



KARITA SADEHARJU

# Maternal and Childhood Enterovirus Infections and the Risk of Beta-cell Autoimmunity



ACADEMIC DISSERTATION

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“it’s up to you  
if you give it up”

Dedicated

A close-up of handwritten text in black ink on a light-colored, slightly textured background. The text is written in a cursive, slanted style. The word "to" is positioned above the word "Arthur". The "A" in "Arthur" is large and has a prominent loop. The "r" is connected to the "A", and the "t" is also connected. The "H" and "u" are written in a fluid, cursive manner.

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## LIST OF ORIGINAL COMMUNICATIONS

This dissertation is based on the following publications, which are referred to in the text by their Roman numerals (Reports I, II, III and IV).

- I Sadeharju K, Hämäläinen A-M, Knip M, Lönnrot M, Koskela P, Virtanen SM, Ilonen J, Åkerblom HK Hyöty H and the Finnish TRIGR Study group: Enterovirus infections as a risk factor for type I diabetes -virus analyses in a dietary intervention trial. *Clinical and Experimental Immunology* 132: 271-277, 2003.  
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- II Sadeharju K, Knip M, Hiltunen M, Åkerblom HK, Hyöty H and the Childhood Diabetes in Finland Study group: The HLA-DR phenotype modulates the humoral immune response to enterovirus antigens. *Diabetologia* 46: 1100-1105, 2003.
- III Sadeharju K, Lönnrot M, Kimpimäki T, Savola K, Erkkilä S, Kalliokoski T, Savolainen P, Koskela P, Ilonen J Simell O, Knip M and Hyöty H: Enterovirus antibody levels during the first two years of life in prediabetic autoantibody-positive children. *Diabetologia* 44: 818-823, 2001.
- IV Sadeharju K, Knip M, Virtanen SM, Savilahti E, Tauriainen S, Koskela P, Åkerblom HK Hyöty H and the Finnish TRIGR Study group: Maternal enterovirus antibodies in breast milk protect the child from enterovirus infections – Results from the TRIGR (Pilot 2) trial. Submitted for publication.

The publishers of the original articles have kindly granted permission to reprint the Reports.

## ABBREVIATIONS

|       |  |
|-------|--|
| ATCC  | American Type Culture Collection                     |
| AUC   | the area under the curve -method                     |
| BB    | BioBreeding  |
| CAV   | coxsackie A virus                                    |
| CBV   | coxsackie B virus                                    |
| cDNA  | complementary DNA                                    |
| CI    | confidence interval                                  |
| CMV   | cytomegalovirus                                      |
| CRS   | congenital rubella syndrome                          |
| DiMe  | the Childhood Diabetes in Finland Study              |
| DIPP  | the Finnish Diabetes Prediction and Prevention Study |
| EBV   | Epstain-Barr virus                                   |
| EIA   | enzyme immunoassay                                   |
| EIU   | enzyme immunoassay unit                              |
| EV    | enterovirus  |
| GABA  | gamma acino buturic acid                             |
| GAD   | glutamic acid decarboxylase                          |
| GADA  | glutamic acid decarboxylase antibody                 |
| HLA   | human leukocyte antigen                              |
| IAA   | insulin autoantibody                                 |
| IA-2A | tyrosine phosphatase-like protein antibody           |
| ICA   | islet cell antibody                                  |
| IDDM  | insulin-dependent diabetes mellitus                  |
| Ig    | immunoglobulin                                       |
| JDF-U | Juvenile Diabetes Foundation Unit                    |
| NOD   | non-obese diabetic                                   |
| OR    | Odd's ratio  |
| PCR   | polymerase chain reaction                            |

|       |   |
|-------|---|
| PV    | polio virus   |
| RIA   | radioimmunoassay                                      |
| RNA   | ribonucleic acid                                      |
| RT    | reverse transcriptase                                 |
| RU    | relative unit   |
| RV    | rotavirus   |
| TRIGR | Trial to Reduce IDDM in the Genetically at Risk Study |



## ABSTRACT

### **Karita Sadeharju: Maternal and childhood enterovirus infections and the risk of beta-cell autoimmunity**

Type 1 diabetes is a chronic disease, which is caused by the destruction of insulin producing pancreatic beta-cells. Beta-cells are destroyed by immune mediated mechanisms and the disease is classified as an autoimmune disease.

The pathogenesis of the disease is multifactorial. Genetic susceptibility modulates the risk of type 1 diabetes, and HLA locus includes the major genetic susceptibility determinants which are connected to increased or decreased risk of type 1 diabetes. Previous studies have suggested that enterovirus infections are potential risk factors for type 1 diabetes. In the present study the effect of HLA-DR alleles, associated with either the decreased or increased risk of type 1 diabetes, on enterovirus immunity was studied. The role of enterovirus infections in the pathogenesis of type 1 diabetes was evaluated comparing children's and their mothers' enterovirus antibody levels and frequency of enterovirus infections from the pregnancy to the age of 24 to 36 months in prospective birth-cohort series. In addition, the effect of maternal antibodies (acquired transplacentally or in breast milk) on child's enterovirus infections was analysed. Study subjects were from three different studies; DiMe, DIPP and TRIGR.

Main results: Enterovirus infections were common showing a peak incidence at the age of 18 months (follow-up until the age of 3 years). Enterovirus viremia was the most frequent at the age of 9 months. Infections were more frequent among boys than girls. Children with diabetes associated autoantibodies had more enterovirus infections and higher levels of enterovirus antibodies than matched control children. They also had their enterovirus infections earlier than control children. Children whose mothers had particularly high enterovirus antibody levels in breast milk had fewer enterovirus infections than other children during the first year of life. Children with either the HLA-DR3 or -DR4 risk allele for type 1 diabetes had higher enterovirus antibody levels than children carrying the protective HLA-DR2 allele.

The results suggest that HLA-DR alleles, which mediate increased susceptibility to type 1 diabetes, are associated with strong humoral immune responses against enteroviruses. The

pathogenetic significance of this finding is not known. These children may have had more enterovirus infections or their humoral immune response to each enterovirus infection may be stronger. Increased frequency of enterovirus infections in autoantibody-positive subjects suggests that enterovirus infections may be associated with the induction of beta-cell autoimmunity in genetically susceptible children. Breast-feeding seems to protect the child against enterovirus infections.

## LYHENNELMÄ

### **Karita Sadeharju: Äidin ja lapsen enterovirusinfektiot ja beeta-soluautoimmunitetti**

Tyypin 1 diabetes on krooninen, elinikäinen sairaus, jota hoidetaan sairastumisesta lähtien päivittäin useilla insuliinipistoksilla. Sairaus johtuu haimassa sijaitsevien insuliinia tuottavien beetasolujen tuhoutumisesta. Syitä tämän autoimmuunisairauden kehittymiseen ei vielä tiedetä. Sairauden kehittyminen on monitekijäinen prosessi. Geneettinen alttius sairauden kehittymiselle liittyy tiettyihin HLA-alleeleihin sekä tiettyihin muihin geeneihin. Lisäksi tarvitaan jokin ulkoinen altiste. Enterovirusinfektioita pidetään aikaisempien tutkimusten perusteella yhtenä mahdollisena riskitekijänä tyypin 1 diabeteksen kehittymiselle.

Tässä tutkimuksessa haluttiin selvittää, vaikuttavatko diabetekselle altistavat HLA alttiusgeenit enteroviruksia kohtaan suuntautuvaan immuunivasteeseen. Enterovirusten osuutta tyypin 1 diabeteksen kehittämisessä tutkittiin vertaamalla diabetekseen sairastuvien lasten ja verrokkilasten ja näiden äitien enterovirusvasta-ainetasoja sekä enterovirusinfektioiden esiintyvyyttä. Lisäksi tutkittiin äidiltä välittyvien (mm. rintamaidon kautta) enterovirusvasta-aineiden suojavaikutusta lapsen infektoihin. Nämä lapset osallistuivat kolmeen eri tutkimukseen, DiMeen, DIPPiin tai TRIGRIin.

Päätulokset olivat seuraavat: Lapsilla, joilla todettiin diabetekseen liittyvä HLA riskialleeli (HLA-DR3 tai -DR4), oli korkeammat enterovirusvasta-ainetasot kuin suojaavan alleelin (HLA-DR2) omaavilla lapsilla. Enterovirusinfektiot olivat yleisiä infektoita, eniten infektoita löytyi 18 kuukauden iässä olevilta lapsilta (seuranta-aika oli 3 vuoden ikään saakka). Pojilla oli enemmän infektoita kuin tytöillä. Lapsilla, joille kehittyi tyypin 1 diabetekseen liitettäviä autovasta-aineita, oli enemmän enterovirusinfektioita ja korkeammat enterovirusvasta-ainetasot kuin heidän verrokeillaan. Lisäksi he sairastivat infektoita nuoremmalla iällä. Lapset, joiden äidillä oli korkea enterovirusvasta-ainetaso rintamaidossa, sairastivat vähemmän enterovirusinfektioita kuin muut lapset ensimmäisen ikävuoden aikana.

Näiden tulosten perusteella voidaan todeta, että HLA-DR riskialleelin omaavilla lapsilla on voimakas immuunivaste enteroviruksia kohtaan. Tutkimuksen tulokset viittaavat siihen, että enterovirusinfektioiden esiintyminen on ajallisesti yhteydessä beetasoluautoimmunitetin syntyyn. Äidin korkeat enterovirusvasta-ainetasot ja rintaruokinta näyttävät suojaavan lasta enterovirusinfektioilta.

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A handwritten signature in black ink, appearing to read 'Kari Korpela', is shown on a light-colored background.

21.1.2005,  
Seinäjoki, Finland.

## INTRODUCTION

Type 1 diabetes is caused by a selective destruction of beta-cells. Beta-cells produce insulin in the islets of Langerhans in the pancreas. Destruction of beta-cells is mediated by immune mechanisms and reflected by diabetes-associated autoantibodies in blood. The lack of insulin causes an increase in blood sugar levels (hyperglycaemia). Once the beta-cells have been destroyed, they cannot regenerate and attempts to replace these cells by islet cell transplantation have not yet become possible as routine treatment. Thus the disease is chronic and still causes many complications, like atherosclerosis, retinopathy, nephropathy and neuropathy, even though the treatment has improved a lot in recent years.

Many of the new type 1 diabetes patients are children and teenagers and nowadays even the number of younger children is increasing (Tuomilehto et al. 1999). In Finland the incidence of type 1 diabetes is the highest in the world.

The genes influence the risk of getting type 1 diabetes. Certain HLA-allele combinations are associated with increased risk for type 1 diabetes while others protect against this disease. Environmental factors, like microbial infections, toxins and dietary factors are potential risk factors in the pathogenesis of the disease. Currently, enterovirus infections are considered as one of the most probable candidate for environmental trigger of the disease. The knowledge of this destruction process is still scant and more research is needed to prevent new patients in future.

## **LITERATURE REVIEW**

### **ENTEROVIRUSES**

#### **Structure**

Enteroviruses are small, icosahedral RNA viruses, belonging to the family of picornaviruses. They have no lipid envelope, and the virus is therefore insensitive to organic solvents. They are acid stable (stability over a pH range of 3 to 10), consisting of an outer protein shell and an inner RNA code. Enteroviruses have a single-stranded RNA genome comprising a 5' nontranslated region, a long open reading frame that encodes a protein of approximately 2100 amino acid residues, a short 3' nontranslated region, and a polyadenylated tail. The polyprotein has four structural proteins: VP1, VP2, VP3, and VP4 (Hellen and Wimmer 1995).

Based on the antigenic properties, the human enteroviruses were initially grouped into polioviruses (PV), coxsackieviruses A (CAV), coxsackieviruses B (CBV), echoviruses, and the more recently identified enteroviruses (EV) 68 to 71. A new enterovirus classification based on molecular data (phylogenetic relationship) has recently been implemented as an alternative to this antigenic classification (Hyypiä et al. 1997, Table 1). This classification groups human enteroviruses into five species shown in Table 1. The enteroviruses previously classified as echoviruses 22 and 23 now constitute a new genus, Parechoviruses, in the family Picornaviridae (Mayo and Pringle 1998). Recently, new candidates for enterovirus serotypes have been found, suggested to be named numerally enterovirus 73 (EV73), enterovirus 74 (EV74) etc. (Oberste et al. 2001, Oberste et al. 2002, Norder et al. 2003, Oberste et al. 2004).

**Table 1.** Human enteroviruses and parechoviruses (Hyypiä et al. 1997)

|                                |  |
|--------------------------------|--|
| Polioviruses (PV)              | PV1, PV2, PV3  |
| Human enteroviruses –A (HEV-A) | CAV 2-8,10,12,14,16, EV71  |
| HEV-B                          | CAV9, CBV 1-6, all Echoviruses (Echovirus 1-7, 9, 11-21, 24-27, 29-33), EV69 |
| HEV-C                          | CAV 1,11,13,15,17-22,24  |
| HEV-D                          | EV68, EV70   |
| Parechoviruses                 | Parechoviruses 1-3   |

### **The course of an enterovirus infection**

Enterovirus infection starts when the virus attaches to a cellular receptor. The human poliovirus receptor (PVR) has been identified as a member of the immunoglobulin superfamily (Mendelsohn et al. 1989). Cellular receptors for echoviruses [a decay-accelerating factor (DAF) and  $\alpha_2\beta_1$  integrin (VLA-2)] and for coxsackieviruses [coxsackie-adenovirus receptor (CAR),  $\alpha_v\beta_3$  integrin and intracellular adhesion molecule-1 (ICAM-1), DAF] have also been identified. They also share receptors with other viruses, for example adenoviruses (CAR) (Racaniello 1995).

