibodies react has been determined by using deletion mutants of GAD, and these studies have identified the middle (M) (amino acids 245-449) and carboxyl (C) terminal (amino acids 450-585) -regions of GAD as the prime immunogenic sites for autoantibody recognition (Kaufman et al. 1992, Butler et al. 1993, Kim et al. 1994, Daw et al. 1996). Additional studies using a panel of GAD65 specific monoclonal islet cell antibodies derived from patients with T1D suggest that antibodies recognize conformational epitopes (Richter et al. 1993).

Numerous scholars have reported that 70-80% of patients with newly diagnosed T1D test positive for autoantibodies to GAD65, whereas autoantibodies to the GAD67 isoform are detectable in only 15-20% of affected patients (Grubin et al. 1994, Vanderwalle et al. 1995). In most cases, epitopes are shared between the two GAD isoforms (Falorni et al. 1994). There are variable findings, but most studies have reported that age at diagnosis and HLA genotype affects the frequency of GAD65 autoantibodies in patients with recent-onset T1D. In general autoantibodies to GAD65 appear to increase with age and the frequency of these autoantibodies is greatest in HLA-DR3-positive patients (Harrison et al. 1993, Schmidli et al. 1994, Vandenvalle et al. 1995, Hawa et al. 1997, Gorus 1997, Lohmann et al. 1997). The GAD65 autoantibody levels decrease somewhat after diagnosis of T1D (Kaufman et al. 1992), but when compared with other autoantibodies in T1D, they are unusual in that they tend to persist in sera for many years after the clinical disease presentation (Atkinson et al. 1993, Savola et al. 1998b). GAD antibodies are also a hallmark of late autoimmune diabetes in the adult (LADA) (Seissler et al. 1988, Tuomi et al. 1993, Leslie and Pozzilli 1994, Niskanen et al. 1995, Elbein et al. 1997, Turner et al. 1997). Patients with LADA are initially perceived as having type 2 diabetes, but they are often normal-weight and they turn out to need exogenous insulin within 6-12 months after the diagnosis of diabetes.

Epitope analyses of GAD65 autoantibodies of the patients with T1D suggest that at least three major conformational epitopes are recognized. One of the conformational epitopes is located in the middle and two in the C-terminal domain of the molecule, while a fourth linear epitope is located in the N-terminus (Richter et al. 1993, Ujihara et al. 1994, Daw and Powers 1995, Falorni et al. 1996). There is also data suggesting that the epitope recognition of GAD65Ab changes during the preclinical phase of T1D (Falorni et al. 1996). It has been reported that the middle region is an early target of the immune response to GAD65, and that the epitope profile is dynamic in prediabetic individuals during progression to clinical disease with spreading from the immunodominant middle region to the C-terminal region (Söhnlein et al. 2000, Bonifacio et al. 2000).

CELLULAR IMMUNE RESPONSE TO GAD

In contrast to the large number of studies on the humoral immune response to GAD, there are relatively few studies on cell-mediated immune responses to this antigen, and the results published are conflicting, because there are major variations between the assays. T cells from patients with T1D and first-degree relatives may react with GAD and GAD peptides, which also show sequence similarity to the P2-C protein of coxsackie B viruses, but the level of the response is low (Atkinson et al. 1992, Kaufman et al. 1992, Honeyman et al. 1993, Atkinson et al. 1994, Lohmann et al. 1994, Durinovic-Bello et al. 1996). Harrison et al. (1993) reported an inverse correlation between the humoral and cellular immune responses to GAD in high-risk first-degree relatives in such a way that enhanced cellular immunity was associated with progression to overt T1D, whereas high autoantibody titers were detected in subjects who remained unaffected.

GAD65 may be a self-antigen that activates an autoimmune process or merely a bystander, resulting from immunostimulation by antigens released from destroyed cells. Experimental studies on NOD mice support a direct role for GAD65 autoimmunity. These mice show early spontaneous T cell reactivity to GAD65 and modulation of GAD immunity by therapeutic approaches, either oral, intrathymic, intraperitoneal, intranasal or subcutaneous administration of GAD or GAD peptides has been shown to inhibit the disease process and reduce the cumulative incidence of diabetes in these NOD mice (Kaufman et al. 1993, Tisch et al. 1993, Bellmann et al. 1998). Moreover, animal studies have demonstrated the ability of GAD as a self-antigen to transfer insulitis and diabetes, since GAD-reactive $CD4^+$ Th1 cells induced diabetes in NOD mice (Zekzer et al. 1998).

INSULIN

Insulin, an essential autoantigen in T1D (Palmer et al. 1983), is a short protein consisting of 51 amino acids encoded by a gene located on the short arm of chromosome 11 (11p15). Insulin is the only true beta cell specific autoantigen identified so far. As with IA-2 and GAD65, the majority of the insulin autoantibodies recognize conformational epitopes (Leslie et al. 1999), which are located mainly within the B chain of insulin (Fig 4)(Potter and Wilkin 2000). Insulin autoantibodies are the first or among the first markers to appear during the asymptomatic prediabetic

period, and are thereby valuable predictive markers of diabetes in young children, although IAA positivity alone has relatively low predictive power (Ziegler et al. 1999, Kimpimäki et al. 2002). It has been reported that IAA is associated with 5-6% risk of T1D in the general population (Landin-Olsson et al. 1992, Bingley et al. 1997), but the higher the titer of IAA is, the higher is the risk for T1D. IAA levels have been reported to correlate with the loss of beta cell function (Vardi et al. 1998) and with the rate of progression to T1D (Eisenbarth et al. 1992, Atkinson and Eisenbart 2001).

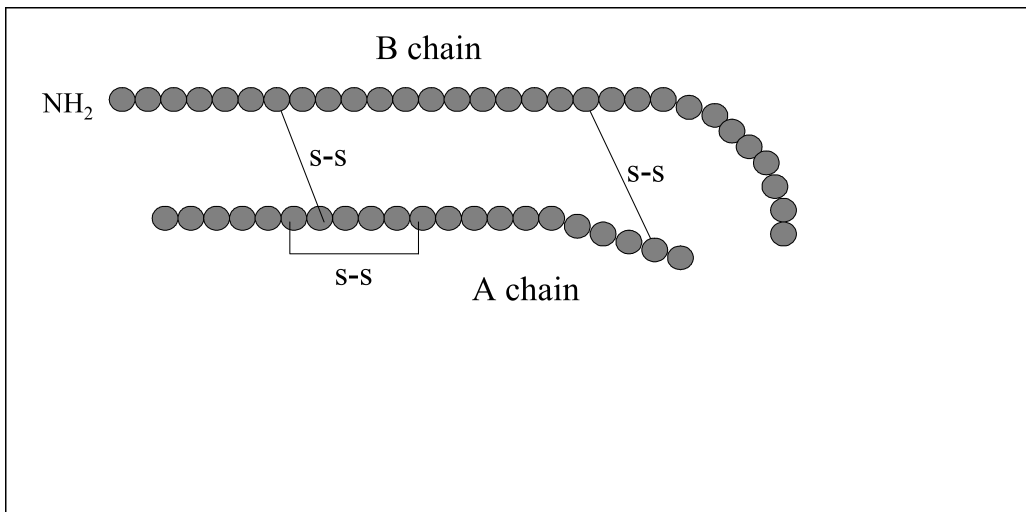

Fig 4. Schematic representation of active insulin. Before secretion from the secretory granules of the β cell, C chain between A and B chain has cleft to form C peptide, and A and B chain together form the active molecule. Based to Potter and Wilkin 2000.

The prevalence of IAA has been observed to vary from 1% to 3% among children from the background population (Schatz et al. 1994, Bingley et al. 1997, Strebelow et al. 1999, Kulmala et al. 2001). In the Finnish DIPP study almost to 5% of children with HLA-conferred susceptibility tested positive for IAA by the age of 2 years (Kimpimäki et al. 2002), while IAA was detected in about 11% of 2-year-old offspring of parents with T1D in the German BABYDIAB study (Ziegler et al. 1999). Among the siblings of children with T1D 1.4 to 6.9% have been observed to have IAA (Krischer et al. 1993, Karjalainen et al. 1996, Verge et al. 1996, Gorus et al. 1997, Kulmala et al. 1998). Among patients with T1D the reported frequency of IAA varies from 28% to 69%, and this variation is at least partly due to a pronounced age effect: young children with newly diagnosed T1D have a markedly higher frequency

of IAA than adolescents or young adults (Hagopian et al. 1995, Vandewalle et al. 1993, Gorus 1994, Komulainen et al. 1999). In addition there are definitely considerable differences between various IAA assays, and the standardization of IAA assays has proved more challenging than the standardization of ICA, GAD65Ab and IA-2Ab assays. An association of IAA with the HLA DR4-DQ8 haplotype and the DQB1*0302 allele has been reported (Ziegler et al. 1991, Bonifacio et al. 1999, Kimpimäki et al. 2001). IAA appears to be characterized by high sensitivity, early appearance, and high frequency of transient antibody positivity.

IAA autoantibodies are a good marker of ongoing autoimmunity, but little is known about the fine specificity of IAA. Previously Dean et al. (1986) and Bodansky et al (1986) have reported them to be either of IgG or IgM class, and in addition there are two more studies about IAA isotypes. Nell et al. (1989) reported that antibodies to exogenous insulin comprise mainly IgG1 antibodies in patients with newly diagnosed T1D, while IgG3 antibodies were more prevalent before the initiation of exogenous insulin. Findings in the offspring of parents with T1D have suggested that the IAA isotypes are mostly IgG1 and in a few cases also IgG4 in preclinical T1D, but the presence of the latter was not associated with protection from clinical disease (Bonifacio et al. 1999).

IA-2/IA-2β

The tyrosine phosphatase-like proteins islet antigens (IA)-2 and IA-2β are major autoantigens in T1D (Lan et al. 1994, Lu et al. 1994, Rabin et al. 1994, Wasmeier and Hutton 1996). Both are enzymatically inactive transmembrane proteins expressed within secretory granules in the central nervous system and in neuroendocrine cells, including pancreatic islet cells (Lan et al. 1994, Wasmeier and Hutton 1996, Solimena et al. 1996).

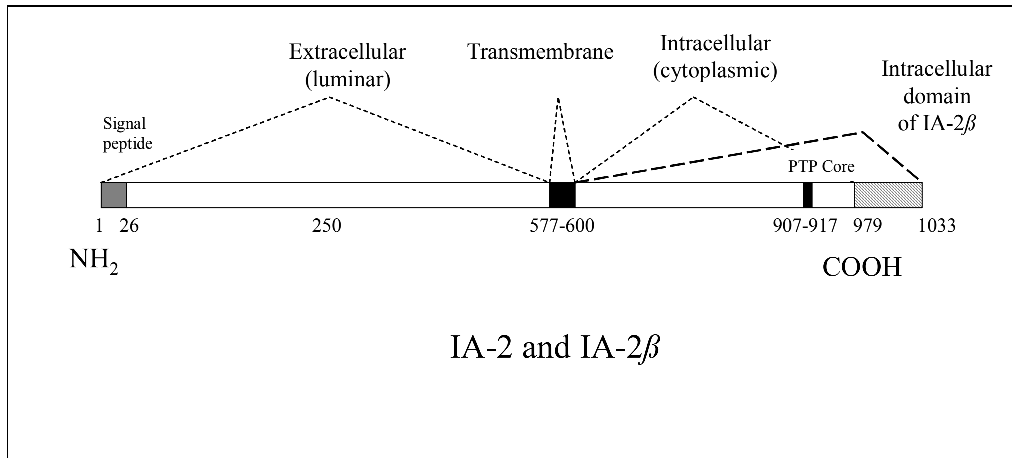

Fig 5. Diagrammatic representation of IA-2 and IA-2β protein. Based to Leslie et al. 1999.

IA-2 and the IA-2β (also known as phogrin) belong to the IA-2 subgroup of the protein-tyrosine phosphatase (PTP) family (Lan et al. 1996, Leslie et al. 1999), and homologs have been found in many animals (Payton et al. 1995, Notkins and Lernmark 2001, Wasmeier and Hutton 2001). The extracellular domains show only modest homology with each other (10-20%), whereas their cytoplasmic-domains are closely related (more than 80%), being characterized by a single PTP-like domain of ~300 amino acids and a short juxtamembrane (JM) portion of 100 amino acids, which links the PTP domain to the transmembrane region (Fig 5) (Wasmeier and Hutton 1996, Bearzatto et al. 2003). The function of these molecules remained a mystery, but recent experiments suggest that they may have a regulatory role in insulin secretion (Ort et al. 2001).

AUTOANTIBODIES TO IA-2 AND IA-2β

Autoantibodies to IA-2 can be detected in a majority of sera from patients with newly diagnosed T1D. Deletion mutants have determined the major antigenic regions of the IA-2 molecule with which the autoantibodies react. These experiments showed that none of the sera from patients with T1D reacted with the extracellular domains; all the sera reacted exclusively with the intracellular domains, and further both to epitopes that were specific to each protein and to epitopes that are shared by both IA-2 and IA-2ß (Lampasona et al. 1996, Zhang et al. 1997, Leslie et al. 1999). Moreover, the

epitopes with which the autoantibodies react are conformationally dependent. Reduction of the disulphide bonds within the intracellular domain of IA-2 results in an almost total loss of reactivity with autoantibodies from patients affected by T1D (Xie et al. 1997). Autoantibodies to IA-2 and IA-2β share common epitopes but may also have distinct epitopes. Responses specific to the IA-2 JM (juxtamembrane) region, the IA-2 PTP-like domain, and the IA-2β PTP-like domain have been identified, likewise autoantibodies that are cross-reactive between the IA-2 and IA-2β PTP-like domains (Lampasona et al. 1996, Hatfield et al. 1997, Notkins et al. 1997, Bonifacio et al. 1998, Kawasaki et al. 1998, Naserke et al. 1998). Most sera that recognize IA-2ß also recognize IA-2, but not all sera that recognize IA-2 recognize IA-2ß (Wasmeier and Hutton 1996). For this reason IA-2 is the protein of choice for most immunoassays (Notkins et al. 1998).

