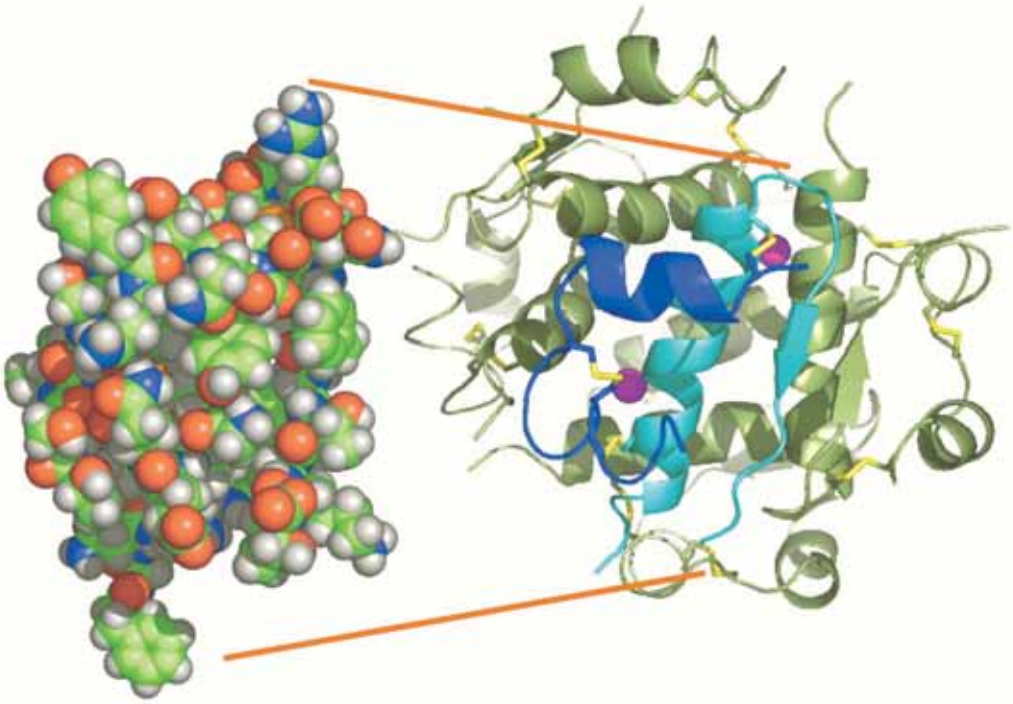

Minna Tiittanen

Immune Response to Insulin and Changes in the Gut Immune System in Children with or at Risk for Type 1 Diabetes

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Laboratory for Immunology,
Department of Viral Diseases and Immunology,
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Helsinki 2006

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IMMUNE RESPONSE TO INSULIN AND CHANGES IN THE GUT IMMUNE SYSTEM IN CHILDREN WITH OR AT RISK FOR TYPE 1 DIABETES

A C A D E M I C D I S S E R T A T I O N

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Laboratory for Immunology, Department of Viral Diseases and Immunology,
National Public Health Institute, Helsinki, Finland
and
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ABSTRACT

Type 1 diabetes (T1D), an insulin-dependent diabetes, is considered to be an autoimmune disease. The cause of T1D is the destruction of insulin-producing β-cells in the pancreatic islets and thus lifelong insulin treatment is necessary. The pancreatic islets are infiltrated with the cells of the immune system and the destruction is considered to be T-cell-mediated. The autoimmune nature of T1D is characterized by the presence of autoreactive T-cells and autoantibodies against β-cell molecules in the circulation of the patients. Autoantibodies to islet cells and to islet cell derived autoantigens, such as insulin, are used together with genetic screening in the prediction of the disease. Insulin is the only β-cell-specific autoantigen associated with T1D but the insulin autoantibodies (IAAs) are difficult to measure with proper sensitivity. T-cell assays for detection of autoreactive T-cells, such as insulin-specific T-cells, have also proven to be difficult to perform.

The genetic risk of T1D is associated with the human leukocyte antigen (HLA) class II gene region as is many other autoimmune diseases. However, genetic risk is only part of the risk of developing T1D: environmental factors also play an important role. The most studied environmental risk factors of T1D are enteroviruses and cow's milk. Both of these factors affect the immune system through the gut and there is increasing evidence suggesting that the gut immune system plays a role in the development of T1D. One hypothesis is that the insulin-specific immune response develops against bovine insulin in cow's milk during early infancy and later spreads to include human insulin, developing into a condition with autoaggressive characteristics.

The aims of this study were to determine whether the separation of immunoglobulin (Ig)G from plasma would improve the sensitivity of the IAA assay and how insulin treatment affects the cellular immune response to insulin in newly diagnosed patients. Furthermore, the effect of insulin concentration in mother's breast milk on the development of antibodies to dietary insulin in the child was also examined. Small intestinal biopsies were also obtained from children with T1D to characterize any immunological changes associated with T1D in the gut.

The isolation of the IgG fraction from the plasma of T1D patients negative for plasma IAAs led to detectable IAA levels that exceeded those in the control children. Thus the isolation of IgG and dissociation of immune complexes may improve the sensitivity of the IAA assay.

The effect of insulin treatment on insulin-specific T-cells was studied by culturing peripheral blood mononuclear cells *in vitro* with both human and bovine insulin. The insulin stimulation *in vitro* induced higher expression of regulatory T-cell markers at the messenger ribonucleic acid (mRNA) level in those patients treated with insulin than in patients examined before initiating insulin treatment. The insulin-induced expression of the regulatory T-cell marker Foxp3 was also demonstrated at the protein level. This finding suggests that insulin treatment in patients with T1D stimulates regulatory T-cells *in vivo* and this may partly explain the difficulties in measuring autoantigen-specific T-cell responses in recently diagnosed patients. The stimulation of regulatory T-cells by insulin treatment may also explain the remission period often seen after initiating insulin treatment.

In the third study we showed that the insulin concentration in breast milk is increased in mothers affected with T1D. The insulin concentration in mother's breast milk also correlated inversely with the levels of bovine insulin-specific antibodies in those children who were exposed to cow's milk proteins in their diet. This finding suggests that human insulin in breast milk induces tolerance to dietary bovine insulin. However, in infants who later developed T1D-associated autoantibodies, the insulin concentration in their mother's breast milk was increased. This finding may indicate that in those children prone to β-cell autoimmunity, breast milk insulin does not promote tolerance to insulin but may in contrast enhance insulin-specific immunity.

In the small intestinal biopsies the presence of several immunological markers, including markers for effector and regulatory T-cells, were quantified with the reverse transcriptase-polymerase chain reaction (RT-PCR). From these markers the expression of the interleukin (IL)-18 cytokine was significantly increased in the gut in patients with T1D compared with children with celiac disease or control children. The expression of regulatory T-cell markers in the intestine was not increased in children with T1D despite the presence of low-grade inflammation. The increased IL-18 expression lends further support for the hypothesis that the gut immune system is involved in the pathogenesis of T1D, whereas no activation of regulatory T-cells is seen in the intestinal immune activation related to T1D.

Keywords: type 1 diabetes, insulin, regulatory T-cells, gut

Minna Tiittanen, Immuunivaste insuliinia kohtaan ja muutokset suoliston immuunijärjestelmässä lapsilla, joilla on tyypin 1 diabetes tai joilla on riski sairastua tyypin 1 diabetekseen
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TIIVISTELMÄ

Tyypin 1 diabetestä (T1D) eli insuliiniriippuvaista diabetestä pidetään autoimmuunitautina, jossa puolustusjärjestelmän solut hyökkäävät kehon omia rakenteita vastaan. T1D:n syy on haimasaarekkeissa olevien insuliinia tuottavien β-solujen tuhoutuminen ja siksi elinikäinen insuliinihoito on tarpeen. Immuunijärjestelmän solut tunkeutuvat haimasaarekkeisiin ja solujen tuhoutumista pidetään T-solujen välittämänä tapahtumana. T1D:n autoimmuunista luonnetta kuvaa β-solujen molekyylejä tunnistavien autoreaktiivisten T-solujen ja autovasta-aineiden läsnäolo potilaiden verenkierrossa. Autovasta-aineita saarekesoluja ja saarekeperäisiä autoantigeeneja, kuten insuliinia, kohtaan käytetään yhdessä geneettisen kartoituksen kanssa taudin ennustamiseen. Insuliini on ainoa β-soluille spesifinen T1D:een liittyvä autoantigeeni, mutta insuliiniautovasta-aine (IAA) määritykseen liittyy ongelmia, esimerkiksi herkkyys jää usein heikoksi. Myös T-solumenetelmät autoreaktiivisten T-solujen, kuten insuliinispesifisten T-solujen, mittaamiseksi ovat osoittautuneet hankaliksi.

T1D:n geneettinen riski liittyy HLA luokka II:n geenialueeseen kuten monella muullakin autoimmuunitaudilla. Kuitenkin geneettinen riski muodostaa vain osan riskistä sairastua T1D:een: ympäristötekijöillä on myös tärkeä merkitys. Tutkituimmat T1D:n ympäristön riskitekijät ovat enterovirusinfektiot ja altistuminen lehmän maidolle varhaislapsuudessa. Molemmat näistä tekijöistä vaikuttavat suoliston puolustusjärjestelmään ja tutkimuksissa onkin enenevässä määrin todisteita viitaten siihen, että suoliston immuunijärjestelmä näyttelee osaa T1D:n kehittymisessä. Yksi hypoteesi on, että insuliinispesifinen immuunivaste kehittyy lehmän maidon naudan insuliinia kohtaan varhaislapsuudessa ja insuliinispesifinen immuunivaste myöhemmin laajenee kattamaan myös ihmisen insuliinin ja kehittyy aggressiiviseksi autoimmuunivasteeksi.

Tämän tutkimuksen tavoitteina oli tutkia parantaisiko immunoglobuliini (Ig) G:n erottaminen plasmasta IAA-menetelmän herkkyyttä ja selvittää kuinka insuliinihoito vaikuttaa soluvälitteiseen immuunivasteeseen insuliinia kohtaan juuri diagnosoiduilla potilailla. Lisäksi äidin rintamaidon insuliinipitoisuuden vaikutusta lapsen insuliinivasta-aineiden kehittymiseen tutkittiin. Myös ohutsuolen koepaloja kerättiin

T1D:stä sairastavilta lapsilta löytääksemme T1D:een liittyviä immunologisia muutoksia suolessa.

IgG:n erottaminen T1D-potilaiden, jotka olivat plasmaltaan IAA negatiivisia, plasmasta johti mitattaviin IAA -tasoihin, jotka olivat korkeammat kuin kontrollilapsilla. Siksi IgG:n eristäminen ja immuunikompleksien hajottaminen saattaisi parantaa IAA-menetelmän herkkyyttä.

Insuliinihoidon vaikutusta insuliinispesifisiin T-soluihin tutkittiin viljelemällä perifeerisen veren mononukleaarisoluja *in vitro* sekä ihmisen että naudan insuliinin kanssa. Insuliinistimulaatio *in vitro* aiheutti korkeamman säätelevien T-solujen merkkiaineiden ekspression lähetti-RNA-tasolla niillä potilailla, jotka olivat saaneet insuliinihoitoa kuin niillä, jotka tutkittiin ennen insuliinihoidon aloittamista. Insuliinin aiheuttama säätelevien T-solujen merkkiaineen Foxp3:n ilmentyminen osoitettiin myös valkuaisainetasolla. Tämä löydös viittaa siihen, että insuliinihoito T1D-potilailla stimuloi säätteleviä T-soluja *in vivo* ja tämä saattaa selittää osittain vaikeudet mitata autoantigeenispesifisiä T-soluvasteita juuri diagnosoiduilla potilailla. Insuliinihoidon aiheuttama säätelevien T-solujen stimulaatio saattaa myös selittää insuliinihoidon jälkeisen remissiovaiheen.

Kolmannessa työssä osoitimme, että äidinmaidon insuliinipitoisuus on koholla T1D:tä sairastavilla äideillä. Myös äidin rintamaidon insuliinipitoisuus korreloi käänteisesti naudan insuliinille spesifisiin vasta-aineisiin niillä lapsilla, jotka olivat ruokavaliossaan altistuneet lehmän maidon valkuaisaineille. Tämä löydös viittaa siihen, että ihmisen insuliini rintamaidossa saa aikaan sietokykyä ravinnon naudan insuliinia kohtaan. Kuitenkin lasten, jotka myöhemmin kehittivät T1D:een liittyviä autovasta-aineita, äitien rintamaidossa oli insuliinipitoisuus koholla. Tämä löydös saattaa tarkoittaa sitä, että lapsilla, jotka ovat alttiita autoimmuniteetille β-soluja kohtaan, rintamaidon insuliini ei saakaan aikaan sietokykyä, vaan voi jopa lisätä insuliinispesifistä immuunivastetta.

Ohutsuolen koepaloista kvantitoitiin useiden immunologisten merkkiaineiden, kuten erilaisten T-solujen merkkiaineiden, esiintymistä käänteistranskriptaasi-polymeraasiketjureaktiolla (RT-PCR). Näistä merkkiaineista interleukiini (IL) -18 sytokiinin ekspressio oli merkittävästi lisääntynyt suolessa T1D-potilailla verrattuna keliakiaa sairastaviin lapsiin ja kontrollilapsiin. Sen sijaan emme havainneet säätelevien T-solujen merkkiaineiden lisääntynyttä ilmentymistä T1D-potilaiden suolinäytteissä. Lisääntynyt IL-18:n ekspressio tuo lisätodisteen sen hypoteesin puolesta, että suolen immuunijärjestelmän aktivaatio liittyy T1D:n patogeneesiin, mutta säätelevien T-solujen aktivaatiota ei T1D:een liittyvässä suolen tulehduksessa nähdä.