After binding to a cell receptor, the viral RNA genome enters into the cellular cytoplasm acting as a template for protein synthesis. The complete viral protein coding region is translated as a single polypeptide. Virus-encoded proteases cleave the polypeptide into three proteins; P1, P2 and P3. P1 includes the four capsid proteins while P2 and P3 code for non-structural proteins of the virus. The same enteroviral RNA molecule is then used as a template for synthesis of negative-stranded RNA molecules, when large amounts of positive-stranded RNA copies are synthesized and used in translation of viral proteins. Enteroviruses are cytolytic viruses that usually lyse the host cell, inhibiting host cell's translation, transcription and protein synthesis (Haller and Semler 1995).

The primary replication occurs in the oropharyngeal and intestinal mucosa. After primary



replication the virus spreads to secondary lymphoid organs causing a viremia. Enteroviruses are mostly transmitted by the faecal-oral route, but respiratory and vertical transmission (from mother to foetus) are also possible. Most of the enterovirus infections are asymptomatic or they may manifest with mild respiratory symptoms. Enteroviruses may also cause a wide variety of diseases, like poliomyelitis, meningitis, encephalitis, myocarditis, gastroenteritis etc, altogether over 20 clinically recognised syndromes. In certain case enteroviruses may also cause persistent infections (Morens and Pallansch 1995).

## **Epidemiology**

Humans are known to be the only natural reservoir of human enteroviruses, although coxsackievirus B5 has also been found to infect swine (Verdaguer et al. 2003). Enteroviruses are found world wide, but seasonality varies by geographical area. In tropical areas enteroviruses are common year-round in or around the rainy season and in temperate climates in summer and autumn (Moore 1982, Melnick 1996). Among people of lower socio-economic status enteroviruses are more prevalent (Jenista et al. 1984).

Enterovirus infections are common in all age groups. In a Finnish study 60% of children had had an enterovirus infection by the age of one year (Juhela et al. 1998). Different age groups have different susceptibilities to enterovirus infections and young children are the most important transmitters of these viruses. They have more severe coxsackievirus B infections while coxsackievirus A, echovirus and poliovirus infections tend to be milder in young children than adults (Morens and Pallansch 1995). In one study the lack of breast-feeding was associated with increased risk of an enterovirus infection in infants (Jenista et al. 1984).

Enteroviral diseases and possibly also enterovirus infections occur more frequently in males than in females (Morens and Pallansch 1995). Complications of enteroviral infections, like central nervous system diseases or carditis, tend to be more severe in males than in females. Several possible explanations for this gender difference have been proposed, but the reason has remained unclear. It may be connected to gender-associated differences in immune responsiveness. Weaker immune response in males may lead to longer virus excretion and higher virus titres (Wong et al. 1977).

### **Immune response to enterovirus infection**

Enterovirus infections induce both humoral and cell-mediated immune responses. Humoral (antibody-mediated) immunity plays an important role in neutralizing the infectivity of the extracellular virus during viremia. Neutralizing antibodies also provide long-term protection against enterovirus infections. The role of cell-mediated immunity (T cells) is not completely understood. It may play a role in clearing intracellular infection and may also help to mount the humoral response. The first exposure to enteroviruses mounts immunological memory response. Secondary exposure of enteroviruses induces more rapid and stronger response (Tracy et al. 1995). Humoral immune response is at first based on antiviral immunoglobulin IgM and later IgG and IgA responses. IgM persists usually for less than 6 months and is a sign of acute or recent infection, while IgG and IgA may persist for years (Tracy et al. 1995).

In newborns the immune system is relatively incompetent and thus the maternal immunological memory is essential for the protection of the infant. Maternal IgG glass antibodies are transported to the foetus through the placenta and maternal IgA antibodies in breast milk acts locally in the infant's gut protecting from viruses. These antibodies transmit the mother's immunologic experience to the foetus and infant, and protect the child for the first 3 to 12 months after birth. During that maternal protection, the child's own immune system matures. However, this protection is serotype specific and targets enterovirus serotypes which the mother has experienced in the past (Zinkernagel 2001).

### **Diagnosis**

Enteroviruses cannot be distinguished from bacterial or other virus infections on clinical symptoms alone and thus laboratory tests are needed to confirm the diagnosis. Earlier immunoglobulins and other viral drugs have been unsuccessfully tried in the treatment of enterovirus diseases (Lin et al. 2002). During recent years a special antiviral treatment for enterovirus infection has been tested in clinical trials. The novel capsid-binding antiviral pleconaril inhibits in vitro replication of most rhinoviruses and enteroviruses. It has been reported that early pleconaril treatment was well tolerated and significantly reduced the duration and severity of symptoms due to picornaviruses in adults

(Hayden et al. 2003). It is also a potential treatment for neonatals and children (Bryant et al. 2004). However, recently liver-associated side effects prohibited this drug FDA approval.

### ***Detection of enteroviruses from clinical samples***

Enteroviruses survive quite well in cerebrospinal fluid, serum, whole blood and faeces, which are the most often used specimens (Pallansch and Roos 2001). Viral infectivity decreases at room temperature in days, but may survive for years at -70°C (Hellen and Wimmer 1995).

Isolation of enteroviruses in cell culture is the traditional diagnostic method. However it is labour intensive, costly, and requires a high level of expertise. In addition some coxsackie A viruses do not grow in cell culture, which further decreases the sensitivity (Lipson et al. 1988).

Reverse transcription polymerase chain reaction (RT-PCR) has been found to be very useful in the detection of enterovirus from clinical samples (Hyypiä et al. 1989, Chapman et al. 1990, Rotbart 1990, Halonen et al. 1995, Lönnrot et al. 1999). Almost all enteroviruses are detected using the primer sequences, which are selected from the relatively conserved 5'noncoding region of the enterovirus genome (Hyypiä et al. 1989, Chapman et al. 1990, Rotbart 1990). This method is sensitive, but false-positive results are possible due to laboratory contaminations (Rotbart 1990).

A new method, based on sequence analysis of the viral genome, has been developed for the identification and classification of enteroviruses. The complete genomes of over 40 human enterovirus serotypes have been sequenced (Oberste et al. 2002). A new serotype of human enteroviruses has recently been identified by comparing the complete VP1 nucleotide sequences; its proposed name is Enterovirus 73 (Oberste et al. 2001). The molecular classification of enteroviruses with the complete VP4 sequences has also been found to be useful in the identification of the enterovirus serotypes (Kubo et al. 2002). These molecular classifications of enteroviruses will be an important technique in the future.

Electron microscopy is not used for enterovirus detection in clinical laboratory practice, because it is labour-intensive and the number of enteroviruses is enough for their detection only in stool samples.

### ***Enterovirus antibody assays***

The neutralization assay is serotype-specific, but time-consuming and laborious. It is highly sensitive and specific but antigenic variation between virus strains used affects test results (Melnick 1996). A fourfold or greater rise in antibody titres between acute-phase and convalescent sera is a classic diagnostic criterion. Neutralization is quite useful for retrospective disease association, but not in clinical laboratory work, where the patient may be suffering from meningitis or other acute disease symptoms. Neutralizing antibodies persist for years.

Antibody assays are complicated because of the number of enterovirus serotypes. Therefore many antigens are usually needed to detect antibody responses. Responses also vary considerably due to cross-reactions between different serotypes (Rotbart and Romero 1995).

Many enzyme immunoassay (EIA) methods have been developed for the detection of enteroviral IgA, IgG and IgM (Frisk et al. 1989, Samuelson et al. 1993, Swanink et al. 1993, Cello and Svennerholm 1994, Bendig and Molyneaux 1996). EIA tests are less laborious than neutralization assays, but their sensitivity varies widely. EIA test measures the amount of antibodies, which bind to several viral antigen epitopes, while neutralization test measures the amount of antibodies, which specifically inhibit the infectivity. So the results from these assays are not completely comparable (reviewed by Rotbart and Romero 1995). The highest sensitivity is seen with broadly reactive antigens, i.e. heat-treated viruses, viral procapsids and synthetic peptide antigens (Roivainen et al. 1991, Hovi and Roivainen 1993), and also using several assays with different antigens in combination (Samuelson et al. 1993, Swanink et al. 1993, Hyöty et al. 1995). Presence of IgM usually indicates acute or recent infection, so it can be used as a clinical diagnostic test, even though the large number of serotypes decreases the specificity of this test.

# **PATHOGENESIS OF TYPE 1 DIABETES**

## **Background**

Diabetes mellitus is a chronic glucose metabolism disorder characterised by insulin deficiency and hyperglycaemia. It is classified into four categories: 1) type 1 diabetes (IDDM = insulin-dependent diabetes mellitus, autoimmune diabetes, juvenile diabetes), 2) type 2 diabetes (adulthood diabetes), 3) gestational diabetes and 4) other specific types like diabetes induced by infections or drugs and diabetes caused by genetic defects of beta-cells (ISPAD Guidelines 2000).

Insulin is a small protein (5808 D), formed in the beta-cells of the pancreas. In healthy subjects its secretion depends on blood glucose level. Insulin lowers the blood glucose level by helping the glucose to transit from blood to cells and adding glucose intake to the cells. Type 1 diabetes is caused by very selective destruction of beta-cells leading to severe insulin deficiency and secondarily to abnormalities in carbohydrate, fat and protein metabolism. Untreated diabetes leads to continuous hyperglycaemia, ketoacidosis and finally to death. The symptoms of the disease are polydipsia, polyuria, weight loss, tiredness and irritability. Patients need life-long replacement therapy with daily parenteral injections of insulin and commit to certain daily routines, like dietary planning and self-monitoring of blood glucose. Even with good care of the disease, patients have increased risk of complications, such as retinopathy, nephropathy, neuropathy and cardiovascular diseases.

## **Epidemiology**

The global variation in the incidence of type 1 diabetes among children before the age of 15 years is very wide and over 350-fold differences have been reported from different populations worldwide: the lowest incidence from China and Venezuela (0.1/100 000 per year) and the highest incidence from Sardinia (36.8/100,000 per year) and Finland (36.5/100,000 per year) during the years 1990 to 1994 (Karvonen et al. 2000).

The incidence of type 1 diabetes is increasing worldwide both in low and high incidence populations (Onkamo et al. 1999). In Finland the incidence was 12/100000 per year in 1953 and has gradually increased being 36/100000 per year in the late 1980s and about 50/100000 per year more

recently (Tuomilehto et al. 1999). This increase has been most marked in the very young children (less than 5 years old). In the year 2001 the incidence was 56/100000 (Karvonen 2004).

Only a few cases are diagnosed before their first birthday. From 1965 to 1996 in Finland 9808 new cases were diagnosed and only 37 (0.4%) were younger than 1 year. However, the incidence was already high in 1-4 year olds in the late 1980s in Finland (31.4/ 100000 per year) (Tuomilehto et al. 1992). In some areas there is a significant male excess (as in Sardinia), but in many areas incidences are close to equal in both genders (Karvonen et al. 2000).

### **Autoimmune process towards beta-cells**

Beta-cell destruction is likely caused by an immune-mediated process. The damage to the cells is focal and restricted to the beta-cells. Many mechanisms have been proposed. It has been shown with NOD mice that autoimmune diabetes can be transferred to a healthy animal with T lymphocytes (Bendelac et al. 1987). These lymphoid cells infiltrate the islets of Langerhans and 3-4 weeks later insulinitis develops. Then these CD4<sup>+</sup> helper- and CD8<sup>+</sup> cytotoxic T-cells are activated and destruction of beta-cells begins (Sinha et al. 1990). Commonly it is thought that damage in the beta-cells develops after an environmental trigger leads to the breakdown of tolerance in genetically susceptible individuals.

In most cases the beta-cell destructive process has been going on for several years before the symptoms of diabetes appear. The subclinical process is reflected by the presence of autoantibodies in serum (Table 2). These include islet cell antibodies (ICA), first found by Bottazzo et al. 1974, which have been demonstrated in 84% of newly diagnosed type 1 diabetes patients (Vähäsalo et al. 1996). In recent decades the ICA assay has played a major role in the identification of risk individuals for the disease. First degree relatives of diabetic patients have ICA in their blood more often than other nondiabetic subjects and the high-risk HLA-DQB1 alleles are positively associated with high ICA levels (Karjalainen et al. 1996). Insulin autoantibodies (IAA) were identified from sera of type 1 diabetic patients before the starting of insulin treatment by Palmer et al. (1983) and were therefore thought to be markers of the autoimmune disease. More than half (54%) of new diabetic patients have IAA in Finland (Sabbah et al. 1999a). Glutamic acid decarboxylase (GAD) is the enzyme that is responsible for the biosynthesis of gamma amino butyric acid (GABA). GABA regulates the insulin secretion and proinsulin synthesis. Glutamic acid decarboxylase antibodies

(GADA) can be detected in more than 70% of new type 1 diabetic patients (Bonifacio et al. 1995). The IA-2 is a protein tyrosine phosphatase-like protein expressed in beta-cells. IA-2 antibodies (IA-2A) have been detected in 86% of young (under 15 years) type 1 diabetic patients at the onset of the disease (Savola et al. 1997). ICA, IAA, GADA and IA-2A have also been found in healthy people, 0.9%, 3.0%, 3.0% and 2.4% of subjects respectively, have these autoantibodies in their blood (Strebelow et al. 1999).