Autoantibodies to IA-2 appear years before the development of clinical disease and are widely used as predictive markers to identify individuals at risk for developing T1D. These autoantibodies can be detected in sera of 60–85% of patients with recent-onset T1D (Hagopian et al. 1995, Verge et al. 1996a, Bingley et al. 1997, Hatfield et al. 1997, Notkins et al. 1997, Kawasaki et al. 1998, Savola et al. 1998a, Leslie et al. 1999) and their detection in sera of nondiabetic individuals together with other islet autoantibodies is highly predictive for future development of the disease (Hatfield et al. 1997, Kulmala et al. 1998). The frequency of IA-2 antibodies varies with age and HLA genotype, being highest in those with disease onset before the age of 15 years (Notkins et al. 1997) and in patients with the HLA DR4-DQ8 haplotype (Genovese 1996, Hawa et al. 1997, Gorus et al. 1997, Lohmann et al. 1997, Kawasaki et al. 1998, Bonifacio et al. 1998).

Antibodies to the conformational epitopes of the intracellular portion of the IA-2/IA-2β molecule have been shown to be the most predictive in relation to T1D (Bonifacio et al. 1998, Kawasaki et al. 1998, Naserke et al. 1998). Those directed against IA-2-specific epitopes appear early, while IA-2*β*-specific antibodies appear relatively infrequently, suggesting that humoral autoimmunity to IA-2*β* is secondary to that to IA-2 and arises via epitope spreading (Hatfield et al. 1997, Bonifacio et al. 1998, Naserke et al. 1998). Recently two major contiguous epitopes of the short IA-2 JM region have been discovered (Bearzatto et al. 2002), which account for most of the Ab

reactivity against the JM region. The JM region of IA-2 appears to be highly immunogenic and it is unique to IA-2, autoantibodies binding to the JM region of IA-2β have not been identified, whereas autoantibodies binding to the IA-2 PTP-like domain often cross-react with IA-2β (Lampasona et al. 1996, Hatfield et al. 1997, Bonifacio et al. 1998).

The unique nature of the immunogenic JM region to IA-2 has implications for the loss of tolerance. Autoantigens, in general, have been detected in human thymus during fetal life (Pugliese et al. 1997), but it is remarkable that the IA-2 JM epitopes could not be detected in human thymus (Diez et al. 2001). Therefore it is possible that IA-2 JM autoreactive T cells could escape deletion or anergy, leading to an increased susceptibility to autoimmunity. So far, only a few reports on T cell epitopes within the IA-2 molecule have appeared (Honeyman et al. 1998, Hawkes et al. 2000), and only a limited number of potential epitopes have been implicated, but T cell epitopes within the JM region are likely. (Honeyman et al. 1998, Peakman et al. 1999, Hawkes et al. 2000)

ICA

Islet cell antibodies (ICA), which were described for the first time in 1974 by indirect immunofluorescence, have represented the golden standard for islet autoantibody analysis for approximately 15 years (Bottazzo et al. 1974, MacCuish et al. 1974). ICA are autoantibodies against the cytoplasmic components of the islet cells (Genovese et al. 1992, Gianani et al. 1992), and the response is of IgG class, further mostly subclass IgG1, but may extend to other subclasses (IgG1+IgG2 and IgG1+IgG3) in 30% of cases (Dean et al. 1983, Bruining et al. 1984, Omar et al. 1987, Millward et al. 1988, Dozio et al. 1994).

The role of ICA as predictive markers has been more extensively studied than that of any other autoantibodies associated with T1D. They have been found to be predictive markers in first-degree relatives of affected patients (Bonifacio et al. 1990) and in a general population with a high background incidence (Karjalainen 1990). It was later discovered that GAD (Atkinson et al. 1993) and IA-2 autoantibodies (Bonifacio et al. 1995) contribute to the ICA-reactivity in sera of patients with newly diagnosed T1D, and in addition, this ICA-response comprises reactivity to other, as yet unidentified

beta cell antigens distinct from GAD and IA-2. ICA are most frequent (70%-90%) among children with T1D at the clinical onset of the disease (Lendrum et al. 1975, Karjalainen et al. 1986, Schatz et al. 1994, Hagopian et al. 1995), but they can be detected several years prior to the onset of T1D (Gorsuch et al. 1981), but when they are the only antibody detected from the sera, their positive predictive value for T1D is low (5-6%) in the general population (Landin-Olsson et al. 1992).

ENVIROMENTAL TRIGGERS OF AUTOIMMUNITY

Although genetic susceptibility is necessary for the development of T1D, the etiology of this disease is multifactorial, and a critical question is what actually triggers the autoimmune cascade. All the evidence shows T1D to be a complex disease affected by exogenous factors in addition to a series of genes. The HLA genes that explain 30-60% of the familial clustering of T1D regulate the immune response by being involved in antigen processing and presentation that are critical elements in the initiation of any immune response, including autoreactivity. The wide global variation in incidence between and within major ethnic groups and the conspicuous increase in the incidence of childhood T1D observed in most developed countries after World War II suggest that environmental factors must be involved in the initiation and/or progression of beta cell destruction leading to T1D. The list of environmental determinants of T1D comprises microbes, dietary factors and toxins among other things. Among exogenous triggers, viruses, and more specifically enteroviruses, are the leading candidates. (Jun and Yoon 2001)

Viral infections have for a long time been implicated in the etiology of T1D. There is epidemiological evidence supporting the hypothesis that at least some cases of T1D are caused by an infectious agent or agents. The earliest observations dating back more than 100 years have shown a temporal relationship between certain viral infections and the development of T1D, and seroepidemiologic studies provide additional support for this model (Harris 1898, Knip and Åkerblom 1999, Jun and Yoon 2001), as the fact that infection with rubella triggers insulitis in children with congenital rubella (Shaver et al. 1985). The search for an infectious etiology of T1D eventually focused on the enteroviruses. In 1979, the most convincing evidence came from Yoon et al. who reported a 10-year-old previously healthy boy presenting in diabetic ketoacidosis within 3 days of onset of flu-like symptoms. He died 7 days

later. Coxsackie B4 virus was isolated from the pancreas post-mortem. Inoculation of the virus into mice produced hyperglycemia, inflammatory cells in the islets of Langerhans, and beta cell necrosis (Yoon et al. 1979). Finally, a temporal relationship was noted by some but not all investigations (Adams 1926, Chueca et al. 1997, Friman et al. 1985) between the peak seasons for enteroviral infections in late summer and peak presentation of clinical diabetes in the fall and winter. However, the evidence that T1D is a chronic immune-mediated disease has changed the emphasis in the search for a viral agent from one that rapidly destroys beta cells to one that may induce a chronic infection or trigger an autoimmune response to beta cell antigens. (Tuomilehto et al. 1996, Neu et al. 1997, Waldhoer et al. 1997)

The search for a unique diabetogenic enterovirus prompted several seroepidemiological case-control studies on patients with recent-onset T1D and subjects with signs of preclinical T1D. In a prospective Finnish study, serologically documented enterovirus infections were shown to be more common in siblings who developed T1D than in those who did not (Hyöty et al. 1995). By using polymerase chain reaction (PCR) technology Finnish investigators found an association between enteroviral infections and ICA seroconversion in children (Hiltunen et al. 1997), and several enterovirus serotypes were associated with prediabetic autoimmune episodes (Roivainen et al. 1998). Lönnrot et al. (2000) confirmed that the presence of enterovirus RNA in serum was a risk factor for increased levels of ICA and GAD antibodies. Enterovirus infections have also been suggested to play a protective role against T1D (Bach 2001). The hygiene hypothesis proposes that the rising incidence of T1D is due to a reduced stimulation of the immune system by early intercurrent infections (Gale 2002). It has been hypothesized that a dramatic decrease in the frequency of enterovirus infections has led to a new epidemiological situation, which causes an increase of serious enterovirus infections such as meningitis or beta cell autoimmunity (Viskari et al. 2002). Similarly, the youngest age group of children, whose incidence of T1D is increasing most strikingly, may be more prone to enterovirus-induced beta cell destruction than before (Viskari et al. 2002) as a consequence of a decrease during infancy in the protection conferred by maternal antibodies transferred either transplacentally or through breast milk. Further it is proposed that there is an analogy to the phenomenon, which was seen with poliovirus – when the frequency of endemic polio infections decreased, poliomyelitis became

more common (Viskari et al. 2002).

Viruses may be involved in the pathogenesis of T1D in several ways (von Herrath et al. 1998), either via a direct beta cell lysis (Yoon et al. 1979, Roivainen et al. 2000), or by triggering an autoimmune reaction against the beta cells (Ludvigsson et al. 1988, Horwitz et al. 1998).

Nutritional and dietary factors have also been implicated as initiators or promoters of T1D, evidence coming from numerous animal and human studies. Breastfeeding of short duration, early introduction of cow's milk (Åkerblom and Knip 1998, Harrison and Honeyman 1999), as well as gluten, N-nitroso compounds, increased linear growth or obesity may increase the risk, whereas vitamin D has been reported as having a possible protective effect in relation to T1D. (Vaarala 2004)

Early introduction of cow's milk to the infant diet may play an important role, as milk proteins comprise in most cases the first source of foreign complex proteins the infant is exposed to (Knip and Åkerblom 1999). So far the results have been partly contradictory. In prospective studies from Australia, the United States and Germany no associations have been observed between cow's milk exposure and the appearance of diabetes-associated autoantibodies in infants and young children, but Kimpimäki et al. (2001) observed that the risk for developing IA-2 antibodies or all four autoantibodies was decreased if the child was exclusively breast fed for more than 4 months. A Finnish study has shown that cow's milk formula induces humoral and cellular immune responses to bovine insulin differing from human insulin by three amino acids in infants with an HLA-conferred susceptibility to T1D (Paronen et al. 2000). In addition, those young children who presented with early signs of beta cell autoimmunity seemed to lack the normal capacity to develop oral tolerance to bovine insulin when continuously exposed, raising the speculation that the immune response mounted against bovine insulin might at some point be diverted to target human insulin (Vaarala et al. 1999).

Another substance, the active form of vitamin D, 1-alpha, 25-dihydroxyvitamin D3, which also has immunological properties is a potent immunomodulator and may play a role in autoimmunity. Vitamin D compounds are known to suppress activated T

cells (DeLuca and Cantorna 2001). Epidemiological studies also suggest that vitamin D supplementation in infancy decreases the risk for T1D (Hyppönen et al. 2001).

FUTURE DIRECTIONS OF TYPE 1 DIABETES

PREDICTION

Screening for multiple antibodies is the most specific and sensitive strategy for identifying subjects at risk for T1D at least among first-degree relatives of the patients. The predictive value of autoantibodies as a primary screening method is not unequivocally defined in the general population, from which 90% of newly diagnosed patients are derived. (Knip 2002)

Among patients with newly diagnosed T1D up to 98% have ICA, IAA, GAD and/or IA-2 antibodies (Savola et al. 1998a). Prospective studies on subjects with a positive family history for T1D conclusively demonstrate that the disease risk strongly correlates with the number of positive antibodies (Bonifacio et al. 1995, Verge et al. 1996b, Kulmala et al. 1998), and accordingly these autoantibodies can be used as predictive markers for the identification of subjects at high risk for T1D (Hagopian et al. 1995, Bingley et al. 1997, Leslie et al. 1999). These estimates show that the likelihood of developing T1D within 5-10 years is 0-10% in the presence of one autoantibody, about 50% in the presence of two autoantibodies, and 60-100% in the presence of three or more autoantibodies among first-degree relatives of patients with T1D (Kulmala et al. 1998, Verge et al. 1996b, Leslie et al. 1999). Positivity for multiple autoantibodies and its high predictive value for future T1D has also been confirmed in the background population by several studies (Bingley et al. 1997, LaGasse et al. 2002).

On the other hand, the appearance of multiple autoantibodies is affected by the HLA genotype, as a strong genetic susceptibility seems to be associated with aggressive, rapidly progressing beta cell destruction (Ilonen et al. 1998), which is reflected by positivity for multiple autoantibodies (Colman et al. 2000, Kulmala et al. 2000a and 2000b) and clinical manifestation of T1D at a young age (Komulainen et al. 1999). A third factor which may provide additional information to predict rapid progression to diabetes is assessment of the insulin secretory capacity by using the first phase insulin response (FPIR) to intravenous glucose. The positive predictive value of a reduced

FPIR is very high, close to 100%, and is a strong indicator of fast progression to overt T1D (Veijola et al. 1995, Keskinen et al. 2002).