Avainsanat: tyypin 1 diabetes, insuliini, säätelevät T-solut, suoli

CONTENTS

ABBREVIATIONS

CBA	cytometric bead array
C_T	threshold cycle
CTLA-4	cytotoxic T-lymphocyte antigen-4
ELISA	enzyme-linked immunosorbent assay
GAD	glutamate decarboxylase
GADA	glutamate decarboxylase autoantibody
HLA	human leukocyte antigen
IA-2	insulinoma-associated protein 2
IA-2A	insulinoma-associated protein 2 autoantibody
IAA	insulin autoantibody
ICA	islet cell antibody
ICOS	inducible co-stimulator
Ig	immunoglobulin
IEL	intraepithelial lymphocyte
IFN	interferon
IL	interleukin
NOD	non-obese diabetic
PBMC	peripheral blood mononuclear cell
RNA/mRNA	ribonucleic acid/messenger ribonucleic acid
RT-PCR	reverse transcriptase-polymerase chain reaction
T1D	type 1 diabetes
TGF	transforming growth factor
Th cell	helper T-cell
TRIGR	Trial to Reduce IDDM in the Genetically at Risk

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Tiittanen M., Knip M. and Vaarala O. Anti-insulin activity in IgG-fractions from children with newly-diagnosed type 1 diabetes and negative for insulin autoantibodies. *Autoimmunity* 37:45-49, 2004

II Tiittanen M., Huupponen JT., Knip M. and Vaarala O. Insulin treatment in patients with type 1 diabetes induces up-regulation of regulatory T-cell markers in peripheral blood mononuclear cells stimulated with insulin *in vitro*. *Diabetes* (December 2006), in press.

III Tiittanen M., Paronen J., Savilahti E., Virtanen SM., Ilonen J., Knip M., Åkerblom HK., Vaarala O. and the Finnish TRIGR Study Group. Dietary insulin as an immunogen and tolerogen. *Pediatr Allergy Immunol* 17:538-543, 2006

IV Tiittanen M., Westerholm-Ormio M., Verkasalo M., Savilahti E. and Vaarala O. Increased expression of IFN-γ in relation to regulatory T-cell marker Foxp3 in jejunal mucosa in celiac disease. (submitted)

1 INTRODUCTION

The first document referring to a medical state resembling diabetes mellitus dates back to 1552 BC in Egypt: the Ebers Papyrus. The first descriptions of sugar in the urine of patients were already found in Hindu medicine around 500 AD, but in 1674 T. Willis in England sparked new interest in diabetes by finding the sweet taste in the urine of patients with diabetes mellitus. In 1776 M. Dobson in England also found sugar in the blood of diabetic patients.

Minkowski and von Mering from the University of Strasbourg found in 1889 that removal of the pancreas from a dog results in diabetes. In 1908 treatment was attempted, using a substance extracted from the pancreas and injected into patients, causing reduced levels of sugar. In 1921 insulin was isolated from a dog´s pancreas and one year later insulin was used successfully to treat a diabetic teenager in Toronto. This medical achievement was recognized by granting the Nobel Prize to Banting and MacLeod in 1923, a year that also saw the beginning of commercial production of insulin.

In 1936 Himsworth suggested that diabetes mellitus falls into two types, and type 1 and type 2 diabetes have been formally recognized since 1979. (History of Diabetes adapted from http://www-unix.oit.umass.edu/~abhu000/diabetes/index.html) Type 1 diabetes (T1D), diagnosed during childhood or adolescence, comprises 5-10% of all diabetes mellitus cases. The autoimmune nature of T1D was proposed in 1965 by Gepts and further confirmed during the 1970s when susceptibility to the disease was associated with the human leukocyte antigen (HLA) gene region, and islet cell-specific autoantibodies and autoreactive T-cells were proven to exist. However, in the 21st century the pathogenesis of T1D remains unknown.

2 REVIEW OF THE LITERATURE

2.1 Epidemiology of type 1 diabetes

Diabetes is one of the most common chronic diseases in childhood and the prevalence of T1D is on the rise (Onkamo et al. 1999). The highest incidence of T1D in children ≤ 14 years of age is in Finland (Podar et al. 2001; Passa 2002). The incidence of T1D in Finland has increased since the mid-1960s by an average of 2.8% per year (Tuomilehto et al. 1995). For example, in 1965 the incidence was 18 per 100 000 children and increased to 48.5 per 100 000 children ≤ 14 years of age in 1998 (Karvonen et al. 1999; Podar et al. 2001).

There are geographical and ethnic differences in the incidence of T1D. A high incidence (> 20 per 100 000 per year in 1994) is found in Finland, Sardinia, Sweden, Norway, Portugal, the United Kingdom, Canada, and New Zealand (Karvonen et al. 2000). Moderately high incidences are found in the European countries and in the United States: e.g. the annual incidence in France was 9.3 per 100 000 in 1995 (Passa 2002) and about 14.8 per 100 000 in 1994 in the United States (Karvonen et al. 2000). In Eastern Europe the incidence is less than in other parts of Europe; e.g. the incidence in Estonia is 2-4 times less than in the Nordic countries (Finland, Sweden, Norway) (Karvonen et al. 2000; Podar et al. 2001). The lowest incidence of T1D (< 1 per 100 000 per year) is found in China and in some areas of South America (Karvonen et al. 2000). A moderately low incidence of T1D (1-10 per 100 000 per year) is found in Asia (e.g. in Japan the incidence is about 1.5 per 100 000 per year [Abiru et al. 2002]), in South America (Aschner 2002), and in Africa (Motala 2002; Motala et al. 2003). Ethnic differences in T1D risk are found in multiracial populations (Diabetes Epidemiology Research International Group 1988; Aschner 2002). For example,

in the United States the incidence of T1D is lower among African-Americans and Hispanics than in white non-Hispanics.

The incidence of T1D is globally increasing and the highest increase in incidence is in children under 5 years of age (Karvonen et al. 1999). In Europe the increase in incidence (from 1989 to 1994) in children 0-4 years of age was 6.3% while for those 5-9 or 10-14 years of age the increases were 3.1% and 2.4%, respectively (EURODIAB ACE Study Group 2000).

2.2 Classification of diabetes in children

The cause of T1D is a deficiency in insulin secretion and thus lifelong insulin treatment is needed in patients with T1D. According to current classifications T1D is divided into two subclasses: type 1A diabetes and a rare type 1B diabetes (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003; American Diabetes Association 2006). Type 1A diabetes results in cell-mediated autoimmune destruction of insulin-producing β-cells in the pancreatic islets. In these children the presence of peripheral autoantibodies is a marker of autoimmune destruction. Type 1A diabetes also has a strong HLA association. Type 1B diabetes is called idiopathic diabetes because the etiology of the β-cell destruction is not known. Patients with type 1B diabetes have no immunological evidence of β-cell autoimmunity and no HLA association. A Japanese group suggested that type 1B diabetes could be further divided into two groups (Imagawa et al. 2000a; Imagawa et al. 2000b). They refer to this novel type as fulminant T1D due to its very rapid onset. Usually the hyperglycemic symptoms last less than one week before the diagnosis. These patients have no autoimmune features in their serum or in the pancreatic islets. The fulminant type of T1D may comprise as much as 10% of the T1D patients in Japan but is very uncommon in caucasoid populations.

Immune-mediated T1D (type 1A) is the most common form of diabetes among children, comprising about 80% of cases worldwide (Rosenbloom et al. 1999). In addition to T1D there are some rare types of diabetes that are diagnosed during childhood or early adulthood (< 25 years of age). These forms are associated with monogenic defects in β-cell function (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003; American Diabetes Association 2006). Maturity-onset diabetes of the young (MODY) is characterized by impaired insulin secretion and divided into its own subtypes according to specific genetic mutations (Mitchell and Frayling 2002). Another form of diabetes caused by a monogenic defect in β-cells is associated with a mutation in mitochondrial deoxyribonucleic acid (DNA) that affects insulin secretion in β-cells (Reardon et al. 1992; Kadowaki et al. 1994). This form of diabetes is maternally transmitted and often associated with deafness. Mitochondrial diabetes is found both in children and in adult-onset patients and the prevalence is about 1.5% of the diabetic population (Gerbitz et al. 1995). Maturity-onset diabetes of the young (MODY) is found mainly among Caucasian populations but mitochondrial diabetes is more prevalent in the Far East where the incidence of autoimmune T1D is very low. Diabetes may in some rare cases also be due to a genetic defect in insulin activity (American Diabetes Association 2006). These cases are associated with mutation of the insulin receptor gene and the symptoms vary from moderate hyperglycemia to severe diabetes.

Type 2 diabetes, which results from insulin resistance with an insulin secretory defect, is known as a disease of adulthood, but recently the incidence of type 2 diabetes has also increased among children and adolescents (Arslanian 2002; Ehtisham and Barrett 2004). It was estimated that in 1994 type 2 diabetes accounted for up to 16% of all new diabetes cases in adolescents in the United States (Pinhas-Hamiel et al. 1996). Type 2 diabetes in youth is strongly associated with diet and

obesity and is much more frequent among particular ethnic groups such as African-Americans, Native Americans, Mexican-Americans, and Arabs.

2.3 Clinical features of autoimmune type 1 diabetes

2.3.1 Diagnostic criteria

The classic symptoms of diabetes include polyuria, polydipsia, and weight loss. If these symptoms are present, the plasma glucose concentration can be measured at any time of the day and a concentration ≥ 11.1 mmol/l is considered diabetic. If the glucose concentration is detected in fasting, a plasma sample of 7.0 mmol/l or over is diabetic. The diagnosis of diabetes can also be performed with an oral glucose tolerance test (OGTT) in which the glucose level should decline to under 11.1 mmol/l within 2 h, but this test is not recommended for diagnosis of children who usually have more severe symptoms and are prone to very high blood glucose values. For an asymptomatic person the diabetic hyperglycemia should be measured on two or more separate occasions (The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus 2003; American Diabetes Association 2006).

Patients who have autoimmune type 1A diabetes are usually diagnosed at a young age (<14 years) with acute onset and are at risk for ketoacidosis at diagnosis. These patients are rarely obese, in multiracial populations are usually white, and most have circulating autoantibodies against β-cell antigens. Their plasma C-peptide levels are low, referring to very low insulin secretion, and no evidence of insulin resistance can be found.

2.3.2 Autoimmunity

The autoimmune features of T1D were first suggested in 1965 when Gepts described insulitis in the pancreatic islets of young patients with T1D (Gepts 1965). The word insulitis is used for the infiltration of cells of the immune system into the pancreatic islets. The infiltrating cells involve CD8-positive cytotoxic T-cells, CD4-positive helper T-cells (Th cells), macrophages and monocytes, natural killer cells, and B-cells, of which the CD8-positive cells seem to predominate in the infiltrations (Bottazzo et al. 1985; Itoh et al. 1993). In 1974 the autoimmune nature of T1D became more evident when both autoantibodies and autoreactive T-cells against β-cells were shown to exist in the circulation of patients with T1D (Bottazzo et al. 1974; MacCuish et al. 1974a; MacCuish et al. 1974b).

T1D is considered to be a T-cell-mediated autoimmune disease. A proportion of the T-cells infiltrating the pancreatic islets are activated, as shown by increased expression of HLA class II molecules and interleukin (IL)-2 receptors on their surface (Bottazzo et al. 1985). Similarly, the islet cells themselves express increased amounts of HLA class I molecules (Bottazzo et al. 1985; Foulis et al. 1987; Itoh et al. 1993). HLA class I and class II molecules are those molecules that present the antigenic epitopes to T-cells and bind to their coreceptors on cytotoxic (CD8+) and on Th (CD4+) cells, respectively. Furthermore, the major gene loci associated with risk of T1D are located within the HLA gene region (Singal and Blajchman 1973; Nerup et al. 1974). The disease can be transferred from a diabetic individual to an unaffected individual by autoreactive cells, as shown in a case report on transfer of T1D between siblings by bone marrow transplantation (Lampeter et al. 1993). Immunosuppressive drugs, which affect specifically T-cells, have a delaying effect on the disease course (Stiller et al. 1984; Bougnères et al. 1988). Treatment with monoclonal antibody against CD3, a molecule expressed in almost all T-cells, in recently diagnosed patients also maintains or improves the patient's own insulin production for at least one year after the diagnosis (Herold et al. 2002). Most likely

the effect of this treatment is due to production of regulatory cells that are capable of suppressing autoreactive T-cells (reviewed by Harlan and von Herrath 2005). A report on the development of T1D in a patient with severe hereditary B-cell deficiency lends further support for the cellular immune response playing an important role (Martin et al. 2001).

Although B-cells are not necessarily required for the development of T1D, autoantibodies to pancreatic islet cells and to individual T1D-associated autoantigens are widely used for the prediction of the disease (Mueller et al. 2002; Kulmala 2003). Patients with recent-onset T1D show humoral immune responses to as many as 14 different β-cell molecules (Lieberman and DiLorenzo 2003). T-cell responses has been demonstrated to seven of these autoantigens which include insulin (MacCuish et al. 1975), glutamate decarboxylase (GAD) 65 (Atkinson et al. 1992), insulinoma-associated protein 2 (IA-2) (Durinovic-Bellò et al. 1996), heat-shock proteins 60 and 70 (Abulafia-Lapid et al. 1999; Abulafia-Lapid et al. 2003), islet cell autoantigen of 69 kDa (ICA69) (Roep et al. 1996), and transcription factor jun-B (Honeyman et al. 1993).