The risk of individual having single autoantibody for developing type 1 diabetes has been reported to be low (Kulmala et al. 1998). About 90% of patients with newly diagnosed type 1 diabetes have at least two autoantibodies (Savola et al. 1998b, Sabbah et al. 2000). The risk of developing type 1 diabetes increases with the number of autoantibodies, being very high in individuals with three autoantibodies, and multiple autoantibodies also reflect an aggressive progression to total beta-cell destruction, which is seen in increasing requirement for exogenous insulin as a treatment (Kulmala et al 1998, Strebelow et al. 1999).

**Table 2.** *Diabetes associated autoantibodies*

|       |                                      | First report           | T1D <sup>#</sup> | GP <sup>§</sup> | Sibs <sup>□</sup> |
|-------|--------------------------------------|------------------------|------------------|-----------------|-------------------|
| IAA   | insulin autoantibody                 | Palmer et. al 1983     | 54%              | 3.0%            | 3.7%              |
| IA-2A | antibody to the IA-2 molecule        | Payton et al. 1995     | 86%              | 2.4%            | 5.3%              |
| ICA   | islet cell antibody                  | Bottazzo et al. 1974   | 84%              | 0.9%            | 7.9%              |
| GADA  | glutamic acid decarboxylase antibody | Baekkeskov et al. 1982 | 73%              | 3.0%            | 7.0%              |

# = detected from newly diagnosed patients with type 1 diabetes (T1D) (Sabbah et al. 1999)

§ = detected from general population (GP) (Strebelow et al. 1999)

□ = detected from siblings of children with type 1 diabetes (Sibs) (Kulmala et al. 1998)

## **Genetic susceptibility**

Genetic susceptibility modulates the risk of type 1 diabetes. Studies in identical twins and the findings of familial clustering provide evidence of the involvement of genetic factors in the aetiology of the disease (Barnett et al. 1981, Dahlquist et al. 1985). However, the predictive power of family history of type 1 diabetes is not very high. In Finland for 11.2% of the children with newly diagnosed type 1 diabetes the same disease was found in their first degree relatives (Tuomilehto et al. 1992). The prevalence of type 1 diabetes in the fathers of diabetic children is higher (5.7%) than in mothers (2.6%) (Tuomilehto et al. 1992). Beta-cell autoimmunity can also be detected more often in the offspring of diabetic fathers than of diabetic mothers (Yu et al. 1995). It is also interesting that the child has a higher risk for type 1 diabetes if her/his mother's diabetes was diagnosed after the birth of the child compared to siblings who were born later, when the mother's diabetes was diagnosed before the pregnancy (Warram et al. 1991).

The most important genes that influence human susceptibility to type 1 diabetes are located within the major histocompatibility complex (MHC), the HLA region on the short arm of the human sixth chromosome. At first the effect of HLA was observed in HLA-DR locus, as DR3 and/or DR4 alleles mediated increased risk and DR2 had a strong protective effect (Nerup et al. 1974, Ilonen et al. 1978). Later the HLA-DQ locus was observed to contain the strongest known risk markers and protective alleles. These genes are not “disease genes“, but “susceptibility genes“, because not all subjects having these genes necessarily contract the disease. (Thorsby and Ronningen 1993) The susceptibility genotypes in Caucasians are mostly DQA1\*0501- DQB1\*02 and DQA1\*0301- DQB1\*0302 and the protective genotype is DQA1\*0102- DQB1\*0602 (Lernmark 1994, Ilonen et al. 1996). In Finland, the absolute risk of having type 1 diabetes is 0.7% in general population, while in HLA-DQB1\*02/\*0302 genotypes the risk is 7% and in DQB1\*0302/X (X= other than \*02, \*0301 or \*0602) genotypes the risk is 2.5% (Ilonen et al. 1996).

The mechanisms by which HLA mediates this effect are not known. It is possible that the risk type HLA alleles increase the immune response to a beta-cell autoantigen by presenting them efficiently to T-cells. These HLA alleles may also have a role in T-cell repertoire selection, antigen recognition and immune activation (reviewed by Deschamps and Khalil 1993).



### **Environmental triggers in the pathogenesis of type 1 diabetes**

The wide global variation and rapidly increasing incidence of type 1 diabetes suggest that environmental factors are important in the aetiology of type 1 diabetes. Viruses have long been suspected to play a role in the pathogenesis of type 1 diabetes. The onsets of type 1 diabetes follows a seasonal pattern typical for virus infections accumulating to the autumn seasons (Gamble et al. 1969). Seasonal variation has also been found in the appearance of autoantibodies, as type 1 diabetes associated autoantibodies usually appear in fall and winter (Kimpimäki et al. 2001a). Viruses are also thought to be the trigger agents in many other chronic autoimmune diseases, like multiple sclerosis, Sjögren's syndrome, juvenile rheumatoid arthritis, autoimmune chronic active hepatitis and systemic lupus erythematosus (Schattner and Rager-Zisman 1990, Talal et al. 1992). The mechanisms by which viruses could cause diabetes are not known. They may cause a direct cytolytic destruction of beta-cells or trigger an autoimmune process against beta-cell autoantigens. The role of molecular mimicry between viral proteins and beta-cell antigens has also been suggested to play a role in the induction of beta-cell autoimmunity (Atkinson et al. 1994).

Dietary factors have been considered to affect the incidence of type 1 diabetes since the beginning of 1980s. The first dietary study was conducted on BB rats, where the rats were divided into two groups. The group fed with casein, corn oil, corn starch and cellulosa type fibre did not develop the disease while 28% from the control group fed normally developed the disease (Scott et al. 1985). Thereafter studies on rats showed that wheat, gluten and soy proteins are potential diabetogenic components in their diet (reviewed by Åkerblom and Knip 1998). The possible role of toxins has also been studied. *N*-nitroso compounds (formed from nitrite) have been found to be diabetogenic. In some studies the intake of nitrite has been found to be greater in children with type 1 diabetes (Virtanen et al. 1994, Helgason and Jonasson 1981, Helgason et al. 1982). Dietary vitamin D supplementation has also been reported to be associated with reduced risk of type 1 diabetes (Hyppönen et al. 2001, Zella et al. 2003). Also exposure to cereals in infancy outside which initial exposure [before the age of 3 months (Norris et al. 2003, Ziegler et al. 2003) or after 7 months (Norris et al. 2003)] increases risk to develop diabetes-associated autoantibodies in susceptible children. Currently especially cow's milk proteins are being studied as potential diabetogenic dietary components (Åkerblom and Knip 1998).

Individuals genetically at risk for type 1 diabetes may have impaired mucosa-mediated tolerance and increased immunity to many dietary proteins. This is supported by the association of type 1 diabetes and celiac disease (Saukkonen et al. 1996). Signs of mucosal inflammation are found to present in jejunal biopsies from type 1 diabetic patients, and organ culture studies indicate a deranged mucosal immune response to gliadin (Auricchio et al. 2004). Although children with type 1 diabetes have shown to have markers of enhanced intestinal immune activation that is different from celiac disease (Westerholm-Ormio et al. 2003). Islet infiltrating and beta-cell autoantigen specific T-cells have shown to express mucosal and gut-associated homing receptors such as  $\alpha 4\beta 7$ -integrin (Hänninen et al. 1993, Paronen et al. 1997). Also in NOD mice (develop spontaneous autoimmune diabetes) have been found T lymphocytes that express gut-associated homing receptor  $\alpha 4\beta 7$ -integrin (Hänninen et al. 1996a, Hänninen et al. 1996b). So it is suggested that there is a link between gut immune system and development of type 1 diabetes (Vaarala 2004). Also immunity to insulin has been suggested to be the mechanism leading to autoimmunity and beta-cell destruction. The first immunization to insulin occurs by exposure to bovine insulin in cow's milk formula and it induces antibodies to insulin in infants at genetic risk for type 1 diabetes (Vaarala et al. 1998, Vaarala et al. 1999).

### ***Mumps virus***

An association between mumps infection and type 1 diabetes was presented as early as in 1898; After mumps a patient developed insulin-dependent diabetes. In 1927 an increase in the number of diabetic patients was reported 2 to 4 years after a mumps epidemic (reviewed by Åkerblom and Knip 1998). Later the association was confirmed in epidemiological studies (Sultz et al. 1975) and experimental cytological studies (Prince et al. 1978).

A restriction of the mumps-specific T-cell response to HLA-DR4-associated elements has been reported, but no differences between healthy and IDDM subjects in T-cell response to mumps virus has been found (Bruserud et al. 1985). Earlier patients with type 1 diabetes had high mumps IgA antibody levels in Finland, but after mumps-measles-rubella (MMR) vaccination were started in 1982, the antibody levels decreased to the normal range (Hyöty et al. 1993). After the implementation of MMR vaccinations in Finland, there was a short plateau in the rising incidence of type 1 diabetes but after a while it started to rise again (Hyöty et al. 1993, Tuomilehto et al. 1999). Altogether, it seems that mumps infections are not a major type 1 diabetes causing agent, but their

role in individual patients cannot be ruled out.

### ***Rubella virus***

Patients with congenital rubella syndrome (CRS) have been infected with the rubella virus transplacentally by their mother during pregnancy. They develop type 1 diabetes more often (10-20%) than healthy children with a latent period of 5 to 25 years (Forrest et al. 1971, Menser et al. 1978, Rabinowe et al. 1986). Patients with CRS are genotypically somewhat like patients with type 1 diabetes, having a high frequency of HLA-DR3 genotypes (Rubinstein et al. 1982). They also have anti-insulin and islet cell antibodies more often (Ginsberg-Fellner et al. 1984) and abnormal carbohydrate metabolism (Rabinowe et al. 1986). In contrast to congenital rubella there is no evidence supporting the role of postnatal rubella infection in the pathogenesis of type 1 diabetes even though a case report has been published on the onset of type 1 diabetes after a recent rubella infection (Sibley 1990).

Several mechanisms of beta-cell destruction caused by rubella virus have been proposed. Rubella virus may infect the beta-cells and alter antigens on the plasma membrane of beta-cells (Yoon 1995). The rubella virus capsid protein and islet cell antigen have a molecular mimicry and that may induce immunological cross-reactions (Karounas et al. 1993). In a recent study no increased frequency of markers for humoral beta-cell autoimmunity was found in patients with CRS, suggesting that diabetes in CRS may be caused by other than autoimmune mechanisms (Viskari et al. 2003).

Nowadays in many countries people are vaccinated against rubella infection and CRS or acute rubella infection is a very rare disease. Thus the connection between type 1 diabetes and this virus cannot explain the increasing numbers of new diagnoses.

### ***Enteroviruses***

Enteroviruses have been suggested as a potential risk factor for type 1 diabetes as long as over 30 years ago, when higher frequency of CBV antibodies was found in patients at the onset of the disease compared to control subjects (Gamble et al. 1969). Nowadays these viruses are one of the main targets in the research intended to identify the environmental risk factors of this disease.

Enteroviruses may cause destruction of pancreatic beta-cells by some of the mechanisms summarized in Table 3. They may cause an acute cytolytic infection in beta-cells. Many

enteroviruses have been shown to be able to infect and destroy human beta-cells in cell culture (Parkkonen et al. 1992, Roivainen et al. 2000). The effect of virus replication on beta-cell survival is found to be dependent on serotype or strain, some cause only little morphological changes, some severe functional damages and some rapid cytolysis (Roivainen et al. 2002). Ylipaasto et al. (2004) reported that seven of 12 newborn infants who died of fulminant coxsackievirus infections had enteroviral RNA in their pancreatic islets, not in exocrine cells, and six of these seven pancreases showed signs of insulinitis. This shows that enteroviruses can in vivo infect human pancreatic beta-cells.

Enteroviruses may also trigger an autoimmune process leading to the destruction of beta-cells. They may induce an immunologic attack against infected pancreatic beta-cells or activate autoreactive beta-cells by “a bystander activation” mechanisms. An infection leads to inflammation, tissue damage, and the release of islet antigen resulting in stimulation of resting autoreactive T cells. (Horwitz et al. 1998) One possible mechanism is molecular mimicry, where sequence homologies between human beta-cells and virus antigens result in an immunological cross-reaction causing beta-cell destruction. GAD65 is one of the beta-cell target antigens in the autoimmune process of type 1 diabetes. The sequence homology between CBV 2C-protein and GAD65 has been found (Kaufman et al. 1992). CBV 2C-protein and GAD65 may induce cross-reactive antibodies (Lönnrot et al. 1996). This cross-reactivity caused by molecular mimicry may lead to the autoimmunity process. Enterovirus infection has been shown to induce humoral responses, that cross-reacted with the known diabetes-associated epitopes in tyrosine phosphatase IAR and heat shock protein 60 (Härkönen et al. 2003). Enteroviruses have also shown to enhance the immunity to a region in preproinsulin (Davydova et al. 2003). Coxsackievirus immunization has been shown to delay the onset of diabetes in NOD mice and this delay was also transferred by splenocytes from coxsackievirus-immunized mice (Davydova et al. 2003). This kind of cross-reactions should also be taken into consideration if vaccines will be developed against enteroviruses.