Screening strategies aim at identifying as early as possible all those individuals who will eventually develop clinical diabetes, and in this respect the appearance of multiple autoantibodies or a diminished FPIR may be too late, since these markers are perceived as markers of relatively advanced preclinical T1D, and one might expect that early intervention would be more effective than treatment in later stages of prediabetes (Knip 2002).

The Immunology of Diabetes Society (IDS) has established guidelines on the assessment of diabetes risk in unaffected first-degree relatives of patients with T1D (Bingley et al. 2001). These guidelines recommend initially testing for GAD65 antibodies combined either with IAA or IA-2 antibodies, or in the case of prospective studies in early childhood it is preferable to include all autoantibody markers in the primary test. Only individuals with elevated titers of two or more markers should be considered as being at high risk for further testing to define the risk more precisely. Second-line genetic testing is of limited value in family members in whom autoantibody levels are known. However, HLA class II typing may be useful in identifying infants for prospective follow-up for the appearance of islet autoantibodies or for recruitment into trials or interventions aimed at primary prevention, i.e. preventing the initiation of autoimmunity. Metabolic testing with assessment of the FPIR in an intravenous glucose tolerance test can be used to identify the subgroup of individuals positive for multiple autoantibodies who are at the greatest short-term risk of progression to diabetes.

So far observations on risk assessment in the general population are limited, which is why international recommendations for screening strategies in the general population are still lacking. Knip considered optimal screening strategies for the identification of at-risk individuals from the general population in 2002. Based on the observations by Hämäläinen et al. 2000, the appropriate age for the initiation of autoantibody screening would be 1.5 years. By that age possible transplacentally transferred maternal antibodies are eliminated from the peripheral circulation of the young child, and only very few individuals are diagnosed with T1D before the age of 2 years

(Komulainen et al. 1999). Genotyping for HLA class II genes cuts the at-risk group to be monitored for the appearance of diabetes-associated autoantibodies down to 13–15% of the total population and still targets 70–80% of future subjects with T1D (Kupila et al. 2001). In addition the analysis of FPIR can also serve as a useful tool to improve the prediction of T1D in the general population. The value of additional markers such as autoantibodies to different epitope regions, autoantibodies with different isotype specificity or Th cells subsets remains to be defined in the assessment of future risk for T1D both among family members and in the general population.

PREVENTION

Successful prevention of the disease is a major goal of diabetes research. The long prediabetic period, increasing knowledge of the disease process, and improved predictive strategies facilitates the establishment of effective preventive measures. So far successful preventive methods do not exist. An immunosuppressive agent, Cyclosporine A, was promising, since it was able to halt insulitis, but because of its toxicity it was withdrawn from clinical trials (Martin et al. 1991). Theoretically, three types of preventive strategies can be applied. Primary prevention covers all tools that aim at preventing the initiation of insulitis in the general population or among those with increased genetic diabetes susceptibility. The intervention is based on the avoidance of such exogenous factors that trigger insulitis, and is implemented before the disease process has started. In principle, this would be an ideal approach, but at the present time it is difficult to apply, since the triggers of the disease process have not been unequivocally defined.

At the moment most intervention trials are focused on secondary prevention in at-risk individuals in whom signs of beta cell autoimmunity have been detected by the presence of disease-associated autoantibodies. Secondary prevention aims at arresting or delaying progressive beta cell destruction at some point before the clinical manifestation of T1D. Tertiary prevention refers to measures initiated after the clinical presentation of the disease, and the intervention is aimed at preserving or restoring beta cell function, or preventing long-term complications of T1D. (Knip 1997)

Both experimental and epidemiological studies have indicated that exposure to cow's milk proteins may be involved in the pathogenesis of T1D. Presently the first extensive primary prevention study “Trial to Reduce IDDM in Genetically at Risk" (TRIGR) is running as a multicenter study in 15 countries, testing the hypothesis whether possessing the initial exposure to dietary complex proteins by weaning to a highly hydrolyzed formula could prevent the development of beta cell autoimmunity and clinical T1D in genetically susceptible newborn infants (Åkerblom 2002).

Several trials aimed at secondary prevention of T1D have been initiated in high-risk individuals with signs of ongoing beta cell autoimmunity. There have been two large multicenter trials, one using oral nicotinamide, a water-soluble group B vitamin that was thought to protect beta cells, and another evaluating the usefulness of insulin in the prevention overt disease in high-risk first-degree relatives. A third trial still going on is the Diabetes Prevention and Prediction (DIPP) Study aimed at delaying T1D by nasal insulin administration in genetically susceptible young children testing persistently positive for at least two diabetes-associated autoantibodies. Parenteral injection of any disease-relevant autoantigen is one experimental strategy used by immunologists to induce antigen-specific T cell tolerance to treat organ-specific autoimmunity (Liblau et al. 1997), and it is assumed that insulin could act either by inducing beta cell rest (and thereby reducing its antigenicity), or by acclimatizing the immune system to insulin (Rabinovitch and Skyler 1998).

The European Nicotinamide Diabetes Intervention Trial (ENDIT) included ICA-positive first-degree relatives up to the age of 40 years, who received daily either a high dose of oral nicotinamide or placebo to test whether the treatment is able to reduce the cumulative incidence of T1D by 50% over a period of 5 years. The trial was completed in 2002 and the outcome showed no difference between the two groups in the progression rate to clinical T1D (The European Nicotinamide Intervention Trial, ENDIT 2004). The American Diabetes Prevention Trial (DPT-1) included two arms, relatives with a high risk of T1D being treated with parenteral insulin and relatives at lesser risk with oral insulin (Schatz and Bingley 2001). This strategy was based on the hypothesis that insulin, as an antigen-specific therapy, may modulate the immune response and induce tolerance. A small pilot trial implied that parenteral insulin therapy in autoantibody-positive family members reduced their

progression rate to overt T1D (Keller et al. 1993). The parenteral administration of insulin turned out, however, to have no protective effect in relation to progression to clinical T1D (DPT-1 2002). The Finnish DIPP study aims at identifying newborn infants who carry increased HLA-conferred susceptibility to T1D (phase I), recognizing at an early stage the appearance of markers of beta cell autoimmunity known to precede the presentation of clinical T1D (phase II) and assessing whether it is possible to delay the manifestation of clinical disease with daily administration of nasal insulin in genetically susceptible young children testing persistently positive for multiple ($\geq$2) diabetes-associated autoantibodies (phase III) (Kupila et al. 2001). The placebo-control double blind intervention trial with nasal insulin is well on its way.

AIMS OF THE PRESENT RESEARCH

The objectives of this work were:

- To characterize the changes in the autoantigen-specific humoral immune responses in the prediabetic phase of T1D
- To evaluate whether the isotype and epitope-specificity of autoantibodies can be used as prognostic markers in evaluation of children at risk to T1D
- To analyze possible differences in the immune responses between autoantibody-positive subjects who progress rapidly to overt T1D and those who progress slowly or remain unaffected.

SUBJECTS AND METHODS

SU BJECTS

THE CHILDHOOD DIABETES IN FINLAND (DiMe) STUDY

The subjects in communications I and II were derived from the Childhood Diabetes in Finland (DiMe) Study, which was a prospective population-based family survey designed to investigate the role of genetic, immunological and environmental factors in the development of T1D (Tuomilehto et al. 1992). From September 1986 to April 1989 all 801 newly diagnosed children with T1D aged 14 years or younger were invited to participate in the study together with their families. Blood samples were drawn from the siblings of the affected children. A blood sample was available from 755 initially unaffected siblings out of 977 (77.3%) younger than 20 years of age. A total of 58 (7.7%) siblings were observed to test positive for GAD65 autoantibody in their first sample obtained close to the time of diagnosis in the index case, while 39 (5.2%) had IA-2 antibodies. Subsequent samples were taken at intervals of 3 months for the first 2 years and thereafter at intervals of 6-12 months according to the study protocol. Thirteen (2.0%) and nineteen (2.5%) siblings seroconverted to GAD65 and IA-2 antibody positivity respectively during prospective observation. Accordingly the initial study cohort for the analysis of epitope- and isotype-specific GAD and IA-2 antibodies comprised 71 siblings testing positive for GAD65 antibodies and 58 siblings positive for IA-2 antibodies at least once.

A subject was included in the current series, if he or she tested positive for GAD65 or IA-2 antibodies, at least in two samples, and if the positive serum samples were still available (Table 3). In addition, GAD65 antibodies had to be detected at a minimum level of 15 relative units (RU) in at least one sample during the follow-up period. All siblings were observed for progression to clinical T1D to the end of December 1997, and accordingly the follow-up lasted for an average of 9.2 years (range 3.2 – 16.2 years) in the non-progressors. The samples were stored at -20 °C until analyzed.

Table 3. Characteristics of the study subjects included in this study

Original communication	Study	Subjects	n	Age at start of study (years), mean (range)	Follow-up time, (years) MEAN (range)
I	DiMe	GAD65Ab-positive siblings	42	9.5 (3.2-16.3).	3.6 (0.4-8.6)
II	DiMe	IA-2/IA-2β Ab-positive siblings	34	9.9 (4.2-17.3)	3.4 (0.5-8.2)
III	DIPP	IA-2/IA-2β Ab-positive children	45	2.2 (1.0-4.8)	2.0 (0.4-4.5)
IV	DIPP	IAA-positive children	45	1.2 (0.3-3.6)	1.1 (0.2 – 2.0)

In order to compare the antibodies against minor epitope clusters and the isotypes between children who did or did not progress to T1D, each progressor was matched with one or two non-progressors for sex, age when the last sample was analyzed (+/- 2 yrs; 68%) and with antibody-positive observation time as well as possible. In addition the HLA DR genotype was taken into account as closely as possible.

Antibodies to GAD67 were observed in 17 subjects, and although the detected levels were generally low these antibodies might contribute to the binding to the chimeric constructs, and therefore we excluded all of them from the analysis of epitope specific GAD65 antibodies. As a consequence of matching progressors to non-progressors, seven GAD67Ab-negative children were also excluded, resulting in a cohort of 18 siblings in the analysis of epitope-specific GAD65 antibodies.

THE DIABETES PREDICTION AND PREVENTION STUDY (DIPP)

The DIPP Study is a large ongoing population-based survey of young children with HLA-conferred diabetes susceptibility whose aim is to study the natural course of preclinical T1D, assess the predictive value of various immune and genetic risk markers in the general population and to develop effective tools for preventing or delaying progression to clinical disease (Kupila et al. 2001). According to the study protocol, all infants born in three participating university hospitals (Oulu, Tampere and Turku) are invited for genetic screening for HLA-DQB1 alleles associated with T1D (phase I). Subjects carrying HLA-DQB1 alleles conferring susceptibility to T1D [DQB1*02/DQB1*0302, DQB1*0302/x (x≠DQB1*0301, DQB1*0602 or DQB1*0603) and males born in Turku with DQA1*05-DQB1*02/x] are observed from birth for the appearance of ICA and other diabetes-associated autoantibodies. Since 1994 more than 90 000 newborn infants have been tested for HLA antigens, and close to 14 500 children carrying the risk genotypes had been invited to take part in the DIPP Study by the end of March 2005. Blood samples are obtained at regular follow-up visits at the ages of 3, 6, 12, 18, 24 months and annually thereafter in Oulu and Tampere, whereas the children in Turku are observed at 3-month intervals for the first 2 years of life and thereafter twice a year (phase II)

Islet cell antibodies (ICA) are used for primary screening of beta cell autoimmunity. If a child seroconverts to positivity for ICA, antibodies to insulin, glutamic acid decarboxylase 65 (GAD65) and the protein tyrosine phosphatase-related IA-2 protein are analyzed in all available samples from birth, and children are followed-up at 3-month intervals in all centers. Families with a child repeatedly testing positive for multiple (≥2) autoantibodies (positivity in at least two consecutive samples) are invited to take part in a randomized controlled intervention trial to assess whether it is possible to delay or prevent the manifestation of clinical diabetes by daily administration of nasal insulin (phase III). The study protocol has been approved by the local Ethics Committees, and the parents of the children have given their written informed consent to participation.

The subjects in Communication III comprised 45 children, who had tested positive for IA-2 antibodies at least twice during the follow-up (Table 3). At the time of analysis, 84 children out of a total of 8433 (1.0%) had tested positive for IA-2 antibodies on at

least one occasion, and 16 of these had progressed to overt T1D during the period of prospective observation. Fifteen of them had an IA-2 antibody-positive serum sample available from more than one time point, and these were included as cases in the study. Among the remaining 56 subjects, we identified 30 children who had remained diabetes-free (non-progressors) and could be matched with the progressors for sex, HLA genotype, and age at the end of observation and IA-2 antibody-positive observation time. The matching concordance achieved was 100% for sex and 80% for genotype. IA-2 antibody subclasses and epitope specificities were analyzed in a total of 465 serum samples starting from the time at which IA-2 antibodies were first detected, or from the previous sample if available. The follow-up continued up to diagnosis, and in each non-progressor up to the sample obtained at the corresponding age.

The series included in Communication IV comprised the first 61 (1.4%) children who had tested positive for IAA on at least one occasion by the end of May 2000, and 20 of them had progressed to overt T1D during the period of prospective observation. Fifteen of the progressors had an IAA-positive serum sample available from more than one time point, and these were included as cases in the present study (Table 3). Among the remaining 41 subjects, we identified 30 children who had remained non-diabetic (non-progressors) and could be matched with the progressors for sex, HLA genotype and IAA-positive observation time. All the 30 non-progressors tested positive for IAA in at least two available samples. The matching concordance achieved was 90% for both sex and genotype. IAA of various IgG subclasses and class IgA were analyzed in the serum samples starting from the time at which IAA were first detected, or from the previous sample, if available. Samples taken after the start of the intervention trial were not included in the present analysis, since the follow-up was observed up to the diagnosis of T1D or up to inclusion in the intervention trial, and for each non-progressor up to the sample obtained at the corresponding age.