For the prediction of T1D only four assays are routinely used: islet cell antibodies (ICAs) are measured basically in the same way as reported in 1974 by using immunofluorescence labeling of frozen pancreatic sections (Bottazzo et al. 1974), insulin autoantibodies (IAAs) (Palmer et al. 1983), GAD autoantibodies (GADAs) (Baekkeskov et al. 1990; Rowley et al. 1992), and IA-2 autoantibodies (IA-2As) (Lan et al. 1996) are measured by radiobinding assays using radiolabeled antigens. In prediction a repeated appearance of multiple autoantibodies is associated with increased risk of T1D and if a subject who is positive for three or four autoantibodies carries the highest HLA risk genotype the risk of T1D is over 90% (Kulmala 2003).

2.4 Risk factors of type 1 diabetes

2.4.1 Genetic risk

More than 20 gene loci are associated with susceptibility to T1D (Maier and Wicker 2005). The most important locus defining the risk of T1D is located within the HLA class II gene region and the HLA class II region can be estimated to be responsible for up to 50% of the genetic risk (Noble et al. 1996). Among the other genes three loci, whose effects are relatively modest on T1D susceptibility, were well characterized: the insulin gene (Bell et al. 1984), cytotoxic T-lymphocyte antigen-4 (CTLA-4) gene (Nisticò et al. 1996) and PTPN22 gene (Bottini et al. 2004).

According to the current view, the major factors for the genetic risk of T1D are HLA-DQA1 and HLA-DQB1. These two loci are in linkage disequilibrium and in most cases the characterization of HLA-DQB1 genes is enough to estimate the risk genotype (Ilonen et al. 2002). However, the genetic risk associated with the HLA class II gene region is more complicated and in addition to HLA-DQ the HLA-DR alleles, among others, have a modifying effect on the risk.

The highest risk genotypes of T1D are DQA1*0301-DQB1*0302 (DQ8) and DQA1*0501-DQB1*0201 (DQ2) (Ilonen et al. 2002; Lambert et al. 2004). The highest risk is associated with the DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201 heterozygote. In addition to the risk alleles, three alleles in the HLA-DQ gene region protect against T1D. These are DQB1*0602, DQB1*0301, and DQB1*0603, of which the DQB1*0602 allele has the strongest protective effect.

The highest risk alleles, DQB1*0302 and/or DQB1*0201, are found in 90-95% of patients with T1D (Ilonen et al. 1996; Ilonen et al. 2002). These risk alleles are also common in the general population: approximately 40-45% of Finns carry one of these alleles and half of them have no protective alleles (Ilonen et

al. 1996; Ilonen et al. 2002). However, the risk of a Finnish newborn of developing T1D is less than 1% (in 1998 the risk was 0.54%) (Hyttinen et al. 2003) suggesting that environmental factors play a major role in the pathogenesis of T1D.

2.4.2 Environmental risk factors

2.4.2.1 Viruses

Several viruses have been connected with the risk of T1D but their role in pathogenesis has been difficult to prove. Congenital rubella infection leads to diabetes in about 20% of affected individuals and the virus is able to infect the fetal pancreas (Menser et al. 1978; Forrest et al. 2002). However, the rubella virus cannot be considered as a risk factor for T1D today, since rubella infections are almost nonexistent due to vaccination programs in developed countries. The same holds for the mumps virus which may also be associated with T1D (Kremer 1947) and which is able to infect human β-cells *in vitro* (Parkkonen et al. 1992). The other viruses occasionally connected with T1D include cytomegalovirus (Ward et al. 1979; Pak et al. 1988), Epstein-Barr virus (Nonaka et al. 1982) and retroviruses (Conrad et al. 1997), but the evidence for their role in the pathogenesis of T1D is scanty.

The most convincing candidates for viral triggers are the enteroviruses. Enteroviruses belong to the picornavirus family and comprise over 70 serotypes including polioviruses, coxsackieviruses A and B, echoviruses and several others (Lukashev 2005). Enteroviral infections are common during childhood and are transmitted through the fecal-oral route. The enteroviruses are resistant to acidic pH and bile and they pass through the stomach to the intestine. Viremic spread to other organs, such as the pancreas, can occur. *In vitro* studies showed that some enteroviruses are also able to infect β-cells (Yoon et al. 1978; Roivainen et al. 2000).

The first evidence for the association of enterovirus infection with T1D was in 1969 when it was shown that patients with recent-onset T1D had higher antibody titers to coxsackieviruses, particularly coxsackievirus B4, than unaffected subjects (Gamble et al. 1969). Coxsackievirus B4 was also isolated from the pancreas of a child who died from diabetic ketoacidosis and this virus was able to cause diabetes in a mouse strain (Yoon et al. 1979). In addition to the increased antibody response to coxsackieviruses, increased viral ribonucleic acid (RNA) in blood (Clements et al. 1995; Andréoletti et al. 1997) and cellular immune response to coxsackieviruses in patients with T1D were reported (Jones and Crosby 1996; Juhela et al. 2000).

In follow-up studies of children with increased risk of T1D, findings of the association of coxsackieviruses with T1D are conflicting. In Finnish children the occurrence of coxsackievirus infections was more frequent, both in pregnant mothers whose children later developed T1D and in the siblings of affected children who were diagnosed with T1D in a follow-up study (Hyöty et al. 1995). Furthermore, the increased enterovirus antibody levels were clustered in the sample at intervals in which β-cell-specific antibodies first appeared (Hiltunen et al. 1997). However, a large Finnish study on the association of enterovirus infection during pregnancy and T1D in offspring did not support the hypothesis that T1D is due to maternal enterovirus infection (Viskari et al. 2002). The Australian and the German BabyDiab studies and the American DAISY study, birth-cohorts, also could confirm no association between coxsackieviruses and T1D (Honeyman et al. 2000; Füchtenbusch et al. 2001; Graves et al. 2003). The Australian BabyDiab study found instead a connection between the appearance or increase of T1D-associated autoantibodies and seroconversion of another virus, rotavirus (Honeyman et al. 2000). The same research group also showed that rotavirus is able to infect animal pancreatic islets *in vitro* (Coulson et al. 2002), but no other group has been able to confirm the connection between rotavirus infection and T1D.

The hypothetic mechanisms by which the viruses could cause the destruction of pancreatic β-cells include both direct viral infection of β-cells, causing lytic destruction, and so-called bystander killing (reviewed by Filippi and von Herrath 2005). The bystander killing hypothesis suggests that viral infection near β-cells would cause a release of immune mediators such as cytokines and nitric oxide which are harmful to β-cells, and the release of self-antigens from damaged β-cells could lead to activation of autoreactive T-cells. A third theory is molecular mimicry in which a viral antigenic epitope shares similarity with the self-protein expressed in β-cells and the immune response to this viral epitope would spread to include the self-antigen. Molecular mimicry may be involved in the association between both coxsackievirus B4 and rotavirus and T1D. P2-C protein of coxsackievirus B4 shares an amino acid sequence similar to that of GAD (Kaufman et al. 1992) and the VP7 protein of rotavirus has similarity with both IA-2 and GAD (Honeyman et al. 1998).

2.4.2.2 Dietary factors

The first reports of the possible effect of a dietary component on the incidence of T1D were published in the early 1980s. In 1981 an Icelandic study suggested that the consumption of foods containing high amounts of N-nitrosocompounds, in this case smoked/cured mutton, at the time of conception was associated with the increased incidence of T1D in offspring (Helgason and Jonasson 1981). The effect of the smoked/cured mutton was later confirmed in an animal study (Helgason et al. 1982). N-nitrosocompounds are also formed from dietary nitrate and nitrite in the gut by reaction with amines and amides. Nitrates and nitrites are found in meat products as food additives and nitrates also in vegetables. In a Swedish case-control study the child´s consumption of foods containing N-nitrosoamines or nitrates or nitrites was associated with increased risk of T1D (Dahlquist et al. 1990). In a Finnish study the daily dietary intake of nitrite was also greater in children with T1D and their mothers compared with control children and their mothers (Virtanen et al. 1994). However, a case-control study from Canada could find no association

between the consumption of particular nitrite- and nitrate-containing meat products and the incidence of T1D (Siemiatycki et al. 1989).

In the 1980s it was shown that manipulation of the protein source in the diet of Bio-Breeding rats, one of the animal models of T1D, also affects the disease process. Elliott and Martin (1984) reduced the incidence of diabetes in Bio-Breeding rats from 50% to 15% by replacing whole proteins with amino acids. Furthermore, when 1% milk powder was added to the manipulated amino acid diet the incidence of diabetes was restored to 52%. Later the protective effect of the amino acid -based diet was also shown in another animal model of T1D, non-obese diabetic (NOD) mice (Elliott et al. 1988; Coleman et al. 1990). Elliott et al. (1988) showed that the addition of intact lactic casein in the amino acid -based diet increased the incidence of diabetes, but in the study of Coleman et al. (1990) intact casein or addition of skim milk to the diet did not have this diabetogenic effect. These animal studies and that of Borch-Johnsen et al. (1984), showing an inverse correlation between the duration of breast-feeding and the incidence of T1D, initiated studies on the association of cow's milk with the incidence of T1D.

Since the first report on the association of breast-feeding and T1D, several studies have been published, both for and against (reviewed by Virtanen and Knip 2003). It is worth mentioning that in a large Finnish multivariate analysis, all the association of T1D with the duration of breast-feeding was explained by its correlation with cow's milk exposure, suggesting that early exposure to cow's milk proteins, and not the protective effect of breast-feeding, is the pivotal factor (Virtanen et al. 1993). Two reports were published on the association of cow's milk consumption in the population with the incidence of T1D in several countries: both found a positive correlation (Scott 1990; Dahl-Jorgensen et al. 1991). However, these population-based case-control studies on cow's milk consumption are inconsistent. In a Swedish study the frequency of milk intake was lower in diabetic children (Dahlquist et al. 1990) but in the Australian and Finnish studies the consumption of cow's milk was

higher in children who developed T1D or in the Finnish study developed T1D-associated autoantibodies (Verge et al. 1994; Virtanen et al. 1998).

Three birth-cohorts, the American DAISY and both Australian and German BabyDiab studies, found no association between breast-feeding or the age of exposure to cow's milk with the development of T1D-associated autoantibodies (Norris et al. 1996; Couper et al. 1999; Hummel et al. 2000). In the Finnish DIPP study, however, infants who were exclusively breast-fed for at least 4 months or who were first exposed to cow's milk at 4 months of age or older had lower risk of developing IA-2As or all four autoantibodies measured (ICAs, IA-2As, GADAs and IAAs) (Kimpimäki et al. 2001a). However, in another study from the DIPP case-control cohort, no association with the duration of breast-feeding and β-cell autoimmunity was seen (Virtanen et al. 2006). Based on findings in the case-control studies, an intervention study for primary prevention of T1D, the TRIGR (Trial to Reduce IDDM in the Genetically at Risk) study, has been initiated. The TRIGR pilot study showed that children with increased risk for T1D (children having a relative with T1D and a risk HLA genotype) generated somewhat lower amounts of T1D-associated autoantibodies if they had received casein hydrolysate -based infant formula in early childhood instead of conventional formula containing intact cow's milk proteins (Åkerblom et al. 2005).

Furthermore, it was shown that children with T1D in Finland, Sweden, and Hungary have increased levels of antibodies to cow's milk and to β-lactoglobulin, a protein included in cow's milk but not in human milk (Savilahti et al. 1988; Dahlquist et al. 1992; Saukkonen et al. 1996). The cellular immune response to the cow's milk proteins β-lactoglobulin and β-casein is also increased in patients with newly diagnosed T1D (Cavallo et al. 1996; Vaarala et al. 1996). These findings may be due to either altered consumption of cow's milk or altered immunological response to cow's milk proteins, or increased permeability of the intestine to dietary proteins in children who develop T1D.

The other dietary factors studied in association with T1D include, among others, vitamin D deficiency and exposure to some plant proteins such as cereal gluten. Treatment with the active form of vitamin D, 1,25 dihydroxyvitamin D_3, can prevent diabetes in NOD mice (Mathieu et al. 1992; Mathieu et al. 1994). It was also reported that adequate vitamin D supplementation in infancy is associated with decreased frequency of T1D both in a multicenter trial involving seven European centers and in a Finnish birth-cohort (The EURODIAB Substudy 2 Study Group 1999; Hyppönen et al. 2001).

Gluten, a protein found in wheat, rye, and barley, is the target protein in celiac disease (reviewed by Jabri and Sollid 2006). An increased prevalence of celiac disease in children with T1D was reported (Mäki et al. 1984; Savilahti et al. 1986; Not et al. 2001) and this may be caused partly by the association of celiac disease with the HLA-DQB1*02 genotype which is the other risk allele for T1D. A wheat-based diet can be diabetogenic in rodent models of autoimmune diabetes and in a multicenter trial involving both Bio-Breeding rats and NOD mice the highest incidence of diabetes occurred in rodents fed with a milk-free, wheat-based diet (Beales et al. 2002). Catassi et al. (1987) showed as early as in the 1980s the presence of an increased celiac disease -associated antibody titer in children with recent-onset T1D. Similar findings of increased humoral immune response to celiac disease -associated antigens have also been published since (Bao et al. 1999; Lampasona et al. 1999). Furthermore, patients with newly diagnosed T1D have enhanced cellular immune response to wheat gluten (Klemetti et al. 1998) and patients with T1D have increased antibody response to the wheat storage protein, Glb1 (MacFarlane et al. 2003). In a German birth-cohort, exposure to gluten-containing food before the age of 3 months was associated with the development of β-cell autoantibodies (Ziegler et al. 2003) and in the American DAISY study too early (before 4 months of age) and too late (7 months or older) exposures to cereals were associated with an increased risk of β-cell autoimmunity (Norris et al. 2003).