**Table 3.** *Possible mechanisms by which enteroviruses may cause damage in insulin producing beta-cells (modified from Hyöty 2004)*

**Possible mechanisms of enterovirus-induced beta-cell damage**

|   |
|---|
| 1. Direct cytopathic effect in beta-cells                                       |
| 2. Antiviral immune response destroys beta-cells                                |
| 3. Bystander activation of an autoimmune response                               |
| 4. Immunological cross-reactivity between viral and beta-cell proteins          |
| 5. Interaction with other risk factors (e.g. cow's milk proteins) (see page 66) |

Persistent infection may be needed for the initiation of the beta-cell damage. Persistent virus infections have been associated with chronic autoimmune diseases, like myocarditis and hepatitis and persistent rubella virus infection may play a role in CRS (congenital rubella syndrome) induced type 1 diabetes (Monif et al. 1965). Enteroviruses can also cause persistent infections as shown in chronic myocarditis and in vitro models (Kandolf et al. 1999). Enterovirus genome has also been shown to persist in the pancreas of mice and this viral persistence was associated with beta-cell destruction and development of type 1 diabetes in mice (See and Tilles 1995). Enterovirus can also cause prolonged infections in islet cells in vitro (Chehadeh et al. 2000a).

Studies of the association between enteroviruses and type 1 diabetes in humans include case reports, twin studies, case-control studies and prospective studies. In a case-report by Yoon et al. (1979) CBV4 was isolated from a child who died because of ketoacidosis at the onset of type 1 diabetes. When transferred to mice this virus caused insulinitis, beta-cell necrosis and finally diabetes-like syndrome. Serological case-control studies have mostly shown increased levels of CBV antibodies in patients with newly diagnosed type 1 diabetes (Hyöty and Taylor 2002). Enterovirus IgM class antibodies indicating recent infection have also been found to be increased in patients with newly diagnosed type 1 diabetes (Barret-Connor 1985, Frisk et al. 1989). However, contradictory results have also been reported, where no difference was found between the patients with type 1 diabetes and controls (Mertens et al. 1983) or even decreased enterovirus antibody levels have been

reported in these patients (Palmer et al. 1982). The presence of enteroviral RNA in the blood at the time of onset of type 1 diabetes was found more often in patients (27-64%) compared to 0-5% control subjects by enterovirus PCR (Clemets et al. 1995, Andreoletti et al. 1997, Nairn et al. 1999, Chehadeh et al. 2000b, Yin et al. 2002). These studies indicate that enterovirus infection (acute or persistent) is frequently associated with the onset of the disease.

Some studies have indicated that maternal enterovirus infection during pregnancy may be associated with type 1 diabetes (Dahlquist et al. 1995, Hyöty et al. 1995), but this was not confirmed in a larger study (Viskari et al. 2002). A temporal relationship between enterovirus infection and seroconversion to autoantibodies associated with type 1 diabetes was found in prospective studies (Hyöty et al. 1995, Hiltunen et al. 1997, Lönnrot et al. 2000, Salminen et al. 2003). These results suggest that enteroviruses may trigger beta-cell autoimmunity and/or potentiate the autoimmunity process.

### ***Other viruses***

Many other viruses have been suspected of having a role in the pathogenesis of type 1 diabetes. Many studies have been done on animal models showing that several different viruses can cause beta-cell damage. However, their role in humans has not been confirmed.

Reovirus has been shown to cause transient type 1 diabetes by an autoimmune mechanism in mice (Onodera et al. 1978). Human beta-cells were also susceptible to reovirus infection in vitro (Yoon et al. 1981). About 30% of diabetes resistant BB rats developed an autoimmune diabetes 2 to 4 weeks after being infected with Kilham's rat virus (Guberski et al. 1991). Cattle have been reported to develop type 1 diabetes after bovine viral diarrhea mucosal disease, and lymphocytic infiltration has been found in their pancreatic islets (Tajima et al. 1992). Some molecular mimicry has been found between Epstein-Barr virus (EBV) and HLA-DQ beta chain peptide (Sairenji et al. 1991). A case report on two patients developing type 1 diabetes after an acute EBV infection has been presented (Parkkonen et al. 1994). Encephalomyocarditis virus and mengovirus cause type 1 diabetes in mice (Yoon 1995).

The human genome contains many retroviral sequences, most of them non-infectious. In the genome of the cells endogenous retroviruses exist as viral DNA and are transmitted vertically to the next generation via germ-line DNA. In NOD mice the endogenous retroviruses are associated with insulinitis and the development of diabetes (Suenaga and Yoon 1988) and retrovirus particles have

been found in the pancreatic beta-cells of these NOD mice (Yoon 1995). It has been reported that IAA detected from type 1 diabetic patients cross-react with retroviral antigen p73 (Hao et al. 1993) and that the sera of patients bound both to insulin and retroviral protein in 64% of the patients. On the basis of mouse experiments and this cross-reaction between IAA and p73, it has been suggested that retroviruses may be involved in the pathogenesis of autoimmune diabetes. It has also been shown that four patients who died soon after the diagnosis of type 1 diabetes had retrovirus particles and lymphocytic infiltration in the pancreatic islets (Yoon 1995). After analysing the retrovirus sequence and synthesising the peptide, the diabetic patients were studied, and 70% of them were positive for peptide antibodies while less than 1% of non-diabetic controls had the retrovirus peptide antibodies (Yoon 1995). However, later studies made on diabetic patients and nondiabetic controls have shown that human endogenous retroviruses are not associated with the development of autoimmune type 1 diabetes in humans (Kim et al. 1999, Knerr et al. 1999), and the role of retroviruses has remained without confirmation.

Cytomegalovirus (CMV) infection is common in children. At the age of 7 months 27% of children and at the age of 8 years 41% of children had CMV antibodies in Finland (Aarnisalo et al. 2003). A child may have the infection during the perinatal or postnatal period having the close connection to the mother or many of them from the breast milk. Fifty-two per cent of breast-feeding mothers have a latent CMV infection (Vochem et al. 1998). The virus reactivates and replicates in the mammary glands and thus leads to excretion into breast milk. The cytomegalovirus is present in 85% of seropositive women's breast milk. In one study 59% of the infants of seropositive mothers had CMV infection. In all of these children CMV infection was caused by CMV transmission from CMV-positive breast milk (Vochem et al. 1998). CMV can also be transmitted transplacentally.

Some case reports have been published on people having both congenital CMV infection and type 1 diabetes. In one of these the child had the congenital CMV infection and developed diabetes at the age of 13 months (Ward et al. 1979). However, these cases are quite rare and no direct connection between CMV and diabetes has been shown. Children with diabetes associated autoantibodies had high titres of anti-CMV IgG antibodies (Nicoletti et al. 1990) and many newly diagnosed patients with type 1 diabetes had the CMV genome in their lymphocytes (Pak et al. 1988) and they had higher CMV IgA antibodies than their controls (Hiltunen et al. 1995) suggesting that persistent CMV infection may be involved in the pathogenesis of type 1 diabetes (Pak et al. 1988). No evidence has been found to support the theory of CMV infection in utero being an altering factor for developing

type 1 diabetes (Ivarsson et al. 1993, Hiltunen et al. 1995). In the follow-up of children who seroconverted ICA positivity no signs of primary CMV infection were found (Hiltunen et al. 1995). Thus there is no causal evidence for the role of CMV in the pathogenesis of type 1 diabetes.

Rotaviruses (RV) commonly cause childhood gastroenteritis. The peptide sequence of the virus protein of RV is very similar to T-cell epitopes in the islet autoantigens GAD and IA-2. This molecular mimicry may cause islet autoimmunity with T-cell activation to RV. An association between autoantibodies associated with diabetes and RV seroconversion was presented in the Australian Baby Diab Study (Honeyman et al. 2000) but a controversial result was included in the Finnish DIPP Study (Blomqvist et al. 2002). Thus this association remains still unclear.

### ***Breast-feeding***

The first report of the correlation between short breast-feeding period and type 1 diabetes was reported in 1984 (Borch-Johnsen 1984). In a meta-analysis of the first 13 studies on breast-feeding and early exposure to cow's milk, Gerstein concluded that there was only a small protective effect of breast-feeding and risk effect with exposure to cow's milk (Gerstein 1994). The relative risk has been found to be higher in children with HLA susceptibility genes for type 1 diabetes who had early exposure to cow's milk or shorter periods of breast-feeding. (Virtanen et al. 1991, Kostraba et al. 1993, Virtanen et al. 1993, Perez-Bravo et al. 1996). In the Type I Diabetes Prediction and Prevention (DIPP) Study higher risk of seroconversion to diabetes-associated autoantibodies was found in infants who had been breastfed exclusively for less than 2 months and in infants who first received cows' milk younger than 2 months old (Kimpimäki et al. 2001b). There is also studies where no associations with development of autoantibodies and infant feeding have been found (Norris et al. 1996, Hummel et al. 2000).

It is postulated that breast milk protects the infant against infections and does not contain foreign proteins and so protects the child from type 1 diabetes. It is also thought that it is not the duration of the breast-feeding that influences the development of the disease, but the early exposure of cow's milk (Åkerblom and Knip 1998). But breast-feeding can also be an independent protective factor (Sadauskaite-Kuehne et al. 2004).



### ***Cow's milk proteins***

Several studies have been published suggesting that early exposure of the infant to cow's milk may predispose the child to type 1 diabetes. Cow's milk contains five principal proteins: caseins (70-80%), beta-lactoglobulin, alpha-lactoglobulin, gamma-globulin and bovine serum albumin (BSA). It has been proposed that ingestion of cow's milk in the neonatal period results in the entry of bovine milk proteins into the circulation because the gastrointestinal mucosa is still immature, which, in turn, starts immunological effects and stimulation of the repertoire of cross-reactive T-cells (reviewed by Åkerblom and Knip 1998).

The gut of the 2-3 months old infant is immature immunologically. In a Finnish nutritional intervention project it was found that oral exposure to proteins like cow's milk proteins, resulted in immune responses (Vaarala et al. 1995). Cow's milk proteins are usually the first foreign proteins in the infant's diet in industrialised countries.

In onset type 1 diabetes patients increased levels of IgA to whole cow's milk proteins and beta-lactoglobulin and increased levels of IgG to beta-lactoglobulin have been found (Savilahti et al. 1993). Increased immunity to cow's milk proteins may occur in selected individuals with susceptibility to type 1 diabetes and may reflect the function of mucosa-associated lymphoid tissue. This may result from the lack of growth factors and cytokines present in human milk (Harrison and Honeyman 1999). It has also been proposed that bovine insulin in cow's milk may generate cross-reactive immunity to human insulin (Vaarala et al. 1998).

## **AIMS OF THE PRESENT STUDY**

The aims of the present study are:

1. to study the role of enteroviruses in the pathogenesis of type 1 diabetes by monitoring enterovirus antibody levels and analysing the frequency of enterovirus infections in children who had a genetic risk of type 1 diabetes and turned positive for diabetes-associated autoantibodies in prospective birth cohort studies
2. to evaluate the effect of different HLA-DR antigen combinations associated with either increased or decreased risk of type 1 diabetes on humoral immune response to enterovirus antigens
3. to study the effect of breast-feeding and maternal enterovirus antibodies on children's risk for enterovirus infections

## SUBJECTS

The subjects in Report II participated in the Childhood Diabetes in Finland (DiMe) Study, the subjects in Report III participated in the Type 1 Diabetes Prediction and Prevention (DIPP) Study and the subjects in Reports I and IV participated in the second pilot study of the Trial to Reduce IDDM in the Genetically at Risk (TRIGR) Study (Table 4).

**Table 4.** Summarization of the materials used in Reports I-IV.

| Report | Study | Cases | Control subjects | Case criteria  | Control subjects criteria  | Follow-up                                     |
|--------|-------|-------|------------------|--|--|---|
| I      | TRIGR | 19    | 84               | autoantibody positive children                           | autoantibody negative children matched for the intervention group, gender and HLA-DQB1 alleles | pregnancy, from birth to the age of 24 months |
| II     | DiMe  | 306   | -                | non-diabetic children, whose sibling had type 1 diabetes | -  | one sample of each child, mean age 10.1 years |
| III    | DIPP  | 21    | 104              | autoantibody positive children                           | autoantibody negative children matched for the time of birth, gender and HLA-DQB1 alleles      | pregnancy, from birth to the age of 24 months |
| IV     | TRIGR | 18    | 132              | autoantibody positive children                           | autoantibody -negative children  | pregnancy, from birth to the age of 36 months |

### DiMe Study

The Childhood Diabetes in Finland (DiMe) Study was conducted between September 1986 and April 1989 in all hospitals in Finland treating children with type 1 diabetes (Tuomilehto et al. 1992). The aim of the family study was to determine the familial occurrence of type 1 diabetes and to assess prospectively the effect of genetic and environmental factors on the risk of the disease in non-diabetic

siblings of the probands. Serum samples were obtained at the diagnosis of type 1 diabetes from a total of 780 patients and 765 siblings.