All the young children included in Communications III and IV had increased genetic susceptibility to T1D. Among the IA-2 antibody-positive children 18 (7 progressors) carried the high-risk genotype HLA-DQB1*02/*0302, and 27 (8 progressors) had the moderate risk genotype HLA-DQB1*0302/x, and among the IAA-positive children 16

out of the 45 carried the high-risk genotype HLA-DQB1*02/*0302, 28 had the moderate risk genotype HLA-DQB1*0302/x, and one boy had the HLA-DQB1*02/x genotype.

METHODS

ASSAYS FOR GAD65/GAD67 ANTIBODIES

All samples were analyzed for GAD65 antibodies with a radiobinding assay as previously described (Savola et al. 1998b). Briefly, 2 µl of serum was incubated at +4ºC overnight in 96-well plates with 20 000 cpm of ^{35}S-labeled full-length GAD65 diluted in 50 µl of TBST buffer (50 mM Tris, 150 mM NaCl, 0.1% Tween-20, pH 7.4). After the protein A Sepharose precipitation, unbound radioactivity was washed with excess buffer. Remaining activity was counted with a liquid scintillation counter (1450 MicroBeta Trilux, PerkinElmer Wallac, Turku, Finland). The results were expressed in relative units (RU) based on a standard curve constructed from a dilution of a pool of highly positive samples with a negative sample. The cut-off limit for antibody positivity (5.35 RU) was set at the 99th percentile of 373 non-diabetic Finnish children and adolescents. The disease sensitivity of the GAD65Ab assay was 69% and the specificity 100% based on 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge et al. 1998). In the Diabetes Autoantibody Standardization Program (DASP) workshop in 2002 the disease sensitivity was 82% and the specificity 98% (Bingley et al. 2003), whereas the corresponding figures in the 2003 workshop were 80% and 96%. All samples with antibody levels between the 97.5th and 99.5th percentiles were retested to confirm the antibody status.

In Communication I three different GAD65/GAD67 chimeras were used to analyze epitope-specific antibodies; one ($GAD65_{1-95}/GAD67_{102-593}$) to measure aminoterminal-specific GAD65 antibodies (GAD65-N-Ab), another ($GAD67_{1-101}/GAD65_{96-444}/GAD67_{453-593}$) to determine antibodies specific for the middle part of GAD65 (GAD65-M-Ab) and the third ($GAD67_{1-453}/GAD65_{445-585}$) to quantify carboxyterminal-specific GAD65 antibodies (GAD65-C-Ab), as previously described (Savola et al. 1998b, Bonifacio et al. 2000). In addition GAD67 antibodies were quantified from all samples, in which the GAD65 epitope-specific antibodies were analyzed. Antibodies against the *in vitro* transcribed and translated ^{35}S-labeled products were measured with a radiobinding assay identical to that used to detect

conventional GAD65 antibodies. A serum pool with high levels of epitope-specific GAD65 antibodies was used to construct a standard curve. A standard curve was run on each plate and the antibody levels were expressed in RU. The cut-off limit for positivity was set at the 99th percentile in 104 non-diabetic young Finnish subjects (mean age 10.9 yrs, range 0.5-18.2 yrs) in the assays for GAD67 antibodies (0.91 RU), GAD65-N-Ab (0.55 RU), GAD65-M-Ab (1.51 RU) and GAD65-C-Ab (1.59 RU). All samples from the same subject were analyzed in the same assay run as far as possible.

ASSAYS FOR IA-2 ANTIBODIES IN COMMUNICATIONS I AND II

Antibodies to the protein tyrosine phosphatase-related IA-2 protein were quantified with a specific radiobinding assay as previously described (Savola 1998a). Briefly, 2 µl of serum was incubated at +4ºC overnight in 96-well plates with 10 000 cpm of the ^{35}S-labeled intracellular portion (aa 605-979) of the full-length IA-2 protein diluted in 50 µl of TBST buffer. After protein A Sepharose precipitation, unbound radioactivity was washed away with excess buffer. The remaining activity was counted with a liquid scintillation counter (1450 MicroBeta Trilux). Antibody levels were expressed in relative units (RU) based on a standard curve as for GAD65Ab. The limit for IA-2 antibody positivity was set at 0.43 RU, which represents the 99th percentile in 374 non-diabetic Finnish children and adolescents. The disease sensitivity of this assay was 62% and the specificity 97%, based on 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge et al. 1998). In the 2002 DASP workshop the disease sensitivity of this assay was 62% and the disease specificity 100% (Bingley et al. 2003), while the diseas's sensitivity was 64% and the specificity still 100% in the 2003 DASP workshop. The samples with antibody levels between the 97.5th and 99.5th percentiles were retested to confirm antibody status.

EPITOPE-SPECIFIC IA-2 AND IA-2β ANTIBODIES IN COMMUNICATIONS II AND III

Epitope-specific IA-2 and IA-2β antibodies were analyzed according to a protocol identical to that used for IA-2 antibodies, but using IA-2 $PTP_{687\text{-}979}$, IA-2β $PTP_{741\text{-}1033}$, IA-$2_{389\text{-}779}$ and IA-$2_{601\text{-}682}$/IA-$2\beta_{737\text{-}1033}$ (juxtamembrane region, JM) as radioligands. The epitope constructs were kindly donated by Ezio Bonifacio, Milan,

Italy. The cut-off limits for the positivity of epitope-specific antibodies were based on the 99th percentile in 105 Finnish non-diabetic children and adolescents. These limits were 0.87 RU for IA-2 PTP antibodies, 0.32 RU for IA-2β PTP antibodies, 1.17 RU for IA-$2_{389\text{-}779}$ antibodies and 1.43 RU for IA-$2_{601\text{-}682}$/IA-$2β_{737\text{-}1033}$ antibodies. For the identification of IA-2 $PTP_{687\text{-}979}$ and IA-2β $PTP_{741\text{-}1033}$ specific and cross-reactive antibodies, the sera were preincubated with bacterially expressed IA-2 and IA-2β preparations before performing the radiobinding assay with radiolabeled IA-2 PTP or IA-2β PTP. To identify IA-2 $JM_{601\text{-}682}$ or IA-2β $PTP_{737\text{-}1033}$ specific epitopes the sera were preincubated with the IA-2β $PTP_{741\text{-}1033}$ expression protein before the radiobinding assay with radiolabeled JM. Competition experiments were carried out essentially as described by Bonifacio et al. (1998) and epitope-specific responses were defined as the proportion of specific binding after subtracting the proportion of inhibition by the cross-reactive epitope. Briefly, *Escherichia coli* strain INVαF transformed with plasmid pTRC-His-IA2 PTP or pTRC-His-IA2β PTP were grown to OD600 0.3-0.6 and protein expression induced with 1mM IPTG (isopropyl-β-D-thiogalactopyranodise) for 5 h at room temperature. The cells were harvested by centrifugation and the pellet resuspended in lysis buffer [10 mM HEPES, 150 mMNaCl (pH 7.0), 10 mM benzamide, 0.1% aprotinin, 2% Triton X-100], incubated for 2 h at 4 °C with shaking, centrifuged (15 000 x g, 20 min, + 4°C), and the supernatant used in the inhibition assays. Untransformed *E. coli* lysate was used as a control. The sera, as such or diluted in NHS, were preincubated with 8 µl of the supernatant and incubated for 1 h at 4°C before performing the radiobinding assays as described above. The inhibition percentages were calculated according to the following equation: 100 – [(mean cpm value of inhibited reaction-mean cpm value of NHS)/mean cpm value of total binding- mean cpm value of NHS) x100].

The antibodies were classified into four reactivities according to their direct binding and competition: 1) reactivity to epitopes within the juxtamembrane region of IA-2, 2) reactivity to epitopes in the PTP domain that are unique to IA-2 (specific IA-2 PTP), 3) reactivity to epitopes that are shared between IA-2 and IA-2β (cross-reactive (cr)PTP/βPTP) and 4) reactivity to epitopes in the PTP domain that are unique to IA-2β (specific IA-2β PTP).

ASSAYS FOR IAA

In Communications I and II comprising subjects derived from the Childhood Diabetes in Finland Study, insulin autoantibodies (IAA) were analyzed with a radiobinding assay modified from that described by Palmer et al. (1983). The IAA levels were expressed in nU/mL, where 1 nU/mL corresponds to a specific binding of 0.01% of the total counts. The interassay coefficient of variation was less than 8%. A subject was considered to be positive for IAA when the specific binding was equal to or more than 55 nU/mL (99th percentile in a reference population comprising 105 non-diabetic children and adolescents). The disease sensitivity of the IAA assay was 26%, and the disease specificity 97% based on 140 samples included in the 1995 Multiple Autoantibody Workshop (Verge et al. 1998).

The IAA analyses in the DIPP study (Communications III and IV) were performed with a radiobinding microassay using 5 μl of serum as described previously (Ronkainen et al. 2001). Immune complexes were precipitated with protein A Sepharose (Pharmacia Biotech, Uppsala, Sweden), and the results were expressed in relative units (RU) based on a standard curve run on each plate. The cut-off limit for IAA positivity, i.e. 1.56 RU, represents the 99th percentile in 371 Finnish children and adolescents. The intra-assay and interassay coefficients of variation were less than 8% and less than 12% respectively. The disease sensitivity of the micro-assay was 44% and the specificity 98%, based on the 2002 DASP workshop. The corresponding figures in the 2003 DASP workshop were 42% and 98%, respectively. All samples with IAA titers between the 97.5th and 99.5th percentiles were retested to confirm the antibody status. The samples from children seroconverting to IAA negativity were reanalyzed in the same assay run to confirm the inverse seroconversion.

ISOTYPE-SPECIFIC ANTIBODIES TO GAD65, IA-2 AND INSULIN

Isotype-specific antibodies to GAD65 were analyzed in Communication I with an assay based on the same principles as that used for total GAD65 except that the protein A Sepharose precipitation was replaced by monoclonal subclass-specific antibodies linked to streptavidin agarose (Pierce and Warriner, Chester, UK). The volume of serum used per assay was 5 μl. The biotinylated monoclonal mouse antibodies to human IgG1 (clone G17-1), IgG2 (G18-21), IgG4 (JDC-14), IgA (G20-

359), IgM (G20-127) and IgE (G7-26) and to rat IgM (G53-238) were obtained from PharMingen (San Diego, CA, USA) except for IgG3 (HP6050), which was purchased from Southern Biotech (Birmingham, AL, USA). The streptavidin agarose beads were washed thoroughly with phosphate-buffered saline (PBS, 50 mM phosphate buffer, 150 mM NaCl, pH 7.4) before use, and the biotinylated antibodies were linked to them by incubating 5 µg (10 µg of IAA) of antibody per 15 µl of beads in 50 µl of PBS at +4°C for 1 hour with vigorous shaking. Thereafter the beads were washed once with PBS and twice with TBST buffer. Finally 15 µl of the beads were suspended in TBST buffer (total volume of suspension 50 µl) and used for the precipitation, which was performed at +4°C for 2 hours with vigorous shaking. The results were expressed as SD scores (SDS) calculated from the following equation: SDS = [delta cpm (=IgG subclass or isotype-specific cpm - unspecific anti-rat IgM cpm)-mean delta cpm of control subjects]/SD delta cpm of control samples as described previously (Bonifacio et al. 1999). Forty non-diabetic young Finnish subjects [mean age 9.9 ± 4.0 (SD), range 0.6-16.6 years] were used as controls. The threshold for positivity was set at 3 SDS. An identical assay format was used for the analysis of isotype-specfic IA-2 antibodies using the ^{35}S-labeled intracellular portion (aa 605-979) of the full-length IA-2 protein as the target. The analysis of isotype-specific IAA was also based on the same format using mono-^{125}I-TyrA14-human insulin (Amersham, Little Chalfont, Bucks, UK) as the tracer and a mononclonal anti-human IgG3 antibody (clone G18-3) obtained from PharMingen.

Our laboratory participated in the serum exchange workshop carried out among the laboratories known to measure isotype-specific responses to GAD65 (results presented at the 5th International Congress of the Immunology Diabetes Society, 2001, Chennai, India). Our results were highly concordant with the results of those laboratories, using a method similar to that used in our laboratory. All samples from the same subject were analyzed in the same assay run as far as possible, and a proportion of both the initially positive and negative samples were retested blindly to confirm antibody status and titers.

ISLET CELL ANTIBODIES (ICA)

ICA were quantified by a standard indirect immunofluorescence method on sections of frozen human pancreas from a blood group O donor (Bottazzo et al. 1974). The

end-point dilution titers of the ICA-positive samples were recorded and the results expressed in Juvenile Diabetes Foundation (JDF) units. The detection limit was 2.5 JDF units. All samples initially positive for ICA were retested to confirm antibody positivity. The sensitivity of the ICA assay in our laboratory was 100% and the specificity 98% in the most relevant international standardization workshop (Greenbaum et al. 1992).