Several mechanisms by which dietary components could affect the pathogenesis of T1D have been proposed. The regulation of the gut immune system and oral tolerance may be disturbed in patients with T1D and this hypothesis would fit with the enhanced immune response to dietary proteins found in cow's milk and cereal. We have also proposed a hypothesis that exposure to cow's milk could lead to immunization to bovine insulin. Bovine insulin differs from human insulin by only three amino acids and the immune response to insulin could spread to involve human insulin as well (Vaarala et al. 1998). It cannot be ruled out that the various environmental factors could interact, e.g. breast-feeding could protect from enteric infections as well as enteric infections could increase the immunity to dietary antigens by changing the mucosal immunity. It was shown that children who had cellular immune responses to enteroviruses at 3 months of age had higher humoral immune responses to bovine insulin at 6 and 9 months of age (Vaarala et al. 2002). Vitamin D affects immune cells (Rigby 1988) and reduced levels of vitamin D may enhance anti-viral T-cell responses. In one study Dahlquist et al. (1991) also showed that the frequency of infections and a high intake of foods containing nitrosoamines tended to interact.

2.5 Role of the gut immune system in type 1 diabetes

The most studied environmental factors associated with T1D, enteroviruses and diet, both affect through gut. Gut-associated lymphoid tissue (GALT) is the largest part of the entire immune system. It encounters huge amounts of various nonself particles derived from the diet and microorganisms all the time and its major role is to distinguish between advantageous and harmful nonself molecules. The gut-associated lymphoid tissue (GALT) achieves oral tolerance to useful dietary components and to favorable microflora but still develops a proper immune response

against harmful toxins and pathogens (reviewed by Dubois et al. 2005; Macdonald and Monteleone 2005).

Evidence supporting the view that disturbances in the gut immune system are involved in the pathogenesis of T1D is increasing. As mentioned earlier the diet influences the incidence of autoimmune diabetes in animal models of T1D (Elliott and Martin 1984; Elliott et al. 1988; Coleman et al. 1990). Furthermore gluten-containing, diabetes-promoting diets result in reduced villous height and increased infiltration of T-cells in the small intestine of NOD mice (Maurano et al. 2005) and increased proportions of Th1 cells in the mesenteric lymph nodes of Bio-Breeding rats (Chakir et al. 2005). However, it was also shown that enteropathy, as assessed by increased crypt length, increased proliferative activity of crypt epithelial cells, and increased number of lymphocytes in the mucosae, is present in Bio-Breeding rats soon after weaning despite the consistency of the diet (Graham et al. 2004). Furthermore, the development of insulitis and diabetes can be prevented by early oral administration of probiotics (nonpathogenic, favorable bacteria able to prevent colonization of harmful bacteria) in NOD mice (Calcinaro et al. 2005).

A significant amount of the lymphocytes infiltrating the pancreatic islets express α4β7-integrin, a gut-homing receptor, and also its counterreceptor, mucosal addressin cell adhesion molecule-1 (MAdCAM-1), in the vascular endothelium within the inflamed pancreatic islets in NOD mice (Hänninen et al. 1993; Faveeuw et al. 1994; Hänninen et al. 1996). The development of insulitis and diabetes can be protected by monoclonal antibodies to mucosal addressin cell adhesion molecule-1 (MAdCAM-1) and to the β7 chain (Yang et al. 1997; Hänninen et al. 1998), suggesting that in NOD mice the pathogenesis of diabetes involves the adhesion molecules needed for the migration of lymphocytes into the mucosal lymphoid tissues. Recently, Turley et al. (2005) showed in mice that intraperitoneally injected splenocytes and particulate antigen accumulate in the pancreatic lymph nodes suggesting that antigens and immune cells escaping from the gut can easily reach

this site. The transverse colon was also shown to drain through the pancreatic lymph nodes (Carter and Collins 1974). Through this routing, immunological activation in the gut may modify the presentation of pancreatic self-antigens in the pancreatic lymph nodes as well.

In human studies, as already mentioned, peripheral blood mononuclear cells (PBMCs) of patients with T1D show increased humoral and cellular immune responses to several dietary proteins from cow's milk (Savilahti et al. 1988; Dahlquist et al. 1992; Cavallo et al. 1996; Saukkonen et al. 1996; Vaarala et al. 1996) and from wheat (Catassi et al. 1987; Klemetti et al. 1998; MacFarlane et al. 2003). The densities of T-cells in the intraepithelium and CD25-positive cells in the lamina propria are increased in response to gliadin, a solvent-extracted protein of gluten, in *in vitro* cultures of small intestinal biopsies from patients with T1D (Auricchio et al. 2004). Auricchio et al. (2004) also showed changes in intraepithelial T-cell and lamina propria CD25-positive mononuclear cell densities in patients with T1D with no stimulation. Sblattero et al. (2006) were able to isolate a lymphocyte clone recognizing a celiac disease-associated antigen from a small intestinal biopsy of a patient with T1D and with no signs of celiac disease. The PBMCs of this patient did not recognize the same celiac disease -specific antigen.

In patients with T1D a remarkable proportion of the PBMCs proliferating in response to GAD expresses the gut-homing receptor $\alpha 4\beta 7$-integrin (Paronen et al. 1997), suggesting that the autoantigen-reactive lymphocytes originate from the gut. Increased intestinal permeability and ultrastructural changes in microvilli also appear to be associated with T1D (Carratù et al. 1999; Secondulfo et al. 2004), which may at least partly explain the increased immune response to dietary proteins in patients with T1D. Recently it was shown that increased levels of serum zonulin were associated with increased intestinal permeability in a subgroup of patients with T1D (Sapone et al. 2006). Zonulin is a protein that regulates intercellular tight junctions, thus playing a role in intestinal permeability, and whose levels are up-

regulated in the gut of Bio-Breeding rats (Watts et al. 2005). Furthermore, T1D-associated increase in the expression of HLA, IL-4, IL-1α, and matrix metalloproteinases -1, -3, and -12 by immunohistochemical staining and/or at the messenger RNA (mRNA) level was found in small intestinal biopsies showing derangements in the gut in patients with T1D (Savilahti et al. 1999; Westerholm-Ormio et al. 2003; Bister et al. 2005).

2.6 Insulin as an autoantigen in type 1 diabetes

2.6.1 Humoral immune response to insulin

Insulin is one of the major autoantigens of T1D and has some unique features compared with other autoantigens. Insulin is the major product of pancreatic islet β-cells, which are the specific target of autoimmune destruction. Insulin is the only T1D-associated autoantigen that is exclusively expressed in the β-cells, with the exception of self-antigen -expressing cells in lymphoid tissues such as the thymus, where insulin is expressed at low levels without hormonal importance (Pugliese et al. 2001). The other autoantigens are expressed in other islet cells and in other tissues in addition to the β-cells. Insulin is secreted into the bloodstream and is a ubiquitous antigen in this sense.

In NOD mice over 50% of the T-cell lines obtained from infiltrated islets recognize insulin (Wegmann et al. 1994) and these insulin-specific T-cells can transfer the disease to the recipient mouse (Daniel et al. 1995). In an international workshop on animal models of T1D, it was reported that IAAs are a marker of autoimmunity in NOD mice but the other autoantibody assays, GADAs and IA-2As, are questionable as markers (Bonifacio et al. 2001). It was also shown that if both native forms of insulin are knocked out from NOD mice and compensated for with a mutated form of insulin,

that is not a target of autoreactive cells, the disease is avoided (Nakayama et al. 2005). These studies suggest that insulin is the primary autoantigen in NOD mice.

In humans insulin was the first autoantigen identified to which autoantibodies were proven to exist (Palmer et al. 1983). In birth-cohort studies, IAAs are quite often the first autoantibodies to appear, especially in children who show signs of autoimmunity at a young age (Ziegler et al. 1999; Yu et al. 2000; Kimpimäki et al. 2001b). The age at onset of clinical T1D correlates inversely with IAA titers (Arslanian et al. 1985; Vardi et al. 1988). In addition, the IAA level sometimes fluctuates during the follow-up: after appearance the IAA level usually rises and later declines, which does not commonly occur in other autoantibodies (Ziegler et al. 1999). Further support for the essential role of insulin in the pathogenesis of T1D was published recently by Kent et al. (2005), who showed that T-cell clones isolated from lymph nodes near the pancreatic islets of patients with T1D recognize a particular insulin epitope (A-chain amino acids 1-15). This was the first study in which it was possible to culture T-cells from the target tissue lymph node instead of PBMCs.

In addition to IAAs to endogenous insulin, insulin treatment in patients induces antibodies to exogenous insulin (Berson et al. 1956). IAAs are used as a marker for T1D and insulin antibodies should be distinguished from these. Insulin from different animal species induces insulin antibodies in humans differently and bovine insulin is more immunogenic than for example porcine or human insulin (Kurtz et al. 1980; Reeves and Kelly 1982; Schernthaner 1993). Bovine insulin differs from human insulin by only three amino acids and was used for treatment before the availability of recombinant human insulin. However, exogenous recombinant human insulin also induces insulin antibodies (Fineberg et al. 1983; Schernthaner et al. 1983).

Both IAAs and insulin antibodies are polyclonal and express a range of fine specificities (Potter and Wilkin 2000). IAAs are one of the four autoantibody assays commonly used as a marker for autoimmune destruction, but IAAs also include non-T1D-related specificities. IAAs have been measured with both the liquid-phase

radiobinding assay and solid-phase enzyme-linked immunosorbent assay (ELISA), of which the radiobinding assay is more useful in detecting T1D-associated autoantibodies (Bingley et al. 2003; Bingley and Williams 2004). A microassay (radiobinding assay) is widely used for detecting IAAs (Williams et al. 1997) and its disease specificity is rather good. But the IAA assay has poor sensitivity among the four autoantibody assays: in the Diabetes Antibody Standardization Program the sensitivity of the IAA assay in 23 laboratories ranged from 13-66% (Bingley et al. 2003). Attemps were made to improve the specificity of the IAA assay by measuring the affinity of IAAs in competitive assay: children who are at high risk for T1D have high-affinity IAAs (Schlosser et al. 2005). Another way to improve the IAA assay could be use of the T1D-specific epitope of insulin in the radiobinding assay, but the finding of such an epitope has proven difficult.

2.6.2 Insulin-specific T-cell response

As already mentioned in chapter 2.3.2, T1D is a T-cell-mediated disease. T-cells can be divided into Th cells that express the CD4 receptor, and into cytotoxic T-cells that express CD8. Th cells are further classified based on their cytokine secretion: Th1 cells secrete high levels of interferon (IFN)-γ and IL-2 and provide help for cytotoxic immune responses, and Th2 cells secrete more IL-4, IL-13, and IL-5 and help the humoral immune response (Seder and Paul 1994; Mosmann and Sad 1996). Typically, the Th1 type of cytokine environment down-regulates the Th2 response and vice versa. T1D is considered to be Th1-mediated at the level of the target organ, where CD8 cells are believed to mediate β-cell destruction.

Recently, a novel Th cell lineage was characterized: Th17 cells (Weaver et al. 2006). The development of Th17 cells differs from that of effector Th1 and Th2 cells and from regulatory T-cells. Th17 cells produce IL-17 and IL-6 and their development is dependent on the presence of transforming growth factor (TGF)-β.

In addition to Th1, Th2 and Th17 cells there are different types of regulatory T-cells circulating in the periphery. Thymus-derived, self-antigen-recognizing CD4+CD25^{high} regulatory T-cells express specifically Foxp3 transcription factor, which probably plays an essential role in the development and function of regulatory T-cells (Fontenot et al. 2003; Khattri et al. 2003; Yagi et al. 2004). These regulatory T-cells also constantly express CTLA-4 and glucocorticoid-inducible tumor necrosis factor receptor (GITR). These CD4+CD25^{high} regulatory T-cells express at least the membrane-bound form of TGF-β (reviewed by Fontenot and Rudensky 2005; Sakaguchi 2005). In humans Foxp3-expressing CD4+CD25^{high} regulatory T-cells can be induced from peripheral CD4+CD25- cells on stimulation (Walker et al. 2003; Rao et al. 2005). In addition to these CD4+CD25^{high} regulatory T-cells, there are Th3 and Tr1 types of cells that are able to suppress T-cell responses in the periphery (Cottrez and Groux 2004). Th3 cells are characterized by their secretion of IL-4 and TGF-β and Tr1 cells by their secretion of IL-10. Peripherally induced regulatory cells are distinct from the thymus-derived CD4+CD25^{high}Foxp3+ regulatory T-cells in their specificity to foreign exogenous antigens.

Although T1D is considered as a T-cell mediated disease, the only methods available for measuring possible β-cell damage are based on autoantibodies. There are as yet no generalized widespread autoantigen-specific T-cell assays used for the prediction of T1D as the autoantibody assays. Insulin-specific autoreactive T-cells in patients with T1D and at risk for T1D have been studied since 1975 (MacCuish et al. 1975). MacCuish et al. used the T-cell proliferation test and found differences between the patients and unaffected subjects, although not all unaffected subjects were negative and the stimulation indices were usually low. It was recently proposed that patients with T1D and unaffected subjects differ in the quality of their T-cell responses to islet autoantigens: the patients show polarization to the Th1 phenotype by secreting IFN-γ and healthy subjects show regulatory T-cell phenotype by secreting both IFN-γ and IL-10 in response to islet-derived peptides (Arif et al. 2004).