The study series of the Report II comprised siblings of affected children. These children were grouped according to their HLA-DR allele status. Among the 765 siblings, 20 (11 boys) were homozygous for HLA-DR3, nine (three boys) for HLA-DR2, and 51 (19 boys) for HLA-DR4, whereas 54 (22 boys) were heterozygous for HLA-DR3/ DR4, 56 (25 boys) for HLA-DR2/DRx, 70 (28 boys) for HLA-DR3/DRx, and 46 (27 boys) for HLA-DR4/DRx (x referring to alleles other than DR2, DR3 or DR4). The above 306 siblings were included in the study in Report II and their serum samples were analysed. Their mean age was 10.1 years (range 2.3-19.0). The mean ages of the seven HLA-DR subgroups did not differ.

### **DIPP Study**

The case and control children in Report III were participants of the Type 1 Diabetes Prediction and Prevention (DIPP) Study, which was initiated in Finland in 1994 (Kupila et al. 2001). In this study all babies born at the university hospitals in Turku, Oulu and Tampere were screened for HLA-DQB1 alleles (\*02, \*0301, \*0302, \*0602, \*0603 and \*0604) associated with increased risk for or protection from type 1 diabetes. High risk for developing type 1 diabetes is reported to be in children with HLA-DQB1\*0302/\*02 genotype and moderate risk in children with \*0302/x genotype (x referring to alleles other than \*02, \*0301 or \*0602) (Nejentsev et al. 1999).

The infants carrying the HLA-DQB1\*02/\*0302 or the \*0302/x genotype (x referring to alleles other than \*02, \*0301 or \*0602) were then observed from birth at an interval of 3-6 months over the first 2 years of life and subsequently at an interval of 6-12 months. The follow-up samples were studied for islet cell antibodies (ICA). All samples from children turning positive for ICA were then also analysed for autoantibodies against glutamic acid decarboxylase (GADA), insulin (IAA) and tyrosine phosphatase-related IA-2 protein (IA-2A).

Those 21 children (10 boys) identified as the first ones in the DIPP cohort continuously positive for diabetes-associated autoantibodies were the case subjects in Report III. Eight of them had progressed to Type 1 diabetes by the end of 2001. The last follow-up sample from the remaining 13 children was positive for ICA in all these cases (mean level 54.5 JDFU), IAA in 11 cases (mean level 1.51 RU), GADA in 10 cases (mean level 77.3 RU) and IA-2A in four cases (mean level 16.7 RU).

The last follow-up samples from these 13 cases were thus positive for four autoantibodies in four children, three autoantibodies in six children, two autoantibodies in one child, and one autoantibody in two children (both have now been constantly ICA positive for at least two years).

The case children were born between November 1994 and June 1997, and were followed-up from birth. The mean follow-up time was 20 months (range 9 to 24 months). Samples were drawn at birth (cord blood) and subsequently at 3 to 6-month intervals. Altogether 20 cord blood samples and 123 follow-up serum samples from the case children were available. Nine case children carried the high risk HLA-DQB1\*02/\*0302 genotype (three boys), and 12 cases had the moderate risk HLA-DQB1\*0302/X/x genotype (seven boys).

Three to six (mean five) control children, matched for the time of birth, gender and the HLA-DQB1 alleles, were chosen from the DIPP cohort for each case. The control group comprised 104 children from whom altogether 98 cord blood samples and 562 follow-up serum samples were available. The control children were observed from birth according to the same protocol as the case children. All control children remained constantly negative for ICA.

In addition to the children's samples we also studied serum samples obtained at the end of the first trimester of pregnancy from the mothers of 20 case children and 103 control children.

### **TRIGR Study**

The study population comprised 208 children and their mothers from families with at least one member with type 1 diabetes, the newborn infants having an HLA-DQ genotype indicating increased genetic risk [HLA-DQB1\*0302/\*02, \*0302/\*0603, \*0302/\*0604, \*0302/x or DQB1\*02/y-DQA1\*05/z (x referring to alleles other than \*02, \*0301 or \*0602, y referring to alleles other than \*0301, \*0302, \*0602 or \*0603, z referring to alleles other than \*0201)] (Nejantsev et al. 1999). The families participated in the second pilot study of the TRIGR [Trial to Reduce IDDM (insulin dependent diabetes mellitus) in the Genetically at Risk] Project in Finland (Åkerblom et al. 1999). This pilot study was aimed at evaluating the possible effect of the elimination of cow's milk proteins in early infancy on the appearance of diabetes-associated autoantibodies by the age of 2 years. The study design was double blinded, and the newborn infants were randomized into two groups. When supplementary milk feeding was started, those in the intervention group received a casein hydrolysate formula (Nutramigen®, Mead Johnson & Comp., Evansville, IN, USA) until the age of 6-8 months,

while the infants in the control group were given a regular formula (Enfamil®, Mead Johnson & Comp.). The control formula included 20 % casein hydrolysate formula in order to eliminate the taste and smell difference between the two study formulas. The infants were born between April 1995 and November 1997 in 15 hospitals all around Finland. In addition to the cord blood sample, serum samples were obtained from the children at follow-up visits at the ages of 3, 6, 9, 12, 18, 24 and 36 months. Maternal serum samples had been taken at the end of the first trimester of pregnancy and at the time of delivery. All sera were stored at -20°C until analysed. Written informed consent was obtained from the mother before enrollment. The study was approved by the Joint Ethics Committees of the participating hospitals.

For Report I we selected 103 children from the TRIGR cohort, including 19 index cases and 84 control subjects. The 19 index cases developed signs of progressive  $\beta$ -cell autoimmunity, i.e. positivity for type 1 diabetes-associated autoantibodies by the age of 2 years. Eight of them had progressed to clinical type 1 diabetes by the end of January 2004. Three to five control infants were chosen for each index case (mean four, total 84) according to a nested case-control design matching for the intervention group, gender and HLA-DQB1 genotype. The control infants were observed according to the same protocol as the index cases but remained constantly negative for all four autoantibodies analysed. Analyses were done on children's serum samples taken until the age of two years. A total of 141 samples was available from the case children and 616 samples from control children.

Report IV included 150 infants and their mothers from the same TRIGR cohort, including 18 index cases. From these 150 mothers a breast milk sample was available. The 18 index cases were the subjects who have before the age of 36 months developed signs of progressive beta-cell autoimmunity, i.e. positivity for Type 1 diabetes-associated autoantibodies. Eight of them had progressed to clinical type 1 diabetes by the end of January 2004. Analyses included children's samples until the age of three years, average 27.6 months. Maternal serum samples had been taken at the end of the first trimester of pregnancy, at the time of delivery and three months after delivery during breast-feeding. Breast milk samples were taken at the time of delivery (mean two days (range 0-3 days) after and three months after delivery (1.9-4.0 months)). Only one breast milk sample was available from 66 mothers, both samples from 84 (56%) mothers. Fifty-five mothers had type 1 diabetes.

In Report I were 19 children, who had diabetes-associated autoantibodies and 18 of these

were also included into Report IV. One index child's mother didn't give the breast milk sample and thus the child was excluded from Report IV. In Report IV there were 132 autoantibody -negative children from TRIGR study, 58 of these also included into Report I. Alltogether 76 children from Report I (n=103) included also into Report IV.

## METHODS

### Antibody analyses

#### *Adenovirus antibodies*

IgG and IgA class antibodies against adenovirus hexon protein were measured using enzyme immunoassay (EIA).

Microtitre plates (Nunc Immunoplate, Nunc, Glostrup, Denmark) were coated with the antigen at 1.8 µg/ml concentration in carbonate buffer (pH 9.4). Serum samples were analysed in 1/100 (IgA) and 1/2000 (IgG) dilution in PBS supplemented with 1% bovine serum albumin and 0.05% Tween 20. Binding of antibodies was documented using peroxidase-conjugated anti-human IgG and IgA (P214 and P216 respectively, Dako, Copenhagen, Denmark).

All serum samples of a single child were analysed in the same assay in all reports. The results of the EIA tests were expressed in enzyme immune units (EIU), which show the relative antibody reactivity of the sample compared to the reactivities of positive and negative reference sera included in each assay.

#### *Enterovirus antibodies*

IgG and IgA class antibodies against purified coxsackievirus B4 (CBV4), echovirus 11 (EV11), poliovirus 1 (PV1) and a synthetic enterovirus peptide antigen [sequence KEVPALTAVETGAT-C derived from an immunodominant region of the capsid protein VP1 (Roivainen et al. 1991), known to be a common antigenic determinant for several enteroviruses (Hovi and Roivainen 1993)] were measured using enzyme immunoassay (EIA) (Hyöty et al. 1995). The purified CBV4 and EV11 were incubated at +56°C for 15 min to expose antigenic determinants common for various enterovirus serotypes.

Microtitre plates (Nunc Immunoplate, Nunc, Glostrup, Denmark) were coated with the antigen at 1.0 µg/ml (CBV4, EV11, PV1) and 2.5 µg/ml (bovine serum albumin-conjugated peptide) concentrations in carbonate buffer (pH 9.4). Serum and breast milk samples were analysed in 1/100 (IgA), 1/500 (EV11 IgG) and 1/2000 (other IgG assays) dilution in PBS supplemented with 1% bovine serum albumin and 0.05% Tween 20. Binding of antibodies was documented using



peroxidase-conjugated anti-human IgG and IgA (P214 and P216 respectively, Dako, Copenhagen, Denmark).

All serum samples of a single child were analysed in the same assay in all reports. The results of the EIA tests were expressed in enzyme immune units (EIU), which show the relative antibody reactivity of the sample compared to the reactivities of positive and negative reference sera included in each assay. All samples were analysed in two pits and if the difference between these two results was more than 5%, the sample with earlier and the next one were reanalysed.

IgM class enterovirus antibodies were measured in Report I against a mixture of three enterovirus antigens (coxsackievirus B3 (CBV3), coxsackievirus A16 (CAV16) and EV11) using a capture EIA method, which is a modification of capture radioimmunoassay (RIA) (Hyöty et al. 1995). Monoclonal anti-human IgM antibody-sensitised microtitre plates were purchased from Medix Biochemica (Kauniainen, Finland). Sera were incubated for 90 min at +37°C in 1/100 dilution of PBS + 1% bovine serum albumin + 0.05% Tween 20. After washings, the mixture of heat-treated enterovirus antigens (10 µg/ml for each antigen) was incubated for 60 min at +37°C, and then comparable mixture of biotinylated detection antibodies was added (10 µg/ml for each antigen, 60 min at +37°C). These detection antibodies were produced by immunising rabbits with purified heat-treated CBV3, CAV16 and EV11. The IgG fraction of rabbit hyperimmune sera was purified in a fast protein liquid chromatography system using a protein A column (Pharmacia Fine Chemicals, Uppsala, Sweden) and then coupled with biotin according to standard procedures. After washings, streptavidin-horseradish peroxidase conjugate (Life Technologies, Gaithersburg, MD) was added (30 min at +37°C) and, as the final step, the colour reaction of the orto-phenylenediamine-dihydrochloride substrate (30 min at +37°C) was recorded at 942nm. The samples giving absorbance values that exceeded the cut-off level for antibody positivity (three times the level obtained with PBS instead of the antigen) were reanalysed using host mock-infected cell lysate as antigen and were considered negative if this reactivity exceeded 50% of that obtained with the virus antigen.

Neutralizing antibodies were measured against all coxsackie B serotypes 1-6 (ATCC reference strains) by a standard plaque neutralisation assay (Roivainen et al. 1998) in Report I. The viruses were treated with serial fourfold dilutions of sera for 60 min at +36°C and overnight at room temperature. The serum-treated virus was administered to monolayers of Green monkey kidney cells, and the amount of infectious virus measured by counting the plaques after 46 hours of incubation at +36°C. The reciprocal of the last serum dilution able to block virus infectivity by 80% was taken as

the neutralization titre, in which a fourfold or higher increase was considered significant.

### ***Mumps virus antibodies***

IgA and IgG class serum antibodies to the mumps virus were determined with a solid phase modification of an enzyme immunoassay (EIA). Purified mumps virus antigen was prepared from infected embryonated chicken eggs (Julkunen et al. 1983). To purify the mumps antigen, harvested allantoic fluid was first centrifuged (9500g, 20 min at +5°C) and the supernatant was concentrated by a hollow-fibre liquid concentrator (Amicon: Grace Company, MA, USA). The virus concentrate was purified by centrifugation through a 30% sucrose layer on a 50% sucrose cushion (100000g, 2 hours at +4°C). A visible virus band located between the sucrose layers was collected and dialysed against PBS (phosphate buffered saline) and used as antigen in EIA.

Microtitre plates (Nunc-Immunoplate, Nunc, Kamstrup, Roskilde, Denmark) were sensitised by mumps virus antigen in an optimal concentration (1µg/ml) using overnight incubation at +4°C. Serum samples diluted 1/100 in PBS supplemented by 1 % BSA, 0.05 % Tween 20 and 0.5 M NaCl were incubated for 60 min at +37°C. After washings, rabbit anti-human IgG (1/4000) or IgA (1/1000) (Dako Immunoglobulins, Copenhagen, Denmark) was added and allowed to react for 30 min at +37°C. The plates were again washed and alkaline phosphatase conjugated swine anti-rabbit immunoglobulin (Orion Diagnostica, Espoo, Finland) was added in a 1/100 dilution. After incubation for 2 hours at +37°C 0.3 M NaOH stopped the reaction and the colour reaction was recorded by a Titertek Multiskan spectrophotometer (Eflab, Helsinki, Finland). Antibody levels were expressed in enzyme immunoassay units (EIU) expressing the relative antibody activity of the sample compared with known positive and negative reference sera.