HLA TYPING

In Communications I and II HLA typing was performed using conventional HLA serology as described previously (Tuomilehto-Wolf et al. 1989). All HLA A, B, C, and DR specificities recognized by the Nomenclature Committee of the WHO in 1984 were included in the test panel. The DIPP study subjects included in Communications III and IV were genotyped from cord blood samples at the Department of Virology, University of Turku, Turku, Finland to define selected HLA DQB1 alleles (DQB1*02, *0301, *0302, and *0602/3) known to be significantly associated with either susceptibility to or protection against T1D (Ilonen et al. 1996). The technique is based on solution hybridization with lanthanide-labeled oligonucleotide probes detected with time-resolved fluorometry, and is suitable for screening a large number of samples (Sjöroos et al 1995). Males born in Turku carrying the DQB1*02 allele were genotyped for the DQA1 alleles *0201, *0501 and *03 to identify those with the DR3-DQ2 haplotype (DQA1*0501/DQB1*02) who were invited for prospective follow-up starting from the beginning of year 2000.

STATISTICAL ANALYSES

Unpaired and paired Student t-tests were used to compare mean ages and age differences. The Bonferroni correction for multiple comparisons was performed where appropriate. The distribution of autoantibody positive epitopes and isotypes was evaluated between the groups with cross-tabulation and $\chi 2$ statistics or Fisher's exact test. Individual areas under the curve (AUC) throughout the observation period were calculated for total antibodies, for each isotype and for specific epitope responses as previously described (Matthews et al. 1990) to avoid the problems associated with multiple data points. The Mann-Whitney U-test was used to compare the specific antibody titers and AUC values between the groups studied. Kaplan-Meier life-table survival analysis and log-rank statistics were used to assess

progression to clinical diabetes in relation to various epitope and isotype-specific IA-2 antibodies. A two-tailed P value of 0.05 or less was considered to be statistically significant. All the statistical analyses were performed using the SPSS statistical software package, version 10.1 (SPSS, Chicago, IL, USA).

RESULTS

INITIAL RESPONSE TO GAD65 AND IA-2/IA-2β IN SIBLINGS OF CHILDREN WITH TYPE 1 DIABETES (COMMUNICATIONS I AND II)

Antibodies to the middle domain of GAD65 were most abundant in the initial response to GAD65, as 94% (n=17) of the siblings with GAD65Abs had GAD65-M-Abs in their first positive sample. In addition, the initial response comprised GAD65-C-Abs in 83% (n=16) of the siblings usually together with GAD65-M-Abs and in one case alone. GAD65-N-Abs were detected in four subjects (22%), once together with GAD65-M-Abs and in three cases in combination with both GAD65-M-Abs and GAD65-C-Abs. The prevalence of epitope clusters did not differ between the progressors or non-progressors in the first sample (Fig.6).

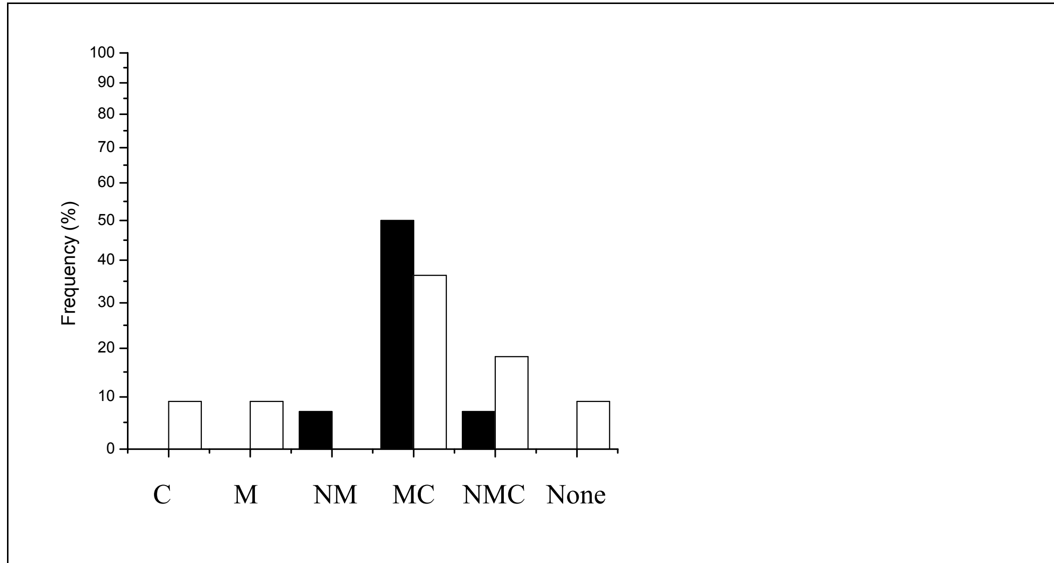

Fig 6. Frequency of various epitope-specific GAD65Ab profiles in the first positive sample in nine siblings who progressed to T1D during prospective observation (■) and in nine siblings who have remained unaffected (□).

In the initial sample 30 of the 34 IA-2 Ab positive siblings studied in Communication II had antibodies to the individual domains of the IA-2/IA-2β molecules, with 13 (38%) children testing positive for the IA-2 JM and IA-2 PTP epitopes, five (15%) for the IA-2β PTP and 28 (82%) for the cross-reactive PTP/βPTP domains, respectively. If a single response was seen it was cross-reactive to IA-2 PTP/βPTP (77%). No initial differences in the frequencies of the antibodies reactive with various epitope regions of IA-2/IA-2β were seen between the progressors and non-progressors (Fig7).

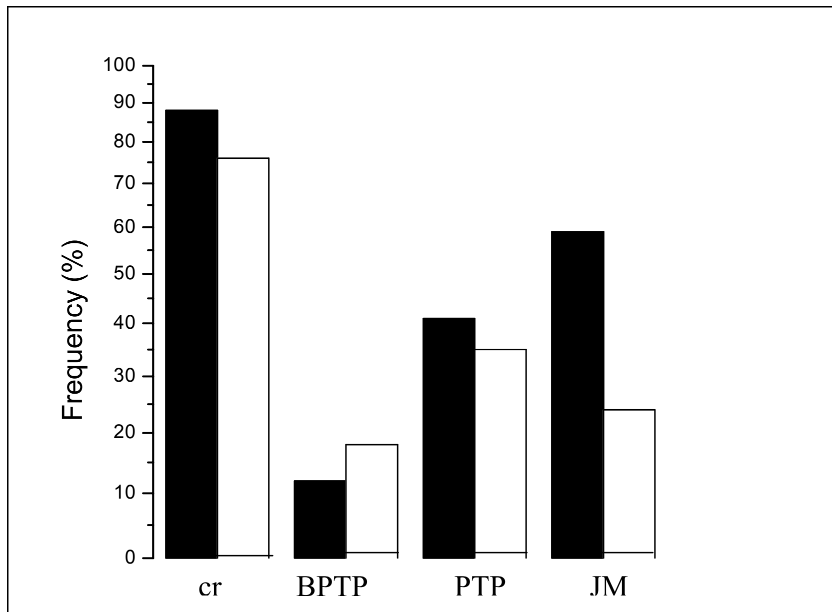

Fig 7. Frequency of various epitope-specific IA-2/IA-2β antibody profiles in the first positive sample in 17 siblings who progressed to clinical T1D during prospective observation (■) and 17 siblings who remained unaffected (□)

The humoral immune response to GAD65 and IA-2 was initially mainly composed of IgG1 antibodies. There were no differences in the distribution or number of the various isotype-specific antibodies between the progressors and the non-progressors in the first antibody-positive sample, although the progressors tended to have IgG2-IA-2 antibodies more often and the non-progressors IgG2-, IgG4-GAD65Abs and IgE-IA-2 more often than the progressors. In general the initial isotype response to GAD65 (Fig 8, panel A) was broader than to IA-2 (Fig 8, panel B), and IgG2, IgG4 and IgM responses behaved differently between antigens, as they were less frequent

(not significantly) among the GAD65Ab-positive progressors but tended to be more common among the IA-2Ab-positive progressors.

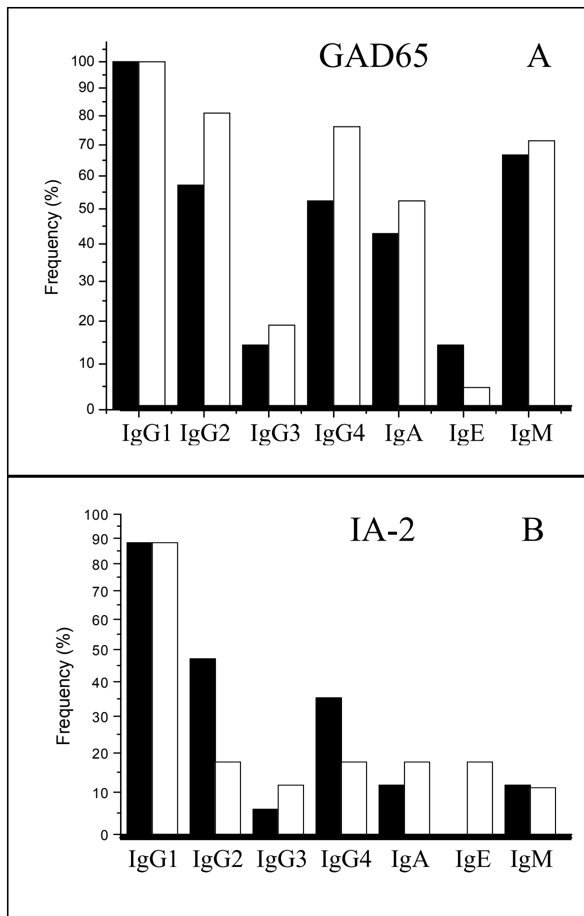

Fig 8. Frequency of various isotype-specificGAD65 antibodies (upper panel A) and the IA-2 antibodies in the first positive analyzed (lower panel B) in siblings who progressed to clinical T1D during prospective observation (■) and siblings who remained unaffected (□).

INITIAL RESPONSE TO IA-2/IA-2β AND INSULIN IN YOUNG CHILDREN WITH GENETIC SUSCEPTIBILITY TO T1D (COMMUNICATIONS III AND IV)

Of the genetically susceptible children who were selected in the study and progressed to T1D 93% (n=14) seroconverted to positivity for IgG1-IA-2 antibodies at the same time as total IA-2 antibodies appeared (at the mean age of 1.9 years). IA-2 JM antibodies appeared very soon thereafter, at a mean age of 2.0 years, whereas the antibodies against other epitope domains and antibodies of other isotypes emerged clearly later within the next year. The non-progressors seroconverted to positivity for

IA-2 antibodies in two phases within a 2-year period. Total IA-2 antibodies, IgG1- and IgG3-IA2 specific antibodies and antibodies that were cross-reactive to epitope regions IA-2 PTP/βPTP appeared first at a mean age of 2.4 years. The next to appear, in the second phase, were IA-2 JM antibodies, followed by IgE, IgA, IgG2, IgM and IgG4-IA-2 and finally antibodies specific to IA-2βPTP.

Antibodies against different epitope domains were initially relatively frequent among the progressors. Isotypes other than IgG1 were rare, and the pattern was similar among the non-progressors. Minor non-significant differences could be seen: The progressors had often reactivity directed to IA-2 JM, whereas the most frequent reactivity was towards IA-2/IA-2β PTP antibodies in the group of the non-progressors (Fig 9).

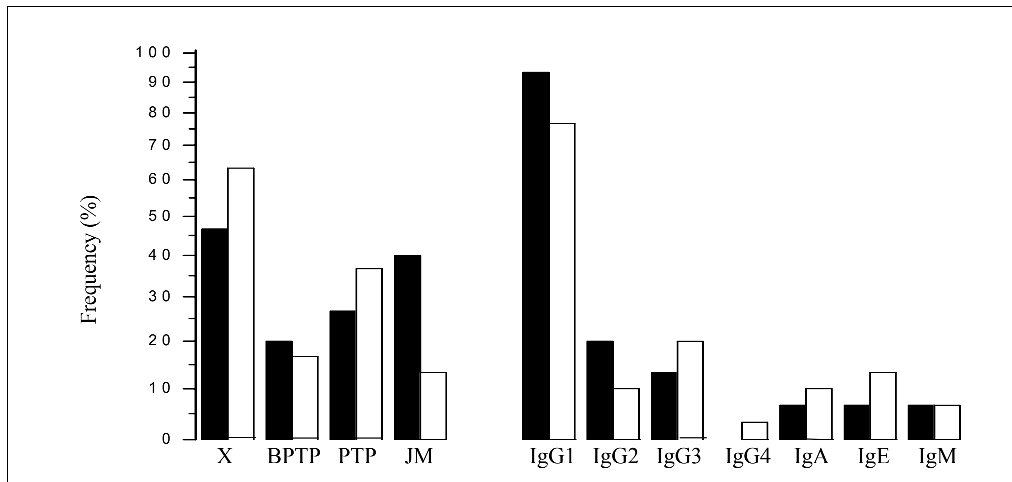

Fig 9. Frequency of various epitope and isotype-specific IA-2 antibodies in the first positive sample in the 15 children who progressed to clinical T1D during prospective observation (■) and at the corresponding age in the 30 children who remained unaffected (□). X = βPTP/PTP cross-reactive antibodies

As expected, IAA appeared earlier at a mean age of 1.04 yrs than the other autoantibodies. Isotypes emerged in the following order: IgG1 at a mean age of 1.30 years, followed by IgG3 (1.31 years) and IgG2 at 1.35 years. IgG4 emerged at a mean age of 1.56 years and IgA at a mean age of 1.2 years. The IAA of the progressors consisted of more isotypes, including IgG3 than the non-progressors (M-W U-test P=0.003).