Animal insulins used as treatment also result in higher T-cell proliferation responses to exogenous insulin than human insulin in a manner similar to that of insulin antibodies (Naquet et al. 1988). However, the humoral immune response to insulin and T-cell response to insulin usually do not correlate (Kurtz et al. 1985; Keller 1990; Mayer et al. 1999). An inverse correlation was also reported (Schloot et al. 1997; Dubois-LaForgue et al. 1999).

In addition to T-cell proliferation tests, several T-cell methods have been tested in the study of insulin-reactive T-cells in peripheral blood. Insulin-specific T-cell lines and a T-cell clone were cultured from PBMCs of patients with T1D (Miller et al. 1987; Schloot et al. 1998; Semana et al. 1999; Alleva et al. 2001). One study group examined the cellular responses to bovine and porcine insulin using a cytotoxicity assay (Richens et al. 1986). An enzyme-linked immunospot (ELISPOT) assay was one of the methods used in attempts to compensate for the difficulties in autoantigen-specific T-cell assays associated with T1D and several studies of insulin- or insulin-peptide-specific enzyme-linked immunospot (ELISPOT) assays were published (Karlsson et al. 2000; Alleva et al. 2001; Arif et al. 2004). One of the novel methods used to find rare insulin-specific T-cells among PBMCs is to make insulin-peptide HLA-tetramers and detect tetramer binding to T-cells by means of flow cytometry (Öling et al. 2005).

However, in most of these insulin-specific T-cell assays the response is not T1D-specific since the unaffected controls also show cellular responses to insulin. Maybe one of the major problems is that the T-cell assays can use only PBMCs and there is no access to the target tissue of T1D. Usually the frequency of antigen-specific T-cells in the circulation is between 1:10 000 and 1:1 000 000 and most likely the proportion of autoantigen-specific T-cells is extremely low. In addition, finding the appropriate form of insulin (from which species, whole insulin or A- and B-chain or proinsulin, in which concentration, etc.) or even the T1D-associated epitope of insulin has been difficult and not yet accomplished.

3 AIMS OF THE STUDY

The aim was to characterize the insulin-specific immune responses in children newly diagnosed with T1D both before and after initiating insulin treatment, to measure the insulin-specific immune response in relation to mother's breast milk insulin content in suckling infants at risk for T1D, and to characterize the immunological changes in the small intestine of patients with T1D. The detailed aim of each publication was as follows:

I The aim of the first publication was to determine whether the formation of insulin-insulin antibody immune complexes could be the reason for the fluctuations in serum IAA levels and poor sensitivity of IAAs.

II In the second publication the effect of insulin treatment on the cellular immune response to insulin was detected both at the effector T-cell and regulatory T-cell levels.

III Since dietary bovine insulin in cow's milk induces immune responses to insulin in small infants, we studied whether a high insulin concentration in mother's breast milk would correlate with reduced humoral insulin-specific immune responses in the infant.

IV In the fourth publication the aim was to characterize the immunological changes in small intestinal biopsies of patients with T1D by screening for common markers of inflammation, effector and regulatory T-cell markers, and markers produced by antigen-presenting cells.

4 SUBJECTS AND METHODS

4.1 Subjects

Publication I

Plasma samples derived from Ficoll-Paque density gradient centrifugation were used in the study. The study included 23 patients with recently diagnosed T1D from the Hospital for Children and Adolescents, Helsinki and from the Jorvi Hospital, Espoo, Finland. The study protocol was approved by the ethics committees of the participating hospitals. A total of 21 of the patients were studied within 10 days after the diagnosis of T1D and two patients 3 weeks after the diagnosis.

In all, 17 patients diagnosed within 10 days after the diagnosis showed negative IAA levels in the plasma and their mean age was 8.5 years (range 1.6 - 12.9 years). Of these newly diagnosed children, 14 tested negative for plasma GADAs and their mean age was 7.7 years (range 0.8 - 13.5 years).

The study included 23 unaffected children without acute infections or autoimmune diseases from the Hospital for Children and Adolescents. All these control children were negative for IAAs and their mean age was 7.3 years (range 1.6 - 14.7 years). 21 of the control children tested negative for GADAs and the mean age of this subgroup of unaffected children was 7.1 years (range 1.6 - 14.7 years).

Publication II

The PBMC samples for the reverse transcriptase-polymerase chain reaction (RT-PCR), for cytometric bead array (CBA) measurements, and for T-cell proliferation tests were taken from 33 newly diagnosed patients with T1D. The blood sample from 12 of these patients was taken at diagnosis before starting exogenous insulin therapy and the mean age in this group 0 was 7.1 years (range 1.9 - 12.5 years). In

all, 21 patients had been treated with insulin for 1-21 days at the time of sampling and this group 1 had a mean age of 7.7 years (range 1.8 - 14.4 years). Ten unaffected children undergoing minor surgery at the Hospital for Children and Adolescents were studied as controls and their mean age was 6.5 years (range 1.2 - 15.8 years).

The PBMC samples for flow cytometry included 12 children with newly diagnosed T1D and four unaffected controls. Seven patients were in group 0, before insulin treatment, and the mean age was 6.5 years (range 1.1 - 12.8 years). Five patients were treated with insulin (group 1) and their mean age was 7.5 years (range 3.6- 13.2 years). The four control children had a mean age of 7.1 years (range 3.5 - 10.1 years).

All the patient samples were from the Hospital for Children and Adolescents, Helsinki and from the Jorvi Hospital, Espoo, Finland. The ethics committees of the hospitals had given approvals for the study.

Publication III

This study included a subgroup of 128 children participating in the TRIGR pilot study. In the TRIGR intervention trial all the children had T1D-associated HLA risk alleles (HLA-DQB1*0302 and/or DQB1*0201) without protective alleles (DQB1*0301, *0602, *0603) and had a first-degree relative with T1D (mother, father, or a sibling). The children were randomized into two groups: after exclusive breast-feeding, the children received either study formula based on hydrolyzed casein (Nutramigen™, Mead Johnson, Evansville, IL, USA) or a regular cow's milk-based infant formula (Enfamil™, Mead Johnson) comprising 80% Enfamil™ and 20% Nutramigen™ during the first 6-8 months of life. The children avoided all cow's milk or cow's meat-containing food during the intervention period until 6-8 months of age. The children were invited to give a blood sample at 3, 6, 9, 12, 24, 36, 48, and 60 months of age during a mean observation period of 4.7 years and the occurrences of ICAs, IAAs, GADAs, and IA-2As were screened (detailed

description of TRIGR pilot study by Åkerblom et al. 2005). Insulin antibodies were measured from the 3-, 6-, 9-, and 12-month plasma samples. From the mothers of TRIGR children in this study breast milk samples were collected at 3-7 days and/or 3 months after delivery (95 samples at 3-7 days and 106 at 3 months).

Publication IV

Small intestinal biopsies from the distal duodenum were obtained from 65 pediatric patients at the Hospital for Children and Adolescents, Helsinki, Finland. The patients were divided into six groups covering two diseases: T1D and celiac disease. Of these patients, 29 children had T1D and were divided into three groups: 12 patients with active celiac disease at the time of sampling (mean age 7.7 years, range 3.3 - 14.3 years), eight T1D patients with normal villous structure but elevated density of intraepithelial lymphocytes (IELs) and/or presence of celiac disease-associated autoantibodies indicating potential celiac disease (mean age 8.1 years, range 4.5 - 15.0 years), and nine patients with normal villous structure and normal density of IELs (mean age 10.6 years, range 3.5 - 18.2 years). The small intestinal biopsy was taken due to positive findings in the annual celiac disease screening test, including immunoglobulin (Ig)A and IgG gliadin, IgA endomysium and tissue-transglutaminase antibodies, or to gastrointestinal symptoms.

In addition to children with T1D, 15 patients with active, untreated celiac disease were studied (mean age 7.0 years, range 1.2 - 12.6 years) and seven children with increased density of IELs and/or celiac disease-associated antibodies indicating potential celiac disease (mean age 8.9 years, range 4.0 - 15.2 years). Furthermore, 14 age-matched children without autoimmune diseases were studied as a control group (mean age 6.2 years, range 1.1 - 14.1 years). The biopsies were taken for growth retardation, gastrointestinal symptoms, positive anti-gliadin antibodies, or any combination of these. The morphology of the jejunum was normal and the endomysium and tissue transglutaminase antibodies were negative in all control children.

The use of biopsy specimens for the study was approved by the ethics committee of the Hospital for Children and Adolescents. Written consent was signed by the parents of the children.

4.2 Methods

Table 1 presents which procedures and methods were used in each publication. The methods are explained shortly in this chapter but the detailed descriptions are printed or referred to in the original publications. All the antibodies used are listed in Table 2.

Separation of IgG fraction

IgG was isolated from plasma, using protein A affinity column (Pharmacia Biotech, Uppsala, Sweden). The IgG fraction was eluted from the column with citric acid pH 3.9, neutralized, and concentrated with an ultrafiltration cell using a membrane with a molecular weight cutoff of 10 000 D. The total concentration of IgG was measured from the IgG fractions and the corresponding plasmas with an automated clinical chemistry analyzer. Both the IgG fraction and the corresponding plasma were diluted to the same IgG concentration which corresponded to the concentration of the plasma diluted 1:2.

IAAs, GADAs, IA-2As, and ICAs

The autoantibodies associated with T1D were measured in the laboratory of Professor Mikael Knip. The methods are referred to in the original publications.

Table 1. Procedures and methods

Procedure	Method	Publication	Reference
Separation of IgG fraction	Protein A affinity chromatography	I	I
Insulin autoantibodies (IAAs)	Radioimmunoassay	I, II, III	Ronkainen et al. 2001
GAD autoantibodies (GADAs)	Radioimmunoassay	I, III	Kulmala et al. 1998
Stimulation of PBMCs	Cell culturing	II	II
Quantitative measurement of mRNAs	Quantitative RT-PCR	II, IV	II, IV
Detection of secreted cytokines	Cytometric bead array (CBA) kit	II	*Becton Dickinson
Detection of Foxp3+ PBMCs	Flow cytometry	II	Roncador et al. 2005
Detection of antigen-specific T-cells	T-cell proliferation test	II	II
Insulin antibodies	ELISA	III	Vaarala et al. 1999
Measuring insulin concentration	ELISA kit	III	*DRG Diagnostics
Detection of Foxp3+ and CD25+ cells in gut biopsies	Immunohisto-chemistry	IV	IV

* According to the manufacturer's instructions

PBMC culture

PBMCs were isolated from heparinized blood with Ficoll-Paque density gradient centrifugation and cultured for 72 hours in U-bottomed 96-well cell culture plates at 2 x 10^5 cells per well in triplicate wells with or without antigens. Tetanus toxoid 20

μg/ml, bovine insulin 300 μg/ml, and human insulin 300 μg/ml were used as antigens. The supernatants were collected for the detection of secreted cytokines, using a CBA, and the cells were collected for the study of mRNA expressions, using quantitative RT-PCR.

Quantitative RT-PCR

Total RNA was extracted from the samples with a commercial kit (GenElute Mammalian Total RNA Miniprep kit, Sigma, St. Louis, MO, USA) and reverse transcription reaction was performed. A quantitative RT-PCR was carried out with commercial primers and probes, using an ABI-Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) in triplicate wells. 18S RNA was used as an endogenous control. PBMCs phytohemagglutinin-stimulated for 48 hours were used as a calibrator sample that was included in every plate. The quantities of the markers were analyzed with a comparative threshold cycle (C_T) method and were presented as relative amounts ($2^{-\Delta\Delta Ct}$): ΔC_T is calculated by subtracting the C_T value of the 18S gene from the C_T value of the marker gene, whereas $\Delta\Delta C_T$ is the difference between the ΔC_T of the analyzed sample and the ΔC_T of the calibrator. Calculation of $2^{-\Delta\Delta Ct}$ then gives a relative amount of the analyzed sample compared with the calibrator.

Cytometric bead array (CBA)

Secreted IFN-γ, IL-5, IL-4, IL-2, and IL-10 were measured with a flow cytometry-based CBA kit (BD Pharmingen, San Diego, CA, USA). The kit is based on the use of beads that are coated with capture antibodies for the cytokines and the beads for each cytokine are distinguished from each other by different intensities of a fluorescence label. After the beads are incubated with the sample supernatants and the cytokines allowed to attach to the antibodies, a detection antibody labeled with

another fluorescence label is added to form a sandwich complex around the cytokine. The intensity of the fluorescence from the capture antibody is proportional to the concentration of the cytokine in the sample.

Table 2. Antibodies used in the detection of IgG concentration (I), flow cytometry (II), insulin antibody ELISA (III), and immunohistochemical stainings (IV).

Antibodies	Conjugate	Source (clone)	Publication
rabbit anti-human IgG	-	Dako	I
mouse anti-human Foxp3	-	Gift from A.H. Banham,UK (150D/E4)	II
goat anti-mouse IgG	Alexa 488	Molecular Probes	II
mouse anti-human CD4	PerCP	Becton Dickinson	II
mouse anti-human CD25	PE	Miltenyi Biotech	II
rabbit anti-human IgG	Alkaline phosphatase	Jackson ImmunoResearch	III
mouse anti-human Foxp3	-	Abcam (236A/E7)	IV
mouse anti-human CD25	-	Dako (ACT-1)	IV

Detection of Foxp3 by flow cytometry

Ficoll-Paque-isolated PBMCs were cultured with or without antigens for 5-6 days in a 24-well cell culture plate at 2 x 10^6 cells per well before staining for flow cytometry. The Foxp3 staining was adapted from a paper by Roncador et al. (2005). The cells were fixed, permeabilized, and treated with DNAse, since Foxp3 is a nuclear protein. The cells were blocked with goat IgG before staining with unlabeled mouse anti-human Foxp3 antibody and Alexa fluor 488-labeled goat anti-mouse IgG was used as a secondary antibody. The cells were still blocked with mouse IgG before staining with labeled mouse anti-human CD4-PerCP and CD25-PE antibodies. The cells were analyzed with FACSCalibur and CellQuest Pro software (Becton Dickinson, San Jose, CA, USA).