### ***Antibodies against beta-cell antigens***

In Reports I, III and IV children's samples were analysed against beta-cell antigens. Islet cell antibodies (ICA) were determined by a standard immunofluorescence method (Bottazzo et al. 1974) with a detection limit of 2.5 Juvenile Diabetes Foundation units (JDF-U). Insulin autoantibodies (IAA) were quantified with a micro-assay (Ronkainen et al. 2001) modified from the method described by Williams et al. 1997 with a cut-off limit of 1.56 relative units (RU) for antibody positivity. Antibodies to the 65 kilodalton isoform of glutamic acid decarboxylase (GADA) were measured with a radioligand assay as described (Petersen et al. 1994, Savola et al 1998a). The cut-off

limit for GADA positivity was 5.35 RU. Antibodies to the protein tyrosine phosphatase related IA-2 molecule (IA-2A) were analysed with a radiobinding assay as described in detail elsewhere (Bonifacio et al. 1995, Savola et al. 1998b). The cut-off limit for IA-2A positivity was set at 0.43 RU. The cut-off limits for IAA, GADA and IA-2A positivity were set at the 99<sup>th</sup> percentile in more than 370 Finnish non-diabetic subjects. All samples with antibody levels between the 95.5<sup>th</sup> and 99.5<sup>th</sup> percentiles were reanalysed to confirm antibody positivity or negativity.

### **Detection of enterovirus RNA**

Detection of enterovirus genome (Reports I and IV) was completed using extraction of viral RNA from samples, the subsequent reverse transcription, polymerase chain reaction (RT-PCR) and hybridization method (Lönnrot et al. 1999).

RNA was extracted from 140µl of serum or milk sample using QIAamp viral RNA kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. The reverse transcriptase (RT) reaction contained extracted sample RNA, RT-buffer, 0.5mM deoxynucleoside triphosphates, 50pmol of the negative-strand primer (4-) and 20U of Moloney murine leukaemia virus transcriptase enzyme. After incubation (60 min at 37°C) cDNA reaction mixture was added to PCR reaction mixture [containing PCR-buffer, 0.2 mM deoxynucleoside triphosphates, 20 pmol of both negative-strand (4-) and positive-strand (Bio-636+) primers and 1U of DNA polymerase enzyme]. Then the tubes were incubated in Thermal cycler for preheating, 40 cyclers and final heating. Separate rooms were used at each step of the RT-PCR work and each analysis included positive and negative controls.

After an incubation the PCR-product and assay buffer (DELFI) were added into streptavidin-coated microtitre wells, and incubated at room temperature with shaking for 30 min. Then the wells were washed with DELFIA Wash Solution. The bound DNA was denatured with NaOH and then nonbiotinylated strands were washed. Hybridization mixture (containing Eu-labelled probe 755, DELFIA assay buffer, 0.85M NaCl and 1% Tween) was added to the wells and the reaction was carried out for 2 hours at 37°C. For the detection of the EU signals DELFIA Enhancement Solution was added to the wells and shaken for 25 min. Time-resolved fluorescence was measured using a VICTOR<sup>TM</sup>1420 Multilabel Fluorometer (EG &G Wallac Oy).

All positive samples were confirmed to be positive by repeated RT-PCR and subsequent

hybridization assay.

### **Diagnostic criteria of enterovirus and adenovirus infections**

Infections were diagnosed by a two-fold or greater increase in the antibody level against any of the antigens and exceeding the cut-off level for seropositivity (15 EIU for IgG and IgA assays) between two sequential serum samples in the EIA analyses. In the capture-IgM assays similar criteria were used (the cut-off level for seropositivity was three times the level obtained with PBS instead of the antigen). In the neutralization assay a four-fold or greater increase in the titre of antibodies was considered significant. The presence of enterovirus RNA in child's or in mother's serum was taken as a marker of current infection. Fulfillment of one or more of these criteria was taken as a marker of acute infection.

### **HLA-DQ typing**

In Report II the HLA genotypes (HLA-DR alleles) were determined by conventional serology (Tuomilehto-Wolf et al. 1989). In Reports I, III and IV the HLA-DQB1 genotype was defined from cord blood specimens by a method based on the polymerase chain reaction for amplifying the gene segment and subsequent hybridization with lanthanide-labelled sequence-specific oligonucleotide probes (Sjöroos et al. 1995). Alleles conferring disease susceptibility (HLA-DQB1 \*0302 and \*02) and those associated with protection (HLA-DQB1 \*0301, \*0602 and \*0603) were defined. Children carrying DQB1 \*02/0302 and DQB1 \*0302/X (X≠ \*0301, \*0602 or \*0603) were included in the follow-up cohort in Report III. Children carrying DQB1 \*02/0302, DQB1 \*0302/X (X≠ \*0301, \*0602 or \*0603) and DQB1 \*02/X genotypes were included in the follow-up cohort in Reports I and IV.

### **Statistical analyses**

In Report I the differences in the infection frequency observed during the follow-up between the case and matched control children were tested using the Score-test (Stata Statistical Software), differences in the occurrence of at least one infection during a given follow-up period between the case and

control children were assessed using the Mantel-Haenszel odds ratio (multiple and varying number of control subjects per case, Stata Statistical Software), differences in the infection frequency between the case and control children in the two formula groups were tested using conditional logistic regression.

In Reports I and III comparisons of the virus antibody levels between the case and control groups were done using the area under the curve -method (AUC –method) (Matthews et al. 1990). The area under the antibody level curve of each case child was compared with the mean area under the antibody level curve of the matched control children. The follow-up periods of the case and the control children were identical. Analyses were made from two periods: from pregnancy to the age of 6 months and from the age of 6 to 24 months, as in IgG antibodies the first period represents mostly maternal antibodies and the second infant's de novo synthesized IgG antibodies.

In Report II the Kruskal-Wallis test was used in comparisons between all HLA-DR groups and comparisons between two HLA-DR groups were made using Mann-Whitney U-test or Chi-square test.

In Report IV the ages of the infection, the frequency of infections and enterovirus antibody levels were compared using the Mann-Whitney U-test and correlations were analysed using Spearman's correlation.

In all Reports a  $p$  value less than 0.05 was considered statistically significant.

## RESULTS

### **Diagnosis of enterovirus infections in prospective series (Reports I and IV)**

Enterovirus infections were analysed during the follow-up measuring enterovirus IgA and IgG antibody levels against CBV4, echovirus 11 and a synthetic enterovirus peptide antigen, IgM class enterovirus antibodies against a mixture of three enterovirus antigens (CBV3, CAV16 and EV11), neutralizing antibodies against coxsackie B serotypes 1-6 and detecting enterovirus RNA from serum samples in Report I. In Report IV enterovirus infections were analysed measuring enterovirus IgA and IgG antibody levels against CBV4, echovirus 11 and a synthetic enterovirus peptide antigen and detecting enterovirus RNA from serum samples.

Enterovirus infections were common in the young children participating the prospective TRIGR study. They were detected in 16% of all sample intervals (Report I) and over 70% of the children had had at least one infection by the age of three years (Report IV). In Report I altogether 107 enterovirus infections were diagnosed in 103 children during the follow-up (mean follow-up time 21 months, range 6-24 months) and in Report IV 149 enterovirus infections were diagnosed in 150 children during the follow-up (mean follow-up time 27 months, range 6-36 months). In Report IV 73 children had one infection, 35 children had two and two had three infections during the follow-up. In Report I the enterovirus infections were most frequent at the age of 12 and 18 months and in Report IV median age at infection was 18 months.

Enterovirus infections were mostly diagnosed according to significant increasing of EV11 IgG levels (46% of infections) and CBV4 IgG levels (20% of infections). Enterovirus RNA positivity was most common before the age of one year and it was seen in 23% of all enterovirus infections. (Report I)

The duration of the IgG responses varied a lot, depending of the serotype causing an infection and against which serotype the antibody response was measured (Report I). If the same serotype that was used as the antigen in the enzyme immunoassay test caused an infection, the response was seen longer than if the serotype was different.

Boys (n=63) had more enterovirus infections than girls (n=40) (1.21 vs 0.80 infections per child during the follow-up,  $p=0.02$ ). Children with HLA-DQB1\*02/X allele had fewer enterovirus infections (0.91 infection per child) than than children with HLA-DQB1\*0302/X allele (1.15

infections per child) and children with both risk alleles (1.11 infections per child) ( $p=0.02$ ). (Report I)

Adenovirus infections were diagnosed in 8% of the sample intervals (49/653) and the peak incidence was at 18 months of age. The frequency of adenovirus infections did not significantly differ between boys and girls (0.52 vs. 0.40 infections respectively, NS) or in different HLA-DQB1 groups (0.56 infections in children with \*02/X allele, 0.41 in children with \*0302/X allele, 0.43 in children with both risk alleles, NS). (Report I)

### **Effect of HLA on humoral immune response to enteroviruses (Report II)**

Enterovirus antibody levels were measured against CBV4, poliovirus 1 and mumps virus from children having seven different HLA-DR antigen combinations. These HLA-DR antigens are associated with either increased (DR3 and DR4) or decreased risk (DR2) of type 1 diabetes. The children participated in the Childhood Diabetes in Finland (DiMe) Study and they were siblings of type 1 diabetic patients.

CBV IgG antibody levels showed significant variation between the seven HLA-DR groups (Kruskal-Wallis test,  $p=0.006$ ). Children with the HLA-DR3 allele (DR3/3, DR3/x), children with the HLA-DR4 allele (DR4/4, DR4/x) and children with both risk alleles had higher levels than children with the protective HLA-DR2 allele (DR2/2, -DR2/x) ( $p=0.01$ ,  $p=0.01$  and  $p=0.008$  for each comparison respectively).

CBV IgA levels did not significantly differ between different HLA-DR groups, but DR3-positive children tended to have higher and DR2-positive children lower antibody levels than other children (NS).

High responders (antibody levels higher than the 75 percentile of all samples analysed) for CBV4 IgG were most frequent among DR3/DR4-heterozygous children (32%) and most infrequent (12%) among children carrying the DR2 allele (DR2/2 and DR2/x, compared to DR3/4  $p<0.02$ ). The frequency of high responders among the children carrying the DR4 allele (DR4/4, DR4/x) was 28 % ( $p<0.03$  compared to those with DR2) and 25 % among the children with the DR3 allele ( $p<0.04$  compared to those with DR2). Overall, 27% of the children, positive for DR3 and/or DR4 were high responders compared to 12% of the children with DR2 ( $p<0.009$ ).

High responders for PV1 IgG were most frequent among DR4-positive children (30%) and most infrequent among DR2-positive children (10 %,  $p<0.05$ ). Twenty-three percent of the DR3-

positive children and 27% of the DR3/DR4-heterozygous children respectively were high responders ( $p<0.1$  and  $p<0.08$  compared to DR2). Altogether 27% of the children positive for either DR3 or DR4 were high responders compared to 10% of the DR2-positive children ( $p<0.03$ ).

High responders for mumps virus IgG were most prevalent among the children carrying the DR2 allele (34% of the DR2-positive children) and least common among children carrying the DR3 allele (18%) ( $p<0.01$ ). The frequency of high responders among the DR4-positive children was 27 % ( $p<0.02$  compared to DR2-positive subjects) and 21 % among the children with both DR3 and DR4 ( $p<0.2$  compared to DR2). No differences were observed in the mumps IgA levels, and the levels were quite low in all seven HLA-DR groups.

Boys had lower CBV4 IgG levels ( $p=0.02$ ) and PV1 IgG levels ( $p=0.02$ ) than girls. This phenomenon was seen particularly in the HLA-DR3/4 ( $p=0.03$ ), HLA-DR3/3 ( $p=0.05$ ) and HLA-DR4/x ( $p=0.03$ ) groups. There was no gender difference in mumps antibody levels.

### **Humoral immune response to enteroviruses in children with diabetes associated autoantibodies (Reports I and III)**

In Reports I and III the enterovirus antibody levels were analysed using the area under the curve - method (AUC). The area under the antibody level curve of each case child was compared with the mean area under the antibody level curve of the matched control children (Fig. 1). In Report I we analysed 103 children from the TRIGR cohort, including 19 index cases and 84 control subjects. Case children were positive for diabetes-associated autoantibodies and their control children were matched for the intervention group, gender and HLA-DQB1 genotype and they were autoantibody-negative. In Report III we analysed 21 case children identified as the first ones in the DIPP cohort continuously positive for diabetes-associated autoantibodies and 104 control children, who were matched for the time of birth, gender and the HLA-DQB1 alleles. In both series the average IgG class enterovirus antibody levels were quite high in maternal samples and antibody levels in maternal samples and cord blood correlated ( $r=0.94$  for CBV4 IgG in Report I) reflecting transplacental transfer of antibodies. After the birth IgG antibody levels decreased during the first 6 months of life being then almost unmeasurable unless the child had an enterovirus infection during that time. IgA antibodies were not detected in cord blood in any of these children. After the age of 6 months the IgA and IgG antibody levels increased after the children acquired their first enterovirus infection.