SPREAD OF GAD65 AND IA-2/IA-2β REACTIVITIES IN SIBLINGS (COMMUNICATIONS I AND II)

GAD65 antibodies specific to the middle region of the molecule remained stable, and if the response was initially restricted to the middle region, it rapidly spread to the C-terminal domain and in a few cases later to the amino (N) -terminal domain. No differences in the number of epitopes between the progressors or non-progressors were seen when comparing the maximal responses, but the median levels of GAD65-M-Ab and GAD65-N-Ab tended to be higher in the progressors than in the non-progressors in contrast to the first sample analyzed. Heterogeneous spreading to additional IgG subclasses and isotypes in the GAD65 response was observed, but without any differences between the progressors and non-progressors. The GAD65Ab response was mainly composed of antibodies of IgG1 subclass. Responses of IgG2-, IgG4-, IgM-, and IgA-GAD65Ab were observed frequently, whereas IgE- and IgG3-GAD65Ab responses were seen more rarely. No significant differences were detected in the integrated levels of various epitope- and isotype-specific GAD65Ab between the progressors and non-progressors.

The profiles of the antibody responses to the IA-2/IA-2β epitope specificities during follow-up were heterogeneous. Most progressors and non-progressors had antibodies to several epitope regions. Spreading of the epitope response to additional IA-2/IA-2β epitope regions was seen. If the initial response comprised cross-reactive PTP/βPTP antibodies, as it mainly did, the spreading of the response resulted in the appearance of specific IA-2 PTP and IA-2 JM antibodies in that order. Specific IA-2β PTP antibodies were seen randomly, and only twice did the response switch to that specificity. Comparing the maximal epitope responses, the frequency of IA-2 JM-specific antibodies turned out to be significantly higher among the progressors than among the non-progressors (Fig 10), whereas the frequencies of other epitope responses were of the same magnitude.

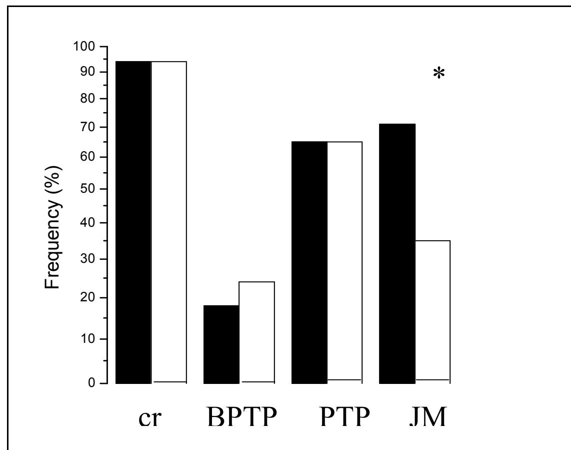

*Fig 10. Frequency of various epitope-specific IA-2/IA-2β antibody profiles during follow-up in 17 siblings who progressed to clinical T1D during prospective observation (■) and 17 siblings who remained unaffected (□) * P ≤0.05 (χ2 statistics).*

The humoral immune response to IA-2 was initially mainly composed of IgG1 antibodies, and inverse seroconversions were rare for IgG1 subclass antibodies. All the siblings experienced seroconversion to positivity for additional isotypes. The non-progressors had IgE-IA-2 antibodies (53% vs. 12%; *P*=0.03) more often than the progressors, but no other significant differences were observed in the distribution of the isotypes during the observation period. There was one significant difference in the integrated levels of isotype-specific IA-2 antibodies between the progressors and non-progressors, the non-progressors having higher levels of IgE-IA-2 antibodies.

The distribution of JM-reactive and IgE-class IA-2 antibodies in progressors and non-progressors during the follow-up showed up to be interesting. The progressors were characterized by an increased frequency of IA-2 JM antibodies and no IgE-IA-2 responses in the absence of an IA-2 JM response. Among the siblings positive for IA-2 JM antibodies, the peak IgE-IA-2 level and integrated IgE-IA-2 response were significantly higher (P<0.01) among the non-progressors than among the progressors.

SPREAD OF IA-2/IA-2βAb AND IAA REACTIVITY IN DIPP CHILDREN (COMMUNICATION III AND IV)

All 15 progressors initially positive for total IA-2 and insulin autoantibodies developed antibodies to various epitopes and isotypes, and accordingly they all had

detectable IgG1-IA-2, IgG1-IAA (Fig 11) and IgG3-IAA (Fig 11) antibodies. Antibodies against cross-reactive IA-2/IA-2β PTP and IA-2 JM epitopes were often present and IgG2-, IgG3-, and IgG4-IA-2 antibodies, and an isotype response comprising IgG2-IAA were also seen often, while other responses were rare. Spreading of the response was considerably more frequent for IA-2 antibodies than for IAA. In communication III most of the IA-2Ab positive progressors (87%) experienced inverse seroconversions, which were observed for all isotypes other than IgG1-IA-2 antibodies. Inverse seroconversions were observed in six IAA-positive children before the clinical diagnosis of T1D, but IgG1- and IgG3-IAA were the most constant isotypes throughout the preclinical period.

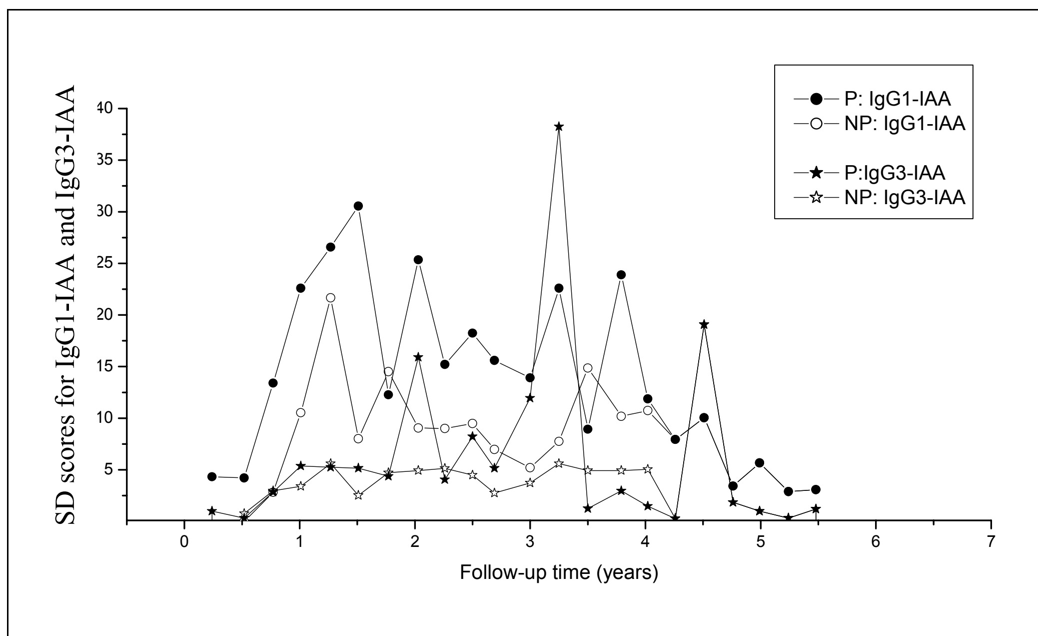

Fig 11. IAA of IgG1 and IgG3 subclasses during prospective observation among those DIPP children who progressed to clinical T1D (P; n=15) and those who remained unaffected (NP; n=30)

The appearance of additional IA-2/IA-2β reactivities during follow-up was also observed among the non-progressors, in whom the increase in the number of positive antibodies was more conspicuous than among the progressors, with an increased frequency of all isotypes except for IgG1. The frequency of IgE-IA-2 antibodies was higher than that seen among the progressors (*P*<0.05), and a significantly higher

proportion of the non-progressors who were positive for IA-2 JM antibodies also had IgE-IA-2 antibodies (83% vs. 11%; *P*=0.002). The frequencies of IgA and IgM-IA-2 antibodies tended to be increased among the non-progressors, and life-table analysis revealed a significantly lower risk of progression to clinical T1D among those with an IgE-IA-2 response ($P<0.05$, Fig 12), while no other differences were observed (data not shown). The integrated IgE-specific IA-2 antibody response was significantly higher among the non-progressors than among the progressors ($P<0.05$), but the levels were low in both groups. Positive seroconversions were also seen among the IAA-positive non-progressors, but the total frequency of isotypes was lower than that seen among IAA-positive children who developed T1D. Both IgG1- and IgG3-IAA appeared earlier in the progressors than in the non-progressors, and the progressors had higher maximal levels of IgG3-IAA during the follow-up than the non-progressors. In addition the frequency (P=0.004) and the integrated levels (AUC) (P=0.002) of IgG3-IAA were higher among the progressors.

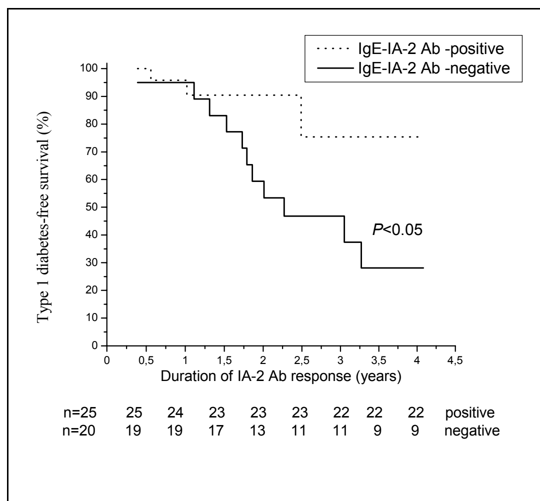

Fig 12. Probability of remaining non-diabetic among 45 IA-2 antibody-positive children IgE-class antibodies positive at least once vs. never during the total observation period. The number of individuals remaining at each time point is indicated under the x-axis. Differences between groups were determined by Kaplan-Meier survival analysis ($P<0.05$; log rank test)

CHARACTERISTICS OF ISLET AUTOANTIBODIES IN PARALLEL ANALYSES

Studies III and IV were partly overlapping, since there were 13 children who were all analyzed for epitope spreading and for Ig isotypes and IgG subclasses and IgG subclass distribution to both IA-2/IA-2β and insulin. We have also serutiuized the humoral immune response to GAD65 in the same 13 children (Ronkainen et al. submitted). Accordingly the opportunity of analyzing the humoral immune response to all three autoantigens in parallel in these 13 children was utilized. Seven of these subjects progressed to overt T1D during the observation period, while six remained diabetes-free.

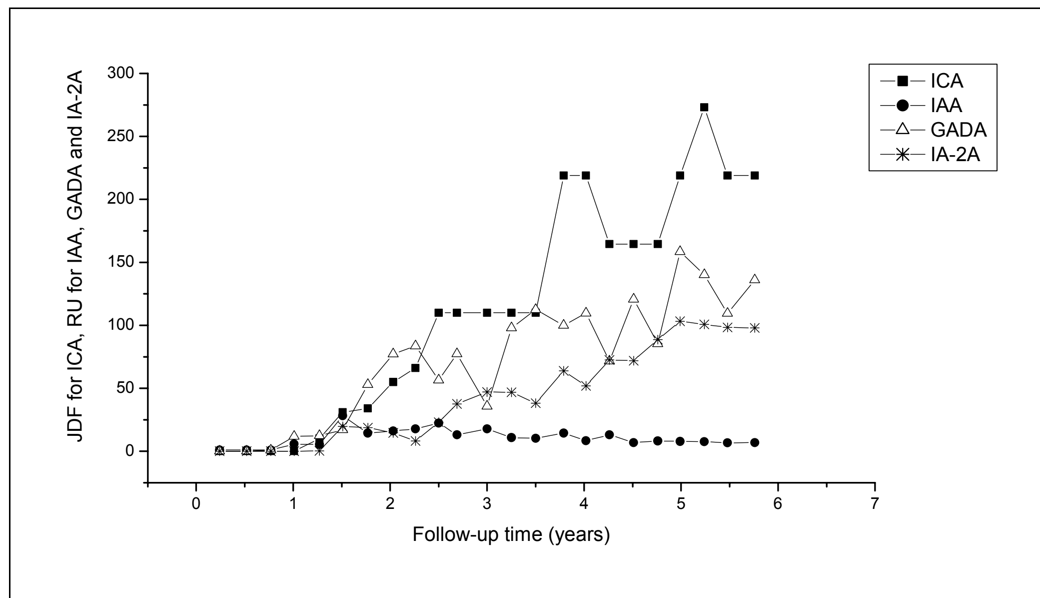

Fig 13. Profile of all diabetes-associated autoantibodies in the 13 DIPP children characterized for epitope- and isotype-specific responses.

Seroconversion to ICA positivity occurred at a mean age of 1.7 years (range 1.0 – 4.5 years), to IAA positivity at 1.4 years (range 0.5 – 3.4 years), to GAD65Ab positivity at 1.5 years (range 0.8 – 4.0 years) and to IA-2 antibody positivity at the age of 2.30 years (range 1.0 – 4.5 years). The appearance of the specific epitope and isotype responses are presented in Table 4, and the detailed characterization of the humoral response is shown individually in Table 5.