T-cell proliferation test

PBMCs isolated with Ficoll-Paque density gradient centrifugation were cultured in U-bottomed 96-well cell culture plates at 1 x 10^5 cells per well in quadruplicate wells with or without antigen. After incubation for 5 days, 1 μCi of tritiated thymidine was added to each well and the cells were harvested 16 - 18 h later to measure the incorporation of radioactivity. Stimulation indices were calculated by dividing the median counts per minute measured from the antigen-stimulated wells by the median counts per minute detected in the wells cultured without antigen.

Insulin antibody ELISA

Insulin-specific antibodies in plasma were measured with a homemade ELISA coated with bovine or human insulin. Human serum albumin was used for residual coating. Alkaline-phosphatase-conjugated rabbit anti-human IgG was used as a secondary antibody.

Procedure to measure insulin concentration

Breast milk samples were centrifuged at 10 000 x g for 30 minutes before measuring the insulin concentration. After centrifugation both cellular debris and the fat layer on the top were discarded. The clear middle layer was used for the analyses. A commercially available ELISA kit was used for the determination of insulin concentration (DRG Diagnostics, Germany).

Immunohistochemistry

Paraffin sections of small intestinal biopsies were used for immunohistochemical staining of Foxp3 and CD25. The slides were first deparaffinized and antigen retrieval was performed using microwave cooking in citrate buffer (pH 6.0) for Foxp3 and in 2mM ethylenediaminetetraacetic acid and 50 mM Tris (pH 9.0) for CD25. Endogenous peroxidase activity was quenched with 0.5% H_2O_2 in methanol. The slides were blocked with horse serum before staining with primary monoclonal mouse anti-human antibodies. The bound antibodies were detected with an avidin-biotin immunoperoxidase kit (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin. The calculated cell densities were expressed as the mean number of positively stained cells/mm^2 in the lamina propria.

Statistical analyses

The non-parametric Kruskal-Wallis test and Mann-Whitney U-test were used for comparisons between the study groups. The spearman rank correlation test was used to analyze correlations between different parameters. Two-tailed p-values < 0.05 were considered significant (SPSS 10.0 for Windows, SPSS Inc, Chicago, IL, USA).

5 RESULTS

5.1 Insulin autoantibody levels in IgG fractions of children with recent-onset type 1 diabetes and negative for plasma insulin autoantibodies (I)

IgG was isolated from the plasma samples of 17 newly diagnosed patients with T1D and of 23 unaffected children who were all negative for plasma IAAs. The IgG concentrations of the IgG fractions and plasmas were measured and the total IgG concentrations did not differ between patients and unaffected controls. Table 3 shows the IgG concentrations in the plasma samples and the IAA levels in the IgG fractions and corresponding plasma samples.

The IAA levels of the IgG fractions were higher in the patients with newly diagnosed T1D than in the control children (p=0.004). (Figure 1.) The cutoff limit of the IAA assay used in this study was 1.55 RU (relative unit). In the IgG fractions this value was exceeded in 8 out of 17 patients (47%), who were negative for plasma IAAs, and in 3 out of 23 (13%) unaffected children.

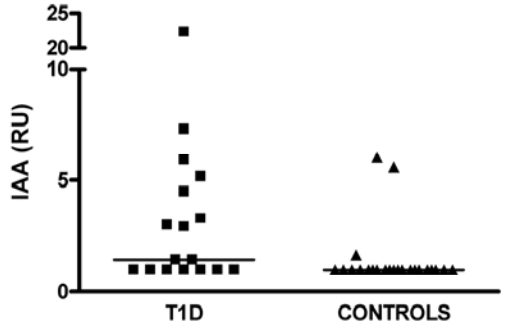

Figure 1. Levels of IAAs in IgG fractions in children negative for plasma IAAs. Median values of patients with recent-onset T1D and control children are shown as horizontal lines.

Table 3. IgG concentrations in plasma (mg/ml) and results of IAAs (RU, relative unit) in plasma samples and in corresponding IgG fractions in children with recent-onset T1D and in unaffected children. The cutoff limit for IAA positivity was 1.55 RU and values exceeding this are bolded.

Diabetic patients			Control children		
IgG conc.	IAA / plasma	IAA / IgG fraction	IgG conc.	IAA / plasma	IAA / IgG fraction
5.00	1.23	**22.35**	10.70	1.00	1.00
6.74	1.00	**2.93**	8.90	1.00	1.00
9.20	1.00	1.00	10.96	1.00	**1.68**
6.06	1.12	1.46	6.16	1.00	1.00
6.52	1.00	1.00	11.54	1.00	1.00
9.18	1.00	1.00	5.80	1.00	**5.58**
9.04	1.00	1.46	9.32	1.00	**6.03**
8.26	1.00	1.00	7.84	1.00	1.00
9.40	1.00	**4.50**	6.80	1.00	1.00
3.14	1.00	1.00	6.16	1.00	1.00
7.94	1.00	**7.29**	9.26	1.00	1.00
6.60	1.00	**3.02**	8.78	1.00	1.00
9.16	1.00	**5.90**	7.78	1.00	1.00
5.82	1.00	**3.31**	6.62	1.00	1.00
6.52	1.00	**5.16**	8.52	1.00	1.00
7.30	1.00	1.00	7.94	1.00	1.00
6.94	1.00	1.00	8.14	1.00	1.00
			7.94	1.00	1.00
			8.56	1.00	1.00
			8.90	1.00	1.00
			5.84	1.00	1.00
			9.54	1.00	1.00
			7.86	1.00	1.00

GADAs were also measured from the plasma samples and the IgG fractions. The levels of GADAs tended to be higher in the IgG fractions than in the corresponding plasma samples, but the GADAs measured in the IgG fractions did not differ between the patients and the unaffected controls negative for plasma GADAs.

5.2 Insulin-induced *in vitro* up-regulation of regulatory T-cell markers in peripheral blood mononuclear cells (II)

PBMCs were obtained from both insulin-treated (group 1) and untreated (group 0) patients with newly diagnosed T1D and from unaffected children. The PBMCs were cultured for 72 hours with bovine or human insulin (or with tetanus toxoid as a control antigen) before detecting the expression of regulatory T-cell markers Foxp3, CTLA-4, and inducible co-stimulator (ICOS), and cytokines TGF-β, IFN-γ, IL-4, and IL-5 at the mRNA level. The secretion of IFN-γ, IL-4, IL-5, IL-2, and IL-10 was measured with a CBA kit.

Both bovine and human insulin induced higher expression of the regulatory T-cell markers Foxp3 ($p=0.040$ and $p=0.055$ for bovine and human insulin, respectively), CTLA-4 ($p=0.035$ and $p=0.009$), and ICOS ($p=0.025$ and $p=0.018$) at the mRNA level in patients with T1D treated with insulin compared with patients with T1D studied before initiating insulin therapy. (Figure 2.) Bovine insulin also induced higher Foxp3 ($p=0.002$, median relative amounts were 10.6 and 4.0), CTLA-4 ($p=0.002$, medians 8.1 and 0.2) and ICOS ($p=0.005$, medians 9.1 and 5.0) mRNA levels in patients on insulin treatment compared with unaffected children.

The bovine and human insulin-induced expression levels of Foxp3, CTLA-4, and ICOS mRNAs correlated with each other ($r=0.44$, $p=0.011$; $r=0.42$, $p=0.016$; $r=0.45$, $p=0.017$; respectively). Bovine insulin-induced Foxp3 mRNA expression correlated with bovine insulin-induced CTLA-4 and ICOS mRNA expressions ($r=0.78$, $p<0.001$ for CTLA-4; $r=0.69$, $p<0.001$ for ICOS), while human insulin-induced Foxp3 mRNA correlated with human insulin-induced CTLA-4 and ICOS mRNA levels ($r=0.71$, $p<0.001$ for CTLA-4; $r=0.55$, $p=0.003$ for ICOS). The human insulin-induced expression of Foxp3 mRNA in insulin-treated patients correlated inversely with HbA1c values detected 5-6 months after diagnosis ($r=-0.66$, $p=0.038$), but bovine insulin-induced Foxp3 mRNA expression was directly correlated with the HbA1c values in patients on insulin treatment ($r=0.74$, $p=0.010$).

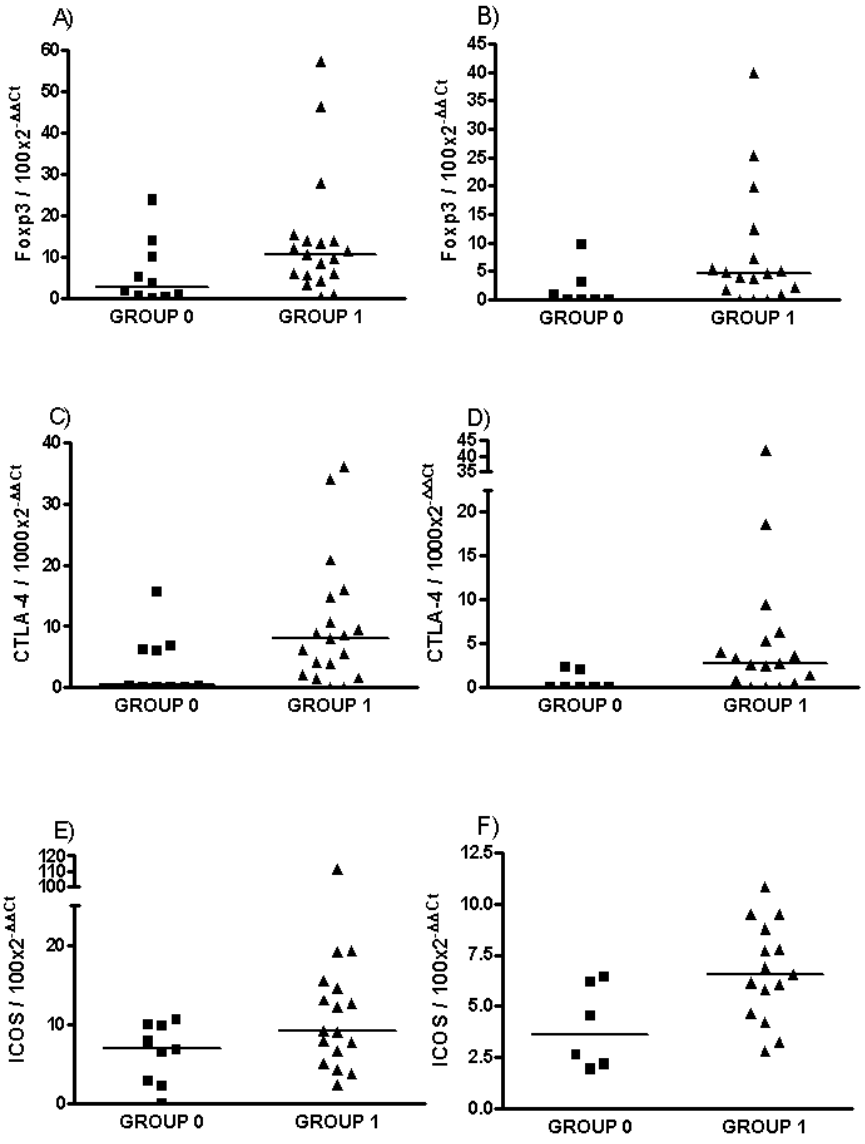

Figure 2. Foxp3-, CTLA-4- and ICOS-specific mRNA expression levels after bovine (A, C, E) and human insulin (B, D, F) stimulations. Samples from T1D patients in group 0 were taken at the time of diagnosis before starting insulin treatment. In group 1 the T1D patients were treated with insulin for 1-21 days. Horizontal lines show medians for each group.

We next analyzed whether the increase in the mRNA levels of regulatory cell markers was due to an increase in the number of regulatory T-cells. The insulin-induced increase *in vitro* in the number of regulatory T-cells, CD4+CD25^{high}Foxp3+ cells, was confirmed by flow cytometric analysis after culturing PBMCs for 5-6 days. The proportions of bovine and human insulin-induced CD4+CD25^{high} cells among CD4+ cells are presented in Table 4. Approximately half of these CD4+CD25^{high} cells expressed Foxp3 (range 42.7%-57.9% after bovine insulin stimulation and 52.0-62.5% after human insulin stimulation), as shown in Figure 3.

Table 4. Proportions of CD4+CD25^{high} cells among CD4+ cells in flow cytometric analysis after bovine or human insulin stimulation in patients with newly diagnosed T1D (group 0 refers to patients before initiating insulin therapy and group 1 to patients on insulin therapy) and in unaffected children. The cells cultured without antigen showed very few CD4+CD25^{high} cells among CD4+ cells in all children (median 0.1%, mean 0.3%). This background is subtracted from the insulin-induced results.