In Report III the enterovirus antibody levels in the children who later developed diabetes-associated autoantibodies (case children) were comparable to those in their control children during pregnancy and the first 6 months of life. However, between 6 and 24 months of age the case children had higher antibody levels than the control children; CBV4 IgG ( $p=0.002$ ), CBV4 IgA ( $p=0.05$ ), EV11 IgG ( $p=0.03$ ), EV11 IgA ( $p=0.005$ ) and peptide IgG ( $p=0.03$ ). In Report I IgG antibody levels to EV11 were higher in case than control children ( $p=0.0009$ ) during the whole follow up including the pregnancy, but antibodies to the other enterovirus antigens (CBV4 and enterovirus peptide) did not differ between the case and control children.

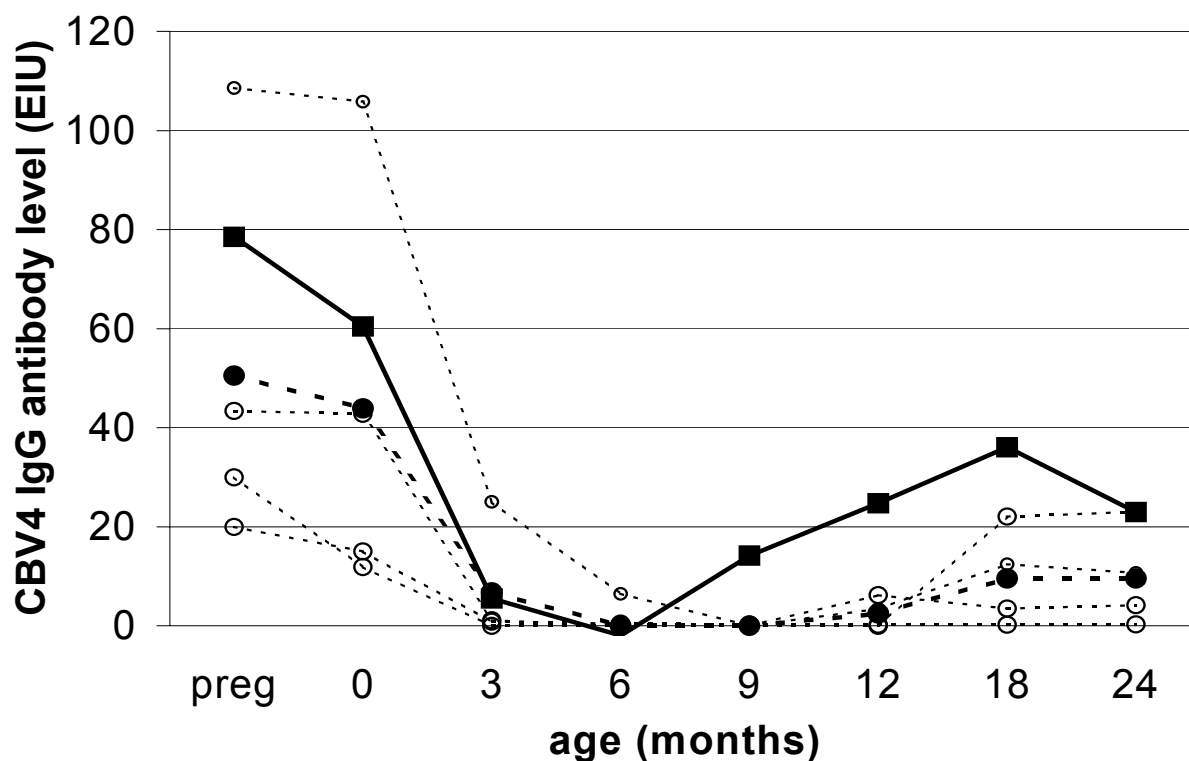


Figure 1.

The level of IgG class antibody to Coxsackievirus B4 in case child, the control children and the mean curve of the control children. In the area under the curve test each case child's curve was compared to control children's mean curve. ■ case child, ○ control child, ● control children mean curve

The difference between the case and the control children in enterovirus antibody level was greater in males than in females and in children with the HLA-DQB1\*0302/x than \*02/\*0302 genotype (Report III). Poliovirus and adenovirus antibody levels did not differ between the case and the control children (Report III).

### **Frequency of enterovirus infections in children who developed diabetes-associated autoantibodies (Reports I and IV)**

The case children in Reports I and IV were positive for diabetes-associated autoantibodies. The control children were matched for the intervention group, gender and HLA-DQB1 genotype. Enterovirus infections were diagnosed by measuring enterovirus IgA and IgG antibody levels against CBV4, echovirus 11 and a synthetic enterovirus peptide antigen and by detecting enterovirus RNA from serum samples. In Report I also IgM class enterovirus antibodies against a mixture of three enterovirus antigens (CBV3, CAV16 and EV11) and neutralizing antibodies against coxsackie B serotypes 1-6 were also analysed.

The overall frequency of enterovirus infections was higher in case than in control children. In Report I the autoantibody positive and negative children had comparable number of diagnosed enterovirus infections (19% vs. 15% of sample intervals), but autoantibody positive children had more often enterovirus RNA in their serum (6.8% vs. 3.2% of the samples,  $p=0.03$ ). The time preceding the first appearance of autoantibodies was analysed separately, and the autoantibody positive children had more enterovirus infections during that period compared to the control children (0.83 vs. 0.29 infections per child,  $p=0.01$ ). Enterovirus RNA was found in 14.0% of the case children's samples and in 8.4% of the control children's samples during this period. ( $p=0.09$ ). Only two children in the whole cohort were positive for enterovirus RNA in two consecutive serum samples and both of them were case children. One of them was repeatedly RNA positive at the age of 9 and 12 months, and the first autoantibody positive sample was taken at the age of 12 months. The other was RNA positive in cord-blood and at the age of 3 months, 12 months before autoantibodies appeared and the child again had enterovirus infection. The frequency of adenovirus infections did not differ between the case and control children (0.26 vs 0.20 per child respectively, NS) during the time period before the seroconversion.

The difference in the frequency of enterovirus infections between the case and control children was seen in males but not in females. In Report I enterovirus RNA was detected in 67% of male cases compared to 24% of male controls ( $p=0.007$ ). In females there was no such difference (14% of the case children and 18% of the control children).

The case children with HLA-DQB1\*0302/X had more enterovirus infections than their control children (1.71 vs 1.03 infections per child,  $p=0.04$ ). Such differences was not observed in other two HLA risk allele groups (DQB1\*0302/\*02 and DQB1\*02/X).

There were 13 CBV infections based on the neutralizing antibody test and all these infections were in control children. Altogether 12% of all infections were diagnosed using these neutralizing antibodies. One child had CBV1, two had CBV2, three had CBV3 and CBV4 and four had CBV5 infection. None of the children had CBV6 infection according to the neutralizing antibody test. The summary of case children's enterovirus infections and autoantibody seroconversions from Report I are presented in Fig. 2.

In Report IV 18 children with autoantibodies had 19 enterovirus infections. They had their infections earlier than the other children (median 12 months vs 18 months,  $p=0.02$ ). All these 18 case children were included in the material of Report I.

Age (months)

| case | F/M | HLA      | Formula |        | Preg | 0 | 3 | 6 | 9 | 12 | 18 | 24 | type 1 diabetes |
|------|-----|----------|---------|--------|------|---|---|---|---|----|----|----|-----------------|
| 1    | F   | *0302/X  | RF      | EV PCR |      | - | - | - |   |    |    |    | 20 months       |
|      |     |          |         | EV ab  | +    | - | - | - |   |    |    |    |                 |
|      |     |          |         | Autoab |      | - | - | 1 |   |    |    |    |                 |
| 2    | M   | *02/X    | RF      | EV PCR |      | - | - | - | + | +  | -  | -  | non-diabetic    |
|      |     |          |         | EV ab  | -    | - | - | - | - | -  | +  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | - | 1  | 4  | 4  |                 |
| 3    | M   | *02/X    | RF      | EV PCR |      | - | - | - | - | -  | -  | -  | non-diabetic    |
|      |     |          |         | EV ab  | -    | - | - | - | - | +  | -  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | - | -  | 2  | 1  |                 |
| 4    | M   | *02/0302 | RF      | EV PCR |      | - | - | - | + | -  | -  | -  | 57 months       |
|      |     |          |         | EV ab  | -    | - | - | - | - | -  | +  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | 1 | 2  | 4  | 4  |                 |
| 5    | M   | *02/X    | RF      | EV PCR |      | - | - | - | - | -  |    |    | 12 months       |
|      |     |          |         | EV ab  | -    | - | - | - | - | -  |    |    |                 |
|      |     |          |         | Autoab |      | - | - | - | 2 | 3  |    |    |                 |
| 6    | M   | *02/X    | RF      | EV PCR |      | - | - | - | - | +  | -  | -  | 36 months       |
|      |     |          |         | EV ab  | -    | - | - | - | - | +  | -  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | 3 | 3  | 4  | 4  |                 |
| 7    | M   | *0302/X  | RF      | EV PCR |      | - | - | - | - | -  | -  | -  | 80 months       |
|      |     |          |         | EV ab  | -    | - | - | - | - | -  | +  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | 1 | 1  | 1  | 2  |                 |
| 8    | M   | *0302/X  | RF      | EV PCR |      | - | - | - | + | -  | -  | -  | 57 months       |
|      |     |          |         | EV ab  | -    | - | - | - | - | +  | +  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | 1 | 1  | 2  | 2  |                 |
| 9    | F   | *02/0302 | RF      | EV PCR |      | - | - | - | - | -  | -  | -  | non-diabetic    |
|      |     |          |         | EV ab  | -    | - | - | - | - | -  | -  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | 1 | 1  | -  | -  |                 |
| 10   | F   | *02/0302 | RF      | EV PCR |      | - | - | - | - | -  | -  | -  | non-diabetic    |
|      |     |          |         | EV ab  | -    | - | - | - | - | -  | -  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | - | -  | -  | 1  |                 |
| 11   | F   | *02/X    | RF      | EV PCR |      | - | - | + | - | -  | -  | -  | non-diabetic    |
|      |     |          |         | EV ab  | +    | - | - | - | - | -  | -  | -  |                 |
|      |     |          |         | Autoab |      | - | - | - | - | 1  | 3  | 4  |                 |

| Age (months) |     |          |         |        |      |   |   |   |   |    |    |    |                 |
|--------------|-----|----------|---------|--------|------|---|---|---|---|----|----|----|-----------------|
| case         | F/M | HLA      | Formula |        | Preg | 0 | 3 | 6 | 9 | 12 | 18 | 24 | type 1 diabetes |
| 12           | F   | *0302/X  | RF      | EV PCR |      | - | - | - | - | -  |    |    | non-diabetic    |
|              |     |          |         | EV ab  | -    | - | - | - | - | -  |    |    |                 |
|              |     |          |         | Autoab |      | - | - | - | - | 1  |    |    |                 |
| 13           | M   | *0302/X  | RF      | EV PCR |      | - | - | - | + | -  | -  | -  | 48 months       |
|              |     |          |         | EV ab  | +    | - | - | - | - | +  | -  | +  |                 |
|              |     |          |         | Autoab |      | - | - | - | - | -  | 1  | 1  |                 |
| 14           | M   | *0302/X  | RF      | EV PCR |      | + | + | - | - | -  | -  | -  | non-diabetic    |
|              |     |          |         | EV ab  | +    | - | - | - | - | +  | -  | -  |                 |
|              |     |          |         | Autoab |      | - | - | - | - | -  | 1  | 1  |                 |
| 15           | M   | *02/0302 | CHF     | EV PCR |      | - | - | - | - | -  |    | -  | 13 months       |
|              |     |          |         | EV ab  | -    | - | - | - | - | +  |    | -  |                 |
|              |     |          |         | Autoab |      | - | - | - | - | 3  |    |    |                 |
| 16           | M   | *02/0302 | CHF     | EV PCR |      | - | - | - | - | +  | -  | -  | non-diabetic    |
|              |     |          |         | EV ab  | -    | - | - | - | - | +  | -  | -  |                 |
|              |     |          |         | Autoab |      | - | - | - | 1 | 1  | 4  | 4  |                 |
| 17           | M   | *0302/X  | CHF     | EV PCR |      | - | - | - | + | -  | -  | -  | non-diabetic    |
|              |     |          |         | EV ab  | +    | - | - | - | + | -  | -  | -  |                 |
|              |     |          |         | Autoab |      | - | - | - | 1 | -  | -  | -  |                 |
| 18           | F   | *0302/X  | CHF     | EV PCR |      | - | - | - | - | -  | -  | -  | 65 months       |
|              |     |          |         | EV ab  | +    | - | - | - | - | -  | -  | -  |                 |
|              |     |          |         | Autoab |      | - | - | - | 3 | 3  | 4  | 3  |                 |
| 19           | F   | *02/X    | CHF     | EV PCR |      | - | - | - | - | -  | -  | -  | non-diabetic    |
|              |     |          |         | EV ab  | +    | - | - | - | + | -  | -  | -  |                 |
|              |     |          |         | Autoab |      | - | - | - | 1 | 2  | 2  | 2  |                 |

Figure 2. Follow-up of the case children in Report I. F/M = Female or Male, HLA = HLA-DQB1 allele, either HLA-DQB1\*02/0302 or \*0302/X or \*02/X, RF = Regular Formula, CHF = Casein Hydrolysed Formula, EV PCR = Enterovirus infection analysed based on RT-PCR test (Enterovirus RNA in blood sample), EV ab = Enterovirus infection analysed based on enterovirus antibody rise, Autoab = how many diabetes-associated autoantibodies are positive, Preg = Pregnancy, type 1 diabetes = the onset of the disease.