Table 4. Mean age (years; range) at seroconversion to antibody positivity during the follow-up period in seven DIPP children who progressed to clinical T1D (P) and in six who remained unaffected (NP).

	IAA		GAD65Ab		IA-2Ab	
	P	**NP**	**P**	**NP**	**P**	**NP**
Total	**1.5 (0.5-3.4)**	**1.2 (0.8-2.0)**	**1.7 (1.0-4.0)**	**1.2 (0.8-2.0)**	**2.2 (1.0-4.7)**	**2.4 (1.0-4.6)**
IgG1	**1.3 (0.2-4.0)**	**1.3 (0.8-2.1)**	**1.7 (1.0-4.0)**	**1.3 (1.0-2.0)**	**2.2 (1.0-4.5)**	**2.3 (1.0-4.6)**
IgG2	**1.5 (0.3-2.4)**	**1.5 (0.5-2.9)**	**2.0 (1.0-4.0)**	**1.6 (1.3-2.0)**	**2.5 (1.5-5.0)**	**3.2 (1.5-4.6)**
IgG3	**1.2 (1.0-2.0)**	**1.4 (0.8-2.1)**	**2.1 (1.0-4.0)**	**1.7 (1.3-2.1)**	**2.8 (1.3-5.7)**	**2.9 (1.5-4.6)**
IgG4	**2.5 (1.0-5.5)**	**2.3 (2.1-2.5)**	**1.7 (1.0-2.5)**	**2.4 (1.0-3.3)**	**2.8 (1.7-4.7)**	**3.3 (1.5-4.3)**
IgA	**1.2 (0.2-3.0)**	**3.3 (3.3-3.3)**	**1.5 (1.5-1.5)**	**2.7 (1.6-3.8)**	**4.0 (2.6-5.5)**	**3.7 (1.5-4.8)**
IgE	**Not detected**		**2.9 (1.8-4.0)**	**2.3 (2.3-2.3)**	**4.7 (4.7-4.7)**	**3.1 (2.0-4.3)**
IgM	**Not detected**		**2.0 (1.7-2.2)**	**1.2 (0.5-1.8)**	**3.3 (1.7-5.2)**	**2.9 (2.0-3.8)**

In this small cohort there were no statistically significant differences in the epitope and isotype-specific profiles between progressors and non-progressors. IAA antibodies appeared first, followed almost simultaneously by GAD65 antibodies, while IA-2 antibodies clearly emerged as the last antibody reactivity (Fig 13). IAA of IgG3 subclass appeared earlier among the progressors, but the total IgG3 response was of the same magnitude. The proportion of children positive for IgG4-GAD65Ab and for IgE-IA-2Ab was higher among the non-progressors, and even through JM-IA-2 epitope specific antibodies did not dominate in the group of progressors, they showed up in a few children as a single epitope response which according our results is a marker of T1D risk. The epitope response was directed into the PTP/βPTP direction among the non-progressors.

*Table 5. The individual epitope and isotype-specific responses to insulin, GAD65 and IA-2 in those children analyzed for their humoral immune response to the three biochemical characterized T1D autoantigens in the DIPP study.(X≠*0301, *0602 or *0603)*

		HLA-DQB1	Epitope and Isotype response		
Subject No	**Sex**	**Genotype**	**IAA**	**GAD65**	**IA-2**
Progressors					
630A	F	*02,*0302	IgG1,4	M,C,N, IgG1,2,3,IgE	X-reac,sPTP,IgG1,2,3,4, IgA, IgE, IgM
2176A	M	*02,*0302	IgG1,2,3,4,A	M,C,IgG1,2,3,4,IgA	X-reac,sPTP,IgG1,2,3,4,IgA, IgM
7801A	F	*0302/x	IgG1,2,3,A	M,C,IgG1,3,4,IgM	JM,IgG1
31875A	M	*0302/x	IgG1,2,3,4,A	M,N,IgG1,IgM	JM,IgG1,2
35160A	F	*0302/x	IgG1,2,3,4,A	M,C,IgG1,2,3	X-reac,sPTP,IgG1,2,3,4
35386A	M	*0302/x	IgG1,2,3,4	IgG1	X-reac,sPTP,JM,IgG1,2,3,4,IgM
36584A	F	*0302/x	IgG1,2,3,4,A	M,C,IgG1,2,4,IgE,IgM	X-reac,sPTP,IgG1,2,4
Non-progressors					
8955A	F	*02,*0302	IgG1,3	M,C,IgG1,2,3,4	X-reac,sPTP,IgG1,2,IgA
3755A	M	*0302/x	IgG1,2,3,4,	M,C,N,IgG1,2,3,4,IgA, IgM	X-reac,sPTP,JM,IgG1,4,IgA
3688A	F	*02,*0302	IgG1,2,3,4,A	M,C,N,IgG1,2,3,	X-reac,sPTP,IgG1,2,3,4,IgA, IgE,IgM
30303A	F	*0302/x	IgG1,3,4,A	M,C,N,IgG1,2,4,IgA, IgM	X-reac,sPTP,IgG1,2,3,IgA,IgE
31537A	F	*0302/x	IgG1,2,3,4	M,C,IgG1,2,3,4,IgE	X-reac,sPTP,IgG1,2,4,IgA,IgE, IgM
35006A	F	*0302/x	IgG1,3	M,N,IgG1,2,3,4,IgA	X-reac,sPTP,IgG1,2,4,IgA,IgE, IgM

DISCUSSION

About 40,000 individuals in Finland are affected by T1D, and about 500 new cases are diagnosed each year in children under 16 years of age (Karvonen et al. 2000). T1D is an incurable disease, and the number of very young children afflicted by T1D continues to increase at an alarming rate. At present, there is no method to prevent or cure it, and the main goal of ongoing research is to generate new knowledge on the natural history of the disease.

The purpose of this study was to evaluate the natural course of humoral beta cell autoimmunity and the impact of the appearance of autoantibody epitopes and isotypes on the development of T1D among Finnish children. The study population comprised genetically susceptible newborn children identified from general population and siblings of children with recently diagnosed T1D. An autoimmune process of varying duration precedes the clinical presentation of T1D, and although the autoantibodies targeting islet antigens may not play any pathogenic role, they have been confirmed as markers of an ongoing autoimmune process and are useful for assessing the risk of developing clinical T1D (Knip 1997, Kulmala et al. 1998). Despite increasing knowledge of specific autoantibodies, genetic disease susceptibility, and the fact that environmental factors play a major role in the etiology of this disease, it is not possible to predict accurately whether a given child will develop T1D or not, or when the clinical manifestation will occur. There is a need to identify additional markers facilitating disease prediction on an individual basis among subjects with an increased risk for progression to overt T1D to allocate the future preventive interventions to the children with a very high risk of disease.

There has been discussion as to whether a Th1/Th2 imbalance could lead to T1D, and further whether the distribution of different isotypes to autoantigens might reflect the polarization of the cellular immune response (Katz et al. 1995, Pilström et al. 1995, Nicholson and Kunchroo 1996, Abbas et al. 1996, Petersen et al. 1999). As organ-specific disorders such as T1D are perceived as Th1-mediated processes, it has been hypothesized that destructive islet cell autoimmunity is Th1-mediated, whereas a Th2 response is non-pathogenic, and that the cytokines regulate the balance between protection (IL-4) and destruction (IL-12) (Garraud et al. 2003, Rapinovitch and Suarez-Pinzon 2003). Modulatory cytokines are also instrumental in immunoglobulin

heavy chain switching, and one may further speculate that as a sign of a non-pathogenic Th2 response the autoantibodies are directed at a single epitope region on a primary antigen and comprise Th2-like isotypes such as IgG4 (IL-4, IL-13) and IgE (IL-4, IL-13. In those unfortunate individuals who eventually progress to clinical T1D the response may subsequently turn into a pathogenic Th1 response, which is associated with spreading of the humoral immune response to other epitopes of any given autoantigen (intramolecular epitope spreading) as well as spreading from one autoantigen to another (intermolecular spreading) and isotype switching with increased synthesis of IgG1 (IL-10, CD40L) and IgG3 (IL-10, CD40L) antibodies.

The coexistence of beta cell autoimmunity and immunoregulation in prediabetes represents a fragile equilibrium. Autoreactive T cells specific for autoantigens are present in most normal individuals but are kept under control by a number of tolerance mechanisms, among which $CD4^{+}CD25^{+}$ T cell mediated regulation propably plays an important role (Lindley et al. 2005). The regulative function may also be mediated partly by the production of Th2 cytokines (IL-4, IL-10), but there are still many unsolved questions left (Bach and Chatenoud 2001).

The present series is unique, since both the isotype and epitope profiles of the immune response to a distinct molecule were studied in the same children and because of the frequent sampling interval starting before (Communications III and IV) or at the time of seroconversion to antibody positivity or thereafter (Communications I and II). Accordingly it was possible to detect even minor changes in the dynamics of the immune response. The children studied were grouped into progressors and non-progressors based on whether they presented with T1D or not during the observation period. Admittedly this classification does not exclude the possibility than some of those classified as “non-progressors” may later manifest T1D. Those IA-2 antibody positive children, now classified as “non-progressors” in Communication III, have an especially high risk for subsequent progression to clinical T1D, since they all already had a susceptible HLA genotype and multiple T1D-associated autoantibodies (including IA-2 antibodies) at a very young age, these being well-known risk factors for T1D. Among the siblings designated as non-progressors the risk of disease is not so obvious, since the follow-up for progression to clinical diabetes continued until the end of December 1997 and thus lasted for almost 10 years. In any case one has to

make the distinction that in these studies the progressors represent those children who progress rapidly to clinical disease, whereas the so-called non-progressors include those children who progress slowly to overt T1D or remain unaffected.

LIMITATIONS OF THE PRESENT WORK

The study cohorts in each of these substudies were relatively small, which resulted in a limited statistical power. The numbers of children who seroconverted to autoantibody positivity, and the children who presented with clinical T1D were too low in relation to the sum variables to reach statistical significance and therefore this research was descriptive rather than hypothesis-driven.

The autoantibodies specific to islet antigens are secondary phenomena reflecting cell-mediated insulitis that plays an instrumental role in the development of beta cell damage, but until now autoantibody responses have been the only useful markers for assessing the risk of developing clinical T1D. Despite its descriptive nature and secondary status with regard to insulitis, this work nevertheless provides novel information on the maturation of the humoral immune response to beta cell antigens that can be used to generate new hypotheses to be tested in future studies.

The methods used for the screening for primary autoantibodies have limitations, which may cause bias in the isotype and subclass results detected and analyzed here. Firstly, to detect ICA fluorescein-conjugated goat anti-human immunoglobulin G is used, which may ignore humoral response other than IgG (e.g. IgM, IgE or IgA). Further, in the determination of IAA, GAD65Abs or IA-2Abs protein A sepharose precipitation is used, which may also have an effect on the results of isotype or IgG subclass specific antibodies. Protein A binds human IgG subclasses 1, 2 and 4, but has no effect on IgG3 or IgA, and the proportion of IgM bound to protein A is about one third of the total level of IgM in serum. It is therefore possible that these analyses, based on the presence of antibodies binding to protein A, have selected for children with antibodies of IgG1, IgG2 or IgG4 isotypes, but children having only antibodies of IgA or IgG3 isotype might have been missed for any further analysis with monolonal isotype specific antibodies (Ibrahim et al. 1993). Althought this may have effect on results, we conclude that bias is small, since Ronkainen et al. (2001) tested

the concordance of IAA between protein A and protein G, which binds all IgG subclasses, and the results obtained were highly concordant.

Secondy, the results of isotype-specific antibodies to GAD65, IA-2 and IAA were expressed as SD scores (SDS) calculated by comparing individual cpm to a mean delta cpm of control subjects, which may have an impact on the results. The results would have been more informative if the cpm of different isotypes had been compared to total antibody or IgG1 individually (ratio), without using the control group. We preferred SDS instead of ratios, since the analysis of total GAD65 and IA-2 antibodies has been done earlier in the DIPP study, and in order to be able to present ratios between isotype specific and total antibodies they would have to be analysed in the same run.

GAD65 ANTIBODY ISOTYPES AND EPITOPE REGOGNITION DURING THE PREDIABETIC PROCESS IN SIBLINGS OF CHILDREN WITH T1D

In Communication I it was noted that the first GAD65Ab response in initially non-diabetic siblings was directed towards both the middle and C-terminal domains of GAD65 in about 90% of subjects. Primary reactivity to GAD65-N-Abs was clearly less common, and it was detected at low titers in only a few siblings in combination with both GAD65-M-Ab and GAD65-C-Ab. As previous reports in GAD65Ab-positive subjects have established (Richter et al. 1993, Falorni et al. 1996, Bonifacio et al. 2000, Söhnlein et al. 2000), these results imply that both the M and C-terminal regions of GAD65 are early, possibly even simultaneous targets of humoral GAD immunity, while the N-terminal region is a secondary epitope. The clear immunodominance of the middle domain at the beginning, and rapid spreading of the response from the middle region to the C and N-terminal domains seen in the study on young genetically susceptible DIPP children (Ronkainen et al. submitted for publication), was not observed here, probably because a majority of the siblings already tested positive for GAD65Ab in the first sample taken, and we do not know for how long these siblings had been positive for GAD65Ab.