Group	Subject	Increase in CD4+CD25^{high} cells after bovine insulin stimulation (%)	Increase in CD4+CD25^{high} cells after human insulin stimulation (%)
0	patient 1	1.1	0
0	patient 2	0.1	0
0	patient 3	2.1	-
0	patient 4	4.7	0
0	patient 5	1.2	0.4
1	patient 6	6.5	1.0
1	patient 7	4.2	1.1
1	patient 8	1.2	0.2
1	patient 9	9.0	1.7
control	control 1	2.6	0
control	control 2	2.3	0.1
control	control 3	0.8	1.4
control	control 4	2.4	1.7

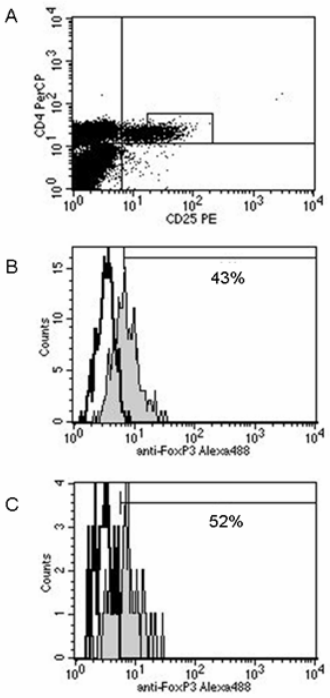

Figure 3. Flow cytometric analysis of CD4+ CD25^{high} Foxp3+ cells in a representative patient with newly diagnosed T1D. Plot A shows how the CD4+CD25^{high} cells are gated for plots B and C. Plot B shows bovine insulin- and plot C human insulin-induced Foxp3 expression. The histograms colored with gray represent the staining with anti-Foxp3 antibody and the uncolored histograms represent staining with a control antibody.

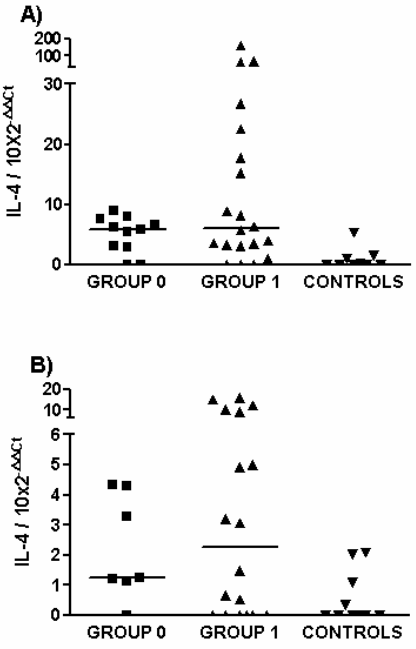

Figure 4. Bovine (A) and human (B) insulin-induced expression of IL-4 mRNA after 72 hours stimulation of PBMCs. Group 0 represents patients with T1D at diagnosis before initiating exogenous insulin therapy and group 1 recent-onset patients after treatment with insulin for 1-21 days. Medians are marked with horizontal lines.

The cytokine-specific mRNA expressions did not differ between the T1D patient groups (before or after initiating insulin treatment). The IL-4 mRNA expression was increased in both patient groups in comparison to unaffected children after bovine ($p=0.007$ for group 0 and $p=0.002$ for group 1) and human insulin stimulation ($p=0.033$ for group 0 and $p=0.057$ for group 1). (Figure 4.) Bovine insulin-induced secretion of IL-4 was also higher in T1D patients treated with insulin than in the control children ($p=0.014$, median levels were 0.8 and 0 pg/ml). The IFN-γ, IL-5, or TGF-β mRNA expressions, as well as the secretion of IFN-γ, IL-5, IL-2, or IL-10, did not differ between the study groups after the insulin stimulations. With a T1D-unrelated antigen, tetanus toxoid, we could find no similar differences between the study groups in the expression of regulatory T-cell markers or IL-4.

In the T-cell proliferation test the study groups did not differ in response to bovine or human insulin or tetanus toxoid. The stimulation indices in proliferation after bovine insulin stimulation correlated with the quantities of bovine-induced IFN-γ, IL-5, Foxp3, and CTLA-4 at the mRNA level ($r=0.54$, $p=0.002$ for IFN-γ, $r=0.62$, $p<0.001$ for IL-5, $r=0.51$, $p=0.003$ for Foxp3, and $r=0.43$, $p=0.016$ for CTLA-4), and IFN-γ and IL-5 at the protein level ($r=0.61$, $p=0.001$ for IFN-γ and $r=0.65$, $p<0.001$ for IL-5) in the total series. In contrast, the human insulin-induced expression of IFN-γ, IL-5, Foxp3, or CTLA-4 did not correlate with the proliferation response induced by human insulin. The proliferation response to human insulin correlated inversely with the quantity of human insulin-induced TGF-β mRNA ($r=-0.41$, $p=0.029$) or with IAAs ($r=-0.42$, $p=0.044$). The IAA levels correlated with the quantities of IL-4 and TGF-β mRNAs in response to human insulin in patients with newly diagnosed T1D ($r=0.46$, $p=0.027$ for IL-4 and $r=0.60$, $p=0.003$ for TGF-β).

5.3 High concentration of insulin in breast milk is associated with decreased humoral immune response to dietary insulin in the offspring (III)

The bovine and human insulin-specific IgG class antibodies were measured with ELISA from blood samples taken at 3, 6, 9, and 12 months of age from the TRIGR children. The breast milk samples from the mothers of the children at 3-7 days and 3 months after delivery were analyzed for insulin content.

The insulin concentrations in breast milk samples varied between 0 and 14 µg/l and no differences were found between the samples taken at 3-7 days or 3 months after delivery. The concentrations of insulin in breast milk were higher in mothers affected with T1D than in nondiabetic mothers in both the 3-7-day samples ($p=0.007$) and in the 3-month samples ($p<0.001$). (Figure 5.)

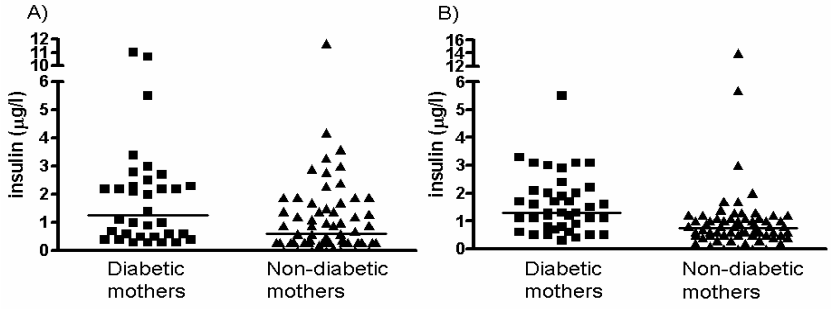

Figure 5. Insulin concentrations in breast milk samples 3-7 days (A) or 3 months (B) after delivery in mothers with or without T1D. The median values are shown with horizontal lines.

The concentration of insulin in breast milk taken 3 months after delivery correlated inversely with the bovine insulin-specific antibody levels detected at 6 months of age in children exposed to cow's milk-based formula (r=-0.39, p=0.013). No association was seen in children who had received casein hydrolysate formula (r=-0.08, p=0.610). At 12 months of age, when all the children were exposed to cow's milk in their diet, an inverse correlation was seen between bovine insulin-specific antibody levels and the concentrations of insulin in breast milk 3 months after delivery in all subjects (r=-0.25, p=0.029). (Figure 6.)

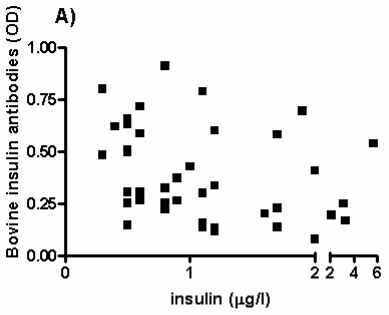

Figure 6. Correlation between insulin concentration in breast milk at 3 months and bovine insulin antibodies in plasma at 6 months (A) or 12 months (B) of age. A): infants in cow's milk formula group and B): all children.

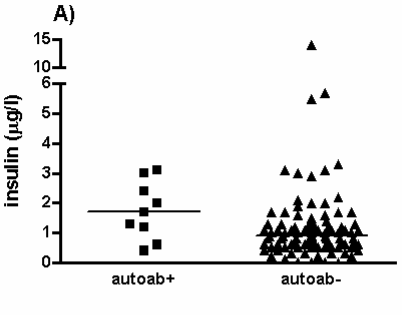

Figure 7. Insulin content in breast milk (at 3 months) in all mothers (A) and in diabetic mothers (B). The groups represent children positive (autoab+) or negative (autoab-) for T1D-associated autoantibodies. Median insulin concentrations are shown.

The breast milk insulin concentrations 3 months after delivery were higher in the mothers of those children who became positive for T1D-associated autoantibodies during the follow-up (p=0.030). The same finding held when only children of mothers with T1D were included in the analysis (p=0.045). (Figure 7.)

5.4 Increased IL-18 mRNA expression in the small intestine of children with type 1 diabetes (IV)

The expression of several immunological markers at the mRNA level was studied in duodenal biopsies of children with T1D and active celiac disease, T1D and potential celiac disease, T1D and normal intestinal mucosae, active celiac disease, potential celiac disease, and control children with normal intestinal mucosae. IL-8 and IL-6 were studied as common markers of inflammation, IL-18 and IL-15 as cytokines expressed by antigen-presenting cells, IFN-γ as a marker for Th1 immune responses, IL-4 as a marker for Th2 immune responses, and Foxp3, TGF-β, and IL-10 as markers for regulatory T-cells. CD25 expression, which is associated with both activated effector T-cells and regulatory T-cells, was also studied.

The expression of IL-18-, Foxp3-, IL-10-, IFN-γ-, and CD25-specific mRNAs differed between the six study groups (in the Kruskal-Wallis test $p<0.001$ for IL-18, IL-10 and IFN-γ; p=0.008 for Foxp3 and p=0.023 for CD25). Those children with T1D with no signs of celiac disease or potential celiac disease expressed higher levels of IL-18 mRNA in small intestinal biopsies than control children with normal mucosae (p=0.035). Pediatric patients with T1D also differed from children with celiac disease, T1D and celiac disease, and potential celiac disease (all p-values were <0.001). In addition, children with T1D and potential celiac disease expressed higher levels of IL-18 mRNA than patients with celiac disease, T1D and celiac disease, and potential celiac disease (all p-values were < 0.001). In patients with active or potential celiac

disease the expression of IL-18 mRNA was lower than in control children with normal mucosae (p=0.004 and p=0.025 respectively). (Figure 8.)

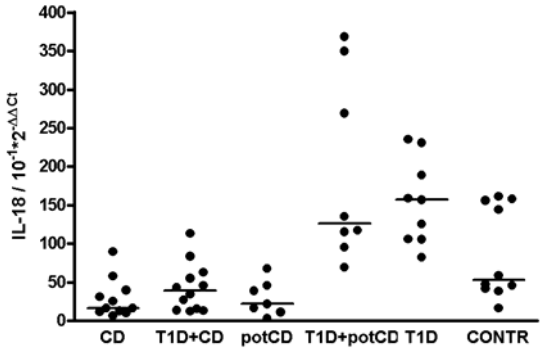

Figure 8. Expression of IL-18-specific mRNA in small intestinal biopsies measured by quantitative RT-PCR. The horizontal lines show medians for each group. CD = celiac disease, potCD = potential celiac disease with elevated levels of TCR γ/δ -expressing IELs and/or presence of celiac disease-associated antibodies, CONTR = control group with normal mucosae.

Pediatric patients with T1D did not differ from the control group in the expression of any other marker tested. The increased expression of Foxp3, IL-10, IFN-γ, and CD25 was associated with celiac disease. The expression of these markers at the mRNA level was confirmed at the protein level by immunohistochemistry only with Foxp3 and CD25, the staining of which correlated with the RT-PCR results. Immunohistochemical staining of cytokines in the paraffin sections was not done.

6 DISCUSSION

6.1 Insulin autoantibodies can be detected in isolated IgG fractions of children with type 1 diabetes and negative for plasma insulin autoantibodies (I)

This study showed that about half of the patients with T1D tested positive for IAAs, when the IAA assay was performed in the isolated IgG fraction instead of plasma in subjects whose plasma tested negative for IAAs. Overall the levels of autoantibodies, both IAAs and GADAs, were increased after IgG fractionation in comparison to plasma. But only with IAAs did the levels detected in patients with T1D exceed significantly the levels detected in the unaffected children.

This finding suggests that insulin may circulate in plasma as insulin-IAA immune complexes that are not detectable in micro-IAA assay. The IgG fraction was eluted with acidic buffer which allows the immune complexes to dissociate, while dialysis during the concentration of the IgG fraction permits a small molecule such as insulin to be discarded. Another possibility is that some unknown molecule in plasma may interfere with the IAA assay and this molecule may be discarded during the fractionation of IgG. However, this phenomenon appears to affect especially IAA assay with poor sensitivity, but not the GADA assay.

The isolation of the IgG fraction in this study is a laborious method that is not suitable for large-scale screening, and thus does not as such offer an improvement in IAA methodology. Furthermore, IgG fractionation may decrease the specificity of the IAA assay, since 3 out of 23 control children became positive for IAAs after isolation of the IgG fraction. However if the IgG fractions were used for measuring IAAs, a new cutoff limit for positivity in the assay should be assessed with a sufficient number of IgG fractions from unaffected children. In conclusion, this finding that the IAA assay performed in plasma or serum samples may result in a

significant proportion of false-negative results may at least partly explain the fluctuation of IAA levels seen in follow-up studies.