None of the GAD65Ab reactivities nor any changes in reactivity over time were associated with progression to T1D or diabetes presentation. Neither were high levels of GAD65-C-Ab able to distinguish progressors from non-progressors, as reported in

a study on adults with latent autoimmune diabetes (LADA) (Falorni et al. 2000). Actually five progressors seroconverted to negativity for GAD65-C-Ab before the manifestation of clinical T1D and the median levels of GAD65-C-Ab decreased towards diagnosis.

We observed that the humoral immune response to GAD65 was mainly composed of IgG1 antibodies in siblings of children with T1D. No significant differences in the distribution of various isotype-specific antibodies were seen, although the non-progressors tended to have IgG2- and IgG4-GAD65Abs more often than the progressors. As a sign of a dynamic process isotype spreading was seen for IgG2-GAD65Abs and was almost significant for IgM-GAD65Abs among the progressors, while the non-progressors experienced a significant isotype spreading for the latter ($P<0.05$). We failed to observe a dominance of Th2-like isotypes in the first samples analyzed, but the frequency of IgM-GAD65Ab was surprisingly high in both groups. We speculate that the high levels of IgG1-GAD65Ab may reflect the dominance of the IgG1 isotype within the humoral immune response in general rather than a polarization of the response into Th1 immunity, whereas the relatively high prevalence of IgM antibodies during the early response may represent Th2 immunity. These considerations, however, are limited by the poorly defined timing of the initial seroconverion to GAD65Ab positivity in most of the siblings, as mentioned earlier.

IA-2 ANTIBODY EPITOPES AND ISOTYPES IN SIBLINGS OF CHILDREN WITH TYPE 1 DIABETES (COMMUNICATION II) AND IN CHILDREN WITH HLA-CONFERRED SUSCEPTIBILITY TO T1D (COMMUNICATION III)

Our observations confirm the original findings of Lampasona et al. (1996) that the cytoplasmic portion of IA-2 is the target of humoral autoimmunity (Hatfield et al. 1997, Notkins et al. 1997, Kawasaki et al. 1998, Bonifacio et al. 1998, Naserke et al. 1998, Achenbach et al. 2004). The number of detectable IA-2 antibody epitope and isotype reactivities was initially slightly higher among those young children who progressed to clinical T1D than among the non-progressors, while unexpectedly, the initial median levels of total IA-2 antibodies were almost equal in the two groups. The number of responses to antigenic domains increased in parallel with the increase in total IA-2 antibody titers, and subsequently remained stable in both groups. Among the progressors the response was primarily directed towards the JM domain and

secondarily towards the PTP/βPTP-like domains of the IA-2 molecule, whereas the order was the reverse among the non-progressors. During the follow-up antibodies specific to the JM domain were constantly detected more often among the progressors, indicating an increased risk of progression to clinical T1D.

The IgG1 antibodies against IA-2 were the first to appear and in a few cases even before the detection of total IA-2 antibodies. IgG2 and IgG3-IA-2 antibodies appeared at the same time as or after IgG1-IA-2, but were usually of lower titers and were present at about the same frequency in both groups. However, the IgG3-IA-2 antibodies, perceived as Th1-associated antibodies (Garraud et al. 2003), appeared earlier than autoantibodies to IgG2-IA-2 among the non-progressors, which suggests that Th1-type immunity was also prevalent in the non-progressors at the time of seroconversion to IA-2 antibody positivity. Regulatory and protective responses may appear later, however, since IgE, IgA, and IgM -IA-2 antibodies emerged in subsequent samples among the non-progressors likely as a sign of an increasing Th2 polarization.

The number of isotypes increased consistently as a function of the duration of the response among the non-progressors, and it was significantly higher among the non-progressors than among the progressors at the time of diagnosis of clinical T1D. Especially the frequencies of those isotypes implicated as reflecting Th2-like immunity, i.e. IgG2, IgG4, IgA and IgE antibodies, were significantly higher. Among the young children recruited from the DIPP Study, the explanation for the appearance of isotypes may be partly physiological, since IgG1 and IgG3 subclasses develop earlier than IgG2, or specially IgG4, but by the age of 12 serum levels reach the adult levels and this could not have any impact on results among the siblings (Meulenbroek 2000). The majority of our siblings already had multiple isotypes or IgG subclasses at the beginning of follow-up, without any significant differences in the distribution or number of the various isotype-specific antibodies between the progressors and non-progressors, but, as expected, the response was dominated by IgG1-IA-2Ab (Dozio et al. 1994, Couper et al. 1998, Bonifacio et al. 1999, Petersen et al. 1999, Hawa et al. 2000, Lohmann et al. 2000, Seissler et al. 2002).

IgG4-IA-2 subclass antibodies were detected in only a small proportion of the first IA-2 antibody-positive samples and usually appeared as the last antibodies during

follow-up, but their frequency increased at a similar rate in both groups. Neither the frequency nor the integrated levels of IgG4-IA-2 antibodies differed between the progressors and non-progressors, as recently reported by Seissler et al. (2002). According to the present life-table analyses, the IgG4-IA-2 response showed no association with a reduced risk of T1D, although the IgG4-IA-2-negative children tended to present with overt diabetes somewhat earlier than the IgG4-IA-2-positive ones. It is possible that the frequency of IgG4-IA-2 antibodies increases among older children who remain diabetes-free for a longer time (Seissler et al. 2002), since repeated long-term antigenic stimulation leads to marked IgG4 response physiologically (Meulenbroek 2000). In addition, the isotype response seems to be antigen-specific, since the IgG4-subclass of GAD65 antibodies turned out to be associated with protection from clinical diabetes even at young age among GAD65Ab-positive DIPP children (Ronkainen 2004, manuscript submitted), whereas IgG4-IAA were unable to distinguish progressors from non-progressors as reported here from the DIPP study (Communication IV) and earlier by the BABYDIAB study (Bonifacio et al. 1999).

The most interesting finding was based on the co-occurrence of antibodies against domain JM of IA-2 and IgE-IA-2 antibodies. The presence of IA-2 JM antibodies was associated with an increased risk of progression to clinical T1D, since such antibodies appeared more frequently and earlier among the progressors than among the non-progressors, and were present at higher levels than most other epitope-specific antibodies during the whole follow-up period until the diagnosis of T1D in the progressors. The non-progressors who were positive for IA-2 JM antibodies usually also had IgE-class IA-2 antibodies, which, according to our data, seem to be linked to relative protection from overt disease, since these were present more than twice as often among the non-progressors than among the progressors during follow-up, and integrated IgE antibody levels were significantly higher among the non-progressors than among the progressors. Only two non-progressors tested positive for IA-2 JM antibodies during the follow-up without being positive for IA-2 antibodies of the IgE class, and in these two cases the titers of the IA-2 JM antibodies were very low and detected only in single samples. Our observation among the progressors that the children positive for IgE-class IA-2 antibodies were somewhat older than those who were negative for IgE antibodies at the time of diagnosis of clinical disease (4.2 years

vs. 3.5 years) supports the hypothesis that an IgE antibody response to IA-2 may be a sign of protective or regulatory antigen-specific autoimmunity in young children.

INSULIN AUTOANTIBODY ISOTYPES IN YOUNG CHILDREN WITH INCREASED GENETIC RISK

The number of detectable IAA isotypes was higher and more stable among those who progressed to clinical T1D. Again there was some heterogeneity in the isotype response, but IgG1-IAA response was the first to appear, whereas IgG4-IAA appeared late. More than 90% of the progressors and two thirds of the non-progressors had an IgG1-IAA response in their first IAA-positive sample. The initial response more often also included IgG2, IgG3, IgG4 and IgA-IAA in the case of the progressors than in the non-progressors.

All the progressors had strong IgG1- and IgG3-IAA responses during prospective observation, while the IgG3-IAA response in particular was less frequent in the non-progressors. On assessing the integrated levels (AUC), we observed IgG3-IAA dominance in six of the progressors (40%) but in only one non-progressor (3%). Observations seen in Communication IV, suggest that a weak or absent IgG3-IAA response, possibly reflecting attenuated insulin-specific Th1 reactivity, could be a protective sign in IAA-positive individuals.

In DIPP Study families with a child persistently testing positive for autoantibodies were invited to take part in a randomized controlled intervention trial to explore whether it is possible to delay or prevent the manifestation of clinical diabetes by daily administration of nasal insulin. In Communication IV samples taken after the start of the intervention trial were not included in the analysis, since nasal insulin may have an impact on in the IAA isotypes, but in the Communication III each child was observed up to diagnosis or to the corresponding age of the progressor despite inclusion in the intervention trial. Among the 45 IA-2Ab positive DIPP children there were only six children (2 progressors) not taking part to the intervention trial. If nasal insulin has the effect of inducing tolerance and proves to have a regulative effect, it may influence the other autoantigens like IA-2Abs as a bystander effect, which must be taken into account here.

EPITOPE SPREADING AND ISOTYPE RESPONSE TO MULTIPLE AUTOANTIBODIES IN YOUNG CHILDREN WITH GENETIC SUSCEPTIBILITY TO T1D

We were able to assess the maturation of the humoral immune response to all three major autoantigens in preclinical T1D, i.e. GAD65, IA-2, and insulin by timing the emergence of various isotypes (IgM, IgE, IgA, IgG subclasses) of antibodies to these antigens, and to observe possible signs of epitope spreading within the molecule after the initial appearance of such antibodies in 13 genetically susceptible young children recruited from the general population within the framework of the DIPP Study. The findings were along the lines observed in the four separate articles, although no statistically significant differences could be seen between the progressors and non-progressors due to the limited number of children. The parallel characterization of the humoral immune responses pointed to an association between risk for clinical T1D and broad antibody responses to IA-2 and insulin, as well as a strong insulin-specific IgG3 response and an isolated response targeting the JM-epitope of the IA-2 molecule. The combined analysis rendered support to the possibility that a broad isotype response to GAD65 and the appearance of IgG4-GAD65 and IgE-IA-2 antibodies may have some protective effect in terms of T1D progression.

SUMMARY

Type 1 diabetes is an incurable chronic disease due to beta-cell destruction, and the number of very young children afflicted by T1D continues to increase at an alarming rate. Despite increasing knowledge of specific pathogenetic factors in T1D, there is a need to identify additional markers facilitating disease prediction on an individual basis among subjects with increased risk for progression to clinical diabetes.

The purpose of this study was to evaluate the natural course of humoral beta cell autoimmunity and the impact of the appearance of autoantibody epitopes and isotypes as a sign of the maturation of the humoral immune response in the development of T1D. The population comprised genetically susceptible children recruited from the general population (the DIPP study) and siblings of children with newly diagnosed T1D (DiMe study). The present series was unique, since to the best of our knowledge, this was the first time these parameters have been analyzed in parallel in the same risk subjects, and the first time that the appearance of such antibodies has been reported in detail based on frequent sequential samples taken from the time of seroconversion in young children with increased risk for T1D. Accordingly it was possible to detect even minor changes in the dynamics of the immune response.

We conclude that the immunological course of preclinical T1D is widely heterogeneous and highly individual, so that the assessment of individual risk remains a challenge. The humoral immune response to GAD65 in siblings of subjects with T1D started out as a simultaneous response to the middle and C-terminal regions of the GAD65 molecule, but if the response was initially directed to the middle epitope cluster, it spread rapidly to the C-terminal domain and, in a few cases, later to the N-terminal domain. The response to GAD65 was mainly composed of antibodies of IgG1 subclass, but other responses of IgG2, IgG4, IgM, and IgA were frequently observed, whereas IgE and IgG3 responses were seen more rarely. Our findings indicated that the analysis of epitope- and isotype-specific GAD65 antibodies did not seem to be capable of separating progressors from non-progressors among GAD65Ab-positive first-degree relatives of children with T1D, the responses being relatively similar in the two groups. From the diabetes prediction point of view our novel finding of the frequent appearance of IgE-IA-2 antibodies in non-progressors during prospective observation may turn out to be valuable. IgE-IA-2 antibodies were

associated with relative protection against progression towards overt diabetes in IA-2 antibody–positive siblings of children with T1D, and among the genetically susceptible young children who progressed to clinical T1D, and especially among children with JM-reactive IA-2 antibodies and/or reactivity to multiple IA-2/IA-2β epitopes, who are otherwise at high risk of progression to overt diabetes. Accordingly, IA-2 antibodies of the IgE class may reflect a regulatory or protective autoimmune response in young children with HLA-conferred susceptibility to T1D.

The present data show furthermore that genetically susceptible young children who progress to clinical T1D were characterized by strong IgG1 and IgG3 responses to insulin. A weak IgG3-IAA response, or the total lack of such as response, was associated with relative protection against clinical disease, since one third of the non-progressors had no detectable IgG3-IAA response during follow-up.

Finally we had an opportunity to explore the epitope and isotype-specific antibodies to GAD65, IA-2/IA-2β and insulin in parallel in 13 genetically susceptible young children. The findings were in line with those observed in the separate studies, showing an association between risk for clinical T1D and broad antibody responses to IA-2 and insulin, as well as a strong insulin-specific IgG3 response and an isolated response targeting the JM-epitope of the IA-2 molecule. The combined analysis showed an association with a broad isotype response to GAD65 and the appearance of IgG4-GAD65 and IgE-IA-2 antibodies and a protective effect against progression to clinical T1D.

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Sanna Hoppu

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ORIGINAL COMMUNICATIONS