6.2 Insulin treatment stimulates regulatory T-cells in patients with newly diagnosed type 1 diabetes (II)

In vitro human and bovine insulin stimulation of PBMCs resulted in an increased expression of the regulatory T-cell markers Foxp3, CTLA-4, and ICOS in those T1D patients who had been treated with exogenous insulin. The PBMCs of children at diagnosis of T1D before receiving insulin therapy showed no increment of regulatory T-cell markers after insulin stimulation. The mRNA expression levels of all three regulatory T-cell markers correlated with each other which confirms the finding. The phenomenon could likewise not be seen with the T1D-unrelated antigen, tetanus toxoid. This finding suggests that exogenous insulin treatment affects the population of regulatory T-cells *in vivo*. In animal studies it was shown that peripheral autoantigen can induce activation of regulatory T-cells (Seddon and Mason 1999; Masteller et al. 2005).

We also showed at the protein level, using flow cytometry, that *in vitro* human and bovine insulin stimulation increases the proportion of CD4+CD25^{high}Foxp3+ cells. However, the same increase was also seen in unaffected children and the number of patients studied was too small to speculate if the increment was higher in those patients on insulin treatment compared with the untreated patients.

In the animal model of T1D, the NOD mouse, regulatory T-cells are able to protect from diabetes (Peng et al. 2004; Masteller et al. 2005). In mice it was also shown that regulatory T-cells are present in insulitis and their protective effect is dependent on the expression of ICOS (Herman et al. 2004). In human studies no difference in the frequency of regulatory T-cells between patients with T1D and unaffected controls was reported (Brusko et al. 2005; Lindley et al. 2005; Putnam et al. 2005).

Two of those studies, however, suggested that the regulatory T-cells of patients with T1D have a defective ability to suppress T-cell proliferation in *in vitro* proliferation tests (Brusko et al. 2005; Lindley et al. 2005). None, however, have addressed the subject of autoantigen-specific regulatory T-cells in patients with T1D.

We used both human and bovine insulin in cell culture. Human insulin is an endogenous antigen and is expressed in the thymus, and thus human insulin-specific thymus-derived regulatory T-cells can exist. Bovine insulin is encountered in the diet and putative bovine insulin-specific regulatory T-cells are peripherally induced to exogenous antigen. Human and bovine insulin differ by only three amino acids and their immunological responses often cross-react, although bovine insulin is a stronger immunogen. In this publication human and bovine insulin-induced expressions of regulatory T-cell markers correlated with each other, but differences in the characteristics of Foxp3 mRNA expression were also found. Human insulin-induced expression of Foxp3 mRNA levels correlated inversely with HbA1c values detected 5-6 months after diagnosis, but bovine insulin-induced Foxp3 mRNA levels were directly correlated with HbA1c values. Bovine insulin-induced Foxp3 expression also correlated with the proliferative response induced by bovine insulin, which could not be found after human insulin stimulation. These correlations suggest that human insulin stimulation induces an increase in regulatory T-cells that are able to suppress human insulin-specific effector T-cells, and regulatory T-cells activated by insulin treatment may explain the remission period seen in many newly diagnosed T1D children after beginning insulin treatment. However, the bovine insulin-induced responses observed in this study did not appear to suppress effector T-cells and bovine insulin could have induced more of an effector T-cell response than a regulatory T-cell response or the bovine insulin-specific peripherally induced regulatory T-cells may have had functional defects.

We also showed that both groups of T1D patients, both before and after initiating insulin treatment, showed increased IL-4 expression in response to insulin

stimulation. Insulin treatment, however, appeared to enhance the IL-4 response. Interestingly, IL-4 activates regulatory T-cells (Maerten et al. 2005). Both the increased regulatory T-cell marker and IL-4 expressions in response to *in vitro* insulin stimulation in newly diagnosed patients may explain the difficulties in developing T1D-associated autoantigen-specific T-cell assays. Likewise, we found no differences in T-cell proliferation between children with T1D and the unaffected controls. The mRNA expression levels of IL-4 and TGF-β mRNAs, both cytokines known to inhibit T-cell proliferation, correlated with the IAA levels of the patients after human insulin stimulation. Furthermore, the IAA levels correlated inversely with the human insulin-induced T-cell proliferation response. This finding suggests that patients with recent-onset T1D have a Th2 type of immune response to insulin associated with IAAs, and the *in vitro* proliferation of T-cells is down-regulated due to the existence of this Th2 response.

The increased IL-4 expression in response to insulin is in agreement with animal studies showing that insulin aerosol treatment in NOD mice induces the formation of insulin antibodies and increases IL-4 secretion from splenocytes (Harrison et al. 1996). Subcutaneous insulin immunization in NOD mice also increases the frequency of IL-4-secreting insulin-specific T-cells and inhibits the proliferative response of T-cells (Tian et al. 1998), while oral insulin treatment in NOD mice up-regulates the amount of IL-4-secreting cells in the pancreatic islets (Ploix et al. 1998). In humans it was shown that intranasal insulin induces increased antibody formation and inhibits the insulin-specific cellular immune response in autoantibody-positive children at risk for T1D (Harrison et al. 2004) and that insulin treatment shifts the insulin antibody response one predominantly of an IgG1 subclass to a Th2-specific IgG4 subclass (Füchtenbusch et al. 2000).

In the present study the insulin-induced enhanced Th2 type of immune response conflicts with the concept of T1D as a Th1-mediated disease, but agrees with the observation of IL-13-secreting insulin-specific T-cell lines derived from the

pancreatic lymph nodes of patients with T1D (Kent et al. 2005). The insulin-induced increase in IL-4 expression also accords with previous publications reporting that stimulated CD4+ T-cells secrete IL-4 and IL-10 in response to preproinsulin epitopes in individuals with T1D-associated autoantibodies (Durinovic-Bellò et al. 2004) and that insulin increases mitogen-induced secretion of IL-4 from PBMCs (Kretowski et al. 1999). In addition, the occurrence of IgG4 subclass IAAs in nondiabetic autoantibody-positive children fits well with our finding, since IL-4 supports formation of IgG4 subclass (Bonifacio et al. 1999). It was suggested that the presence of autoantibodies of the IgG1 subclass represents a low predictive value for T1D but the disease risk increases when the humoral immune response to autoantigens spreads to include the IgG2 and IgG4 subclasses (Achenbach et al. 2004). Thus, the increase in IL-4 expression in response to insulin suggests that the Th2 type of immune response is also involved in the autoimmune response to endogenous insulin in patients with T1D.

6.3 Breast milk insulin promotes oral tolerance to dietary insulin (III)

Insulin is present in breast milk in various species (Kulski and Hartmann 1983; Aranda et al. 1991). We demonstrated that in mothers suffering from T1D the level of insulin in breast milk is higher than in unaffected mothers, which is in agreement with previous results (Jovanovic-Peterson et al. 1989). Patients with T1D use daily insulin injections and their plasma insulin levels are often increased, which probably reflects the concentration of insulin in their breast milk. Due to human insulin treatment, most of the insulin detected in breast milk is most likely of human origin.

We also found that the concentrations of insulin in breast milk were inversely associated with the humoral immune response to bovine insulin. We showed earlier that hydrolyzed casein-based infant formula does not induce as high levels of IgG antibodies to insulin as regular cow's milk-based formula (Vaarala et al. 1999;

Paronen et al. 2000). Indeed, at 6 months of age the inverse association between breast milk insulin and bovine insulin-specific antibodies was detected only in the group of children who were exposed to intact cow's milk proteins in the diet and not in the group of children receiving hydrolyzed casein-based formula. Later, at 12 months of age, the inverse correlation was found in both groups of children in the trial because at that age all the children had received cow's milk after the intervention period. Our finding suggests that human insulin present in breast milk induces tolerance to dietary bovine insulin. When an infant is exposed to cow's milk insulin an immune response to bovine insulin is developed and this response may be down-regulated with human insulin in breast milk.

It is true that bovine and human insulin crossreact in insulin antibody ELISA and as shown by Vaarala et al. (1999) the immune responses to bovine and human insulin measured using ELISA correlate. However, the insulin antibody titers are shown to increase when children are exposed to cow's milk in their diet, and thus the ELISAs measure largely insulin antibodies to dietary bovine insulin.

It was previously shown that the infants of diabetic mothers have decreased insulin-specific immunity (Paronen et al. 2000) and the risk of T1D is lower in the children of diabetic mothers than diabetic fathers (Warram et al. 1984), and these two phenomena could be explained by this tolerogenic effect of breast milk insulin. On the other hand, mother's diabetes may affect child's insulin-specific immune responses in some other way than by increasing insulin concentration in breast milk, for example the fluctuations of mother's blood glucose may stimulate fetal insulin production, which could affect immune responses to insulin.

Interestingly, those children in the present study who later developed T1D-associated autoantibodies behaved differently. The insulin levels in breast milk of the mothers of these children were increased. Perhaps in these children, who were prone to β-cell autoimmunity, the breast milk insulin did not induce tolerance. The derangements in

the gut immune system suggested to be involved in the pathogenesis of T1D may explain the impairment of developing oral tolerance to insulin.

6.4 Immunological changes in the small intestine of children with type 1 diabetes (IV)

The increased mRNA expression levels of IL-18 in duodenal biopsies were strongly associated with T1D patients who showed no signs of celiac disease. The level of IL-18 mRNA was even lowered in patients with celiac disease or potential celiac disease compared with the control group with normal mucosae. The increased expression of regulatory T-cell marker Foxp3 and the cytokines IL-10 and IFN-γ, and CD25 were associated with celiac disease and the expression of these markers was not altered in pediatric patients with T1D.

Both activated macrophages and intestinal epithelial cells are able to produce IL-18 in the gut. A previous finding of increased IL-1α expression in the gut of patients with T1D (Westerholm-Ormio et al. 2003) fits well with this, since both of these cytokines are expressed by activated macrophages. IL-18 is at some level normally expressed in the gut (Takeuchi et al. 1997; Salvati et al. 2002), but its increase was associated with some intestinal disorders such as Crohn´s disease (Monteleone et al. 1999; Pizarro et al. 1999).

In animal studies IL-18 was expressed during the course of insulitis in pancreatic islets (Hong et al. 2000; Frigerio et al. 2002). IL-18 inhibitor is also able to delay the development of an accelerated form of diabetes in NOD mice (Zaccone et al. 2005). In humans the presence of T1D-associated autoantibodies, especially multiple autoantibodies, is associated with an increased level of circulating IL-18 (Nicoletti et al. 2001; Hanifi-Moghaddam et al. 2003). Polymorphisms in the promoter of the IL-18 gene may also be associated with susceptibility to T1D (Kretowski et al. 2002; Ide et al. 2004).

IL-18 is known to affect the induction of oral tolerance and it was shown that the induction of oral tolerance can be abrogated by administration of IL-18 and IL-12 in mice (Eaton et al. 2003). This suggests that the increased IL-18 expression in the gut of patients with T1D may be involved in the impaired induction of oral tolerance, which could explain the enhanced immune response to dietary proteins found in patients with T1D. Unfortunately, immunohistochemical staining for IL-18 was not done in our patient samples. The expression detected only at the mRNA level does not prove the existence of the protein. However, this finding of increased IL-18 mRNA expression in the gut of patients with T1D supports the theory that the gut immune system is involved in the pathogenesis of T1D.

7 CONCLUSIONS

I In the first publication we showed that IAAs measured from plasma or serum may result in a substantial proportion of false-negative results. When the IAAs were detected in the isolated IgG fraction instead, as many as half of the T1D patients negative for IAAs became positive for IAAs. This finding may have been due to the formation of insulin-IAA immune complexes or some other unspecified factor present in plasma, and either the immune complexes or this factor could have been eliminated during the isolation of IgG.

II We found increased expression of regulatory T-cell markers in response to *in vitro* insulin (both human and bovine insulin) stimulation. This was only seen in recent-onset T1D children treated with insulin and not before initiating insulin treatment. Since the T1D-unrelated control antigen showed no similar findings and similar differences between the treated and untreated patients could not be seen in the other markers measured, we conclude that insulin treatment in children with recent-onset T1D stimulates insulin-specific regulatory T-cells *in vivo.* Regulatory T-cells induced by insulin treatment may explain the remission period often seen in newly diagnosed children after beginning the insulin treatment.

Furthermore, we found that *in vitro* insulin stimulation resulted in increased insulin-specific IL-4 expression in children with T1D both before and after initiating insulin treatment, which suggests that the immune response to endogenous insulin also involves the Th2 type of response in patients with T1D. Both insulin-induced increase in regulatory T-cells and increased Th2 immune response may explain the difficulties in studying insulin-specific T-cell responses in newly diagnosed T1D patients.

III Third publication showed that the insulin content in the mother's breast milk modulates the humoral immune response to dietary insulin in the offspring. The high concentration of insulin in breast milk was associated with low levels of bovine insulin-specific antibodies in children, suggesting that human insulin in breast milk may promote tolerance to bovine insulin received from the diet. This finding may explain why the risk of T1D is lower in the children of mothers with T1D than in children of fathers with T1D.

However, in those children who later developed β-cell autoimmunity expressed as T1D-associated autoantibodies, the concentration of insulin in the mothers´ breast milk was significantly higher than in other children. This may indicate that children prone to β-cell autoimmunity cannot promote oral tolerance to dietary insulin, perhaps due to immunological derangements in their gut immune systems.

IV In the fourth publication the expression of IL-18 mRNA was clearly increased in small intestinal biopsies of children with T1D and this increment was not associated with celiac disease or potential celiac disease. In contrast, celiac disease appeared to lower the expression level of IL-18. Expression of IL-18 is a marker for immune activation in the gut and may be involved, among other things, in the development of oral tolerance. However, this finding lends further support for the hypothesis that changes in the gut immune system may play a role in the pathogenesis of T1D.

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