### **Maternal enterovirus infections during pregnancy (Reports I and IV)**

Maternal enterovirus infections during pregnancy have been associated with increased risk of type 1 diabetes in the offspring (Table 5 in page 64).

In Report I enterovirus infections were diagnosed during pregnancy by measuring enterovirus IgA and IgG antibody levels against CBV4, echovirus 11 and a synthetic enterovirus peptide antigen and IgM class enterovirus antibodies against a mixture of three enterovirus antigens (CBV3, CAV16 and EV11) and by detecting enterovirus RNA from serum samples. In Report IV enterovirus infections were diagnosed by measuring enterovirus IgA and IgG antibody levels against CBV4, echovirus 11 and a synthetic enterovirus peptide antigen and detecting enterovirus RNA from serum samples.

In the present study we found that enterovirus infections are frequent during pregnancy as 15% of mothers experienced infection (Report IV). In addition 5% of mothers had an infection during the first months after delivery when the most of the mothers were breast-feeding the baby. In Report I seven mothers of the case children (37%) and 17 mothers of the control children (20%) had an enterovirus infection during pregnancy [odds ratio (OR) 2.4, 95% CI 0.9-7.0]. These same seven mothers of children with autoantibodies (overall 18 mothers) were included in Report IV having a breast milk sample. Comparing them to all 132 mothers of autoantibody-negative children in Report IV, they had more enterovirus infections during pregnancy (39% vs 12% of the mothers,  $p=0.003$ ). Enterovirus RNA was found in eight mothers' serum samples (six during pregnancy and two during breast-feeding) in Report IV. One of these mothers having enteroviral RNA during breast-feeding had type 1 diabetes, and four of six mothers having enteroviral RNA during pregnancy had a child who developed diabetes associated autoantibodies.

In Report IV the maternal IgG class enterovirus antibody levels were lower in serum at delivery compared to 3 months later or early pregnancy (median CBV4 IgG levels 29.2, 40.8 and 44.3 EIU respectively,  $p=0.006$ ). Similar variation was seen in echovirus 11 ( $p=0.001$ ) and in enterovirus peptide ( $p=0.007$ ) antibodies. A strong correlation was seen between IgG enterovirus antibody levels in cord blood and in maternal serum (CBV4  $r=0.94$ , enterovirus peptide  $r=0.90$ , EV11  $r=0.86$ ) at delivery.

#### **Effect of breast-feeding and maternal antibodies on enterovirus infections in children (Report IV)**

Duration of breastfeeding varied considerably. Infants were fed exclusively with breast milk for an average of 2.6 months (range 0-6 months) and 18 % of the children were exclusively breast-fed for longer than 4 months. The average duration of the whole breast-feeding period (exclusive and non-exclusive) was 8.1 months (range 0.15-23 months). Seventy percent of children had breast milk for more than 6 months and 12 % for more than a year. The average duration of breast-feeding (exclusive or total) was the same in autoantibody positive and negative children.

Enterovirus IgA antibody levels against a synthetic enterovirus peptide antigen were analysed from the breast milk samples. Breast milk samples taken a few days after delivery had higher IgA class enterovirus antibody levels than breast milk samples taken three months later (median 37.8 vs. 11.8 EIU,  $p=0.0001$ ). However, antibody levels exceptionally increased clearly in five of the mothers, which may reflect recent or ongoing enterovirus infection in these mothers. In fact, an enterovirus infection was diagnosed during the pregnancy in two of them. This was also found when the mothers who had enterovirus infection during pregnancy were compared to other mothers - the former group had higher IgA class enterovirus antibody levels in breast milk (median 76.4 vs. 37.7 EIU,  $p=0.005$ ).

Breast-feeding was associated with low frequency of enterovirus infections in the child. Children who had breast milk as a single nutrient two weeks or less ( $N=38$ ) had more enterovirus infections in the first year of life compared to children who had breast milk exclusively for over two weeks (0.59 inf/child compared to 0.38 inf/child,  $p=0.04$ ). In older children (one to three years) breast-feeding habits (breast-fed under or over two weeks) showed no correlation to enterovirus infections (0.54 vs 0.49 inf/child, NS). Children who had their first enterovirus infection before the age of 1 year had a shorter period exclusive breast-feeding than children who had no enterovirus infection during their first year of life (median 2 vs 3 months,  $p=0.05$ ), while the total breast-feeding period did not differ (median 8 vs 9 months, NS). Children who had enterovirus RNA in serum before the age of one year had shorter time of exclusive breastfeeding (median 1 vs 3 months,  $p=0.04$ ). Children whose mothers had high enterovirus IgA levels (over 75th percentiles) in breast milk at delivery had fewer enterovirus infections by the age of one year (0.33 vs. 0.67 infections per child,  $p=0.001$ ).

Present of enteroviral RNA was also analysed from breast milk samples using RT-PCR. None of the breast milk samples was positive for enterovirus RNA.

As a clear correlation was seen between enterovirus antibody levels in breast milk and in mothers' serum [serum CBV4 IgA compared to milk IgA at delivery  $p=0.009$  (Fig. 3) and serum CBV4 IgG compared to milk IgA at delivery  $p=0.03$ ], we wanted to analyse if maternal antibodies in breast milk are more important than antibodies transferred from mother to the foetus during the pregnancy in the protection of the child against enterovirus infections. Therefore we analysed the effect of these antibodies in two groups. One group ( $N=38$ ) included children who were exclusively breast-fed for such a short period (less than two weeks), which would not be able to give significant protection to the child (see above) while the other group ( $N=112$ ) included children who were breast-fed for a longer period. In the group with short duration of breast-feeding, the levels of enterovirus antibodies in the maternal serum (or breast milk) had no effect on the frequency of enterovirus infections of the child. However, in the group with longer duration of breast-feeding, the mothers whose children experienced an enterovirus infection by the age of 1 year had lower maternal enterovirus antibody levels both in serum and in breast milk at delivery than the mothers whose children did not experience an infection by that age (medians in serum CBV4 IgG 29.9 vs. 50.2 EIU,  $p=0.007$  and in breast milk IgA 28.8 vs. 38.2 EIU,  $p=0.01$ ). The same was true for the maternal samples taken 3 months after delivery (medians in serum CBV4 IgG 28.2 vs. 68.0 EIU,  $p=0.005$ ).



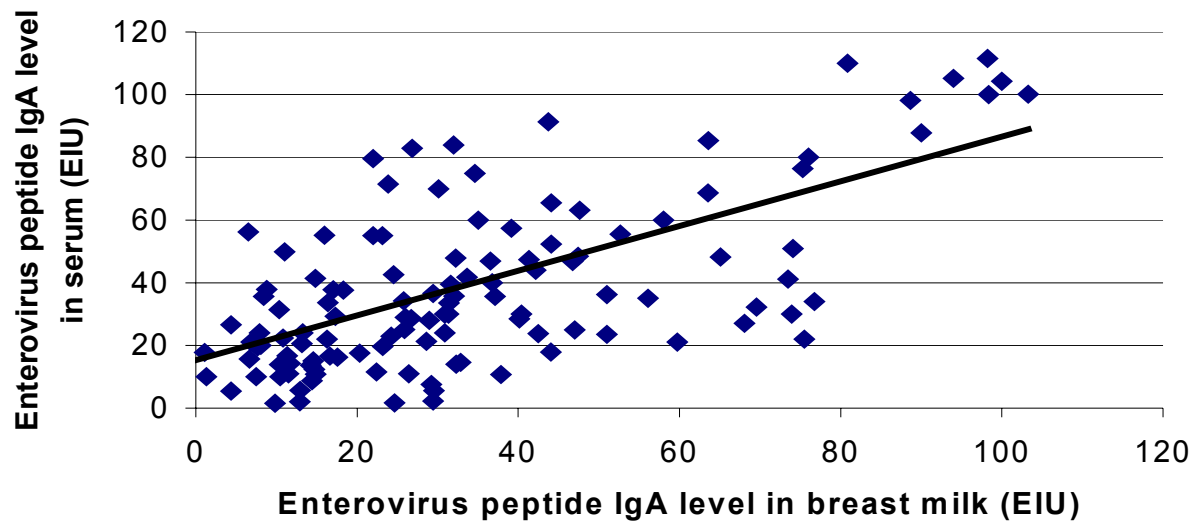


Figure 3. A correlation between enterovirus antibody levels in breast milk and in mothers' serum

Children of mothers who had particularly high CBV4 antibody levels (exceeding the 75th percentiles) at delivery had fewer enterovirus infections before the age of one year compared to children of mothers with lower antibody levels (0.32 vs. 0.56 infections per child;  $p=0.01$ ). The same was true for enterovirus peptide antibodies (0.38 vs. 0.60 infections per child, respectively;  $p=0.001$ ).

Mothers of autoantibody positive children had lower enterovirus antibody levels both in serum and in breast milk at delivery compared to mothers of autoantibody negative children (serum CBV4 IgG 16.0 vs. 34.7 EIU  $p=0.03$  and milk IgA 39.7 vs. 50.7 EIU  $p=0.04$ ).

Duration of breastfeeding was the same in mothers with type 1 diabetes and in unaffected mothers, but mothers with type 1 diabetes ( $N=55$ ) had higher enterovirus antibody levels in breast milk at delivery (median IgA 54.1 vs 34.1 EIU,  $p=0.03$ ) and three months later (median IgA 23.5 vs 8.9 EIU,  $p=0.03$ ) than non-diabetic mothers. Children of mothers with type 1 diabetes had fewer enterovirus infections than children of non-diabetic mothers (0.86 vs. 1.09 infections per child,  $p=0.04$ ) and their infections occurred later (median age at infection: 18 vs. 12 months,  $p=0.004$ ).

### **The effect of infant formula on enterovirus infections (Report I)**

In Report I five of the case children and 15 of the control children were in the intervention group receiving a casein hydrolysate formula. The remaining 14 case children and 69 control children were in the group receiving regular formula. In the casein hydrolysate group case children had 1.4 enterovirus infections per child during the follow-up compared to 1.16 infections per child in the corresponding control subjects receiving the same formula (NS). Case children in the regular formula group had 1.14 enterovirus infections per child compared to 0.96 infections per child in their control children (NS). Conditional logistic regression analysis showed no effect of intervention on the frequency of enterovirus infections ( $p=0.3$ ). All five case children receiving casein hydrolysate formula had at least one enterovirus infection during the follow up compared to 64% (9/14) of case children receiving the regular formula ( $p=0.09$ ) and 72% (18/25) of their control children ( $p=0.1$ ).

## DISCUSSION

### Diagnosis of enterovirus infections

Enterovirus infections are known to be the common cause of a respiratory and gastrointestinal infections. According to the present study, over 70% of children have at least one infection before the age of three years. This is in line with previous observations indicating that enterovirus infections are common in this age-group (Juhela et al. 1998).

As enterovirus infections are usually subclinical and may manifest with various symptoms laboratory tests are needed to confirm the infection. We used several tests to analyse enterovirus infections. According to Report I the infections are seen best using EV11 IgG test; almost half of the infections were diagnosed using this test. There are over 60 human enterovirus serotypes and even if certain cross-reactivity exists between these serotypes, the detection of all serotypes by antibody assays is very difficult. The detection of enterovirus RNA in blood would be the most accurate, but the time that virus in the child is detectable may be very short. In this study only 23% of infections were diagnosed by the presence of viral RNA in serum. The samples were taken from children at 3 to 6 month intervals. Enterovirus infections were most frequently diagnosed at the age 12 to 18 months using serology and at the age of 9 to 12 months using RNA detection. Some children had enterovirus infections soon after birth. Those infections were diagnosed by the presence of enteroviral RNA in sera taken at the age of 3 months and in a few cases viral RNA was already observed in cord blood indicating possible infection in utero. Enteroviruses are known to be able to spread to the fetus. Poliovirus infection of the fetus has been documented, and an increased incidence of premature delivery and fetal deaths have been reported. There is some evidence that some coxsackie- and echoviruses may cross the placental barrier during pregnancy, leading to increased rate of premature abortion and high mortality after delivery (reviewed in Abzug 1995). Maternal antibodies to CBV3 do not protect stillbirth against CBV infection but protect neonates against severe infections. Maternal CBV antibodies transmitted to the offspring through milk also protect neonates against CBV infections (Martino et al. 1995). In the present study was noticed that before the age of 6 months maternal antibodies present in children's serum make it difficult to diagnose infant's infection using serology and at this age the detection of enteroviral RNA is the most appropriate test.

The variation in enterovirus antibody responses was partly due to the difference of the virus

serotype causing the infection and the serotype used as an antigen in the antibody test. If the same serotype was causing the infection than that used as a antigen, the response was longer and clearer than if a different serotype was used as an antigen. There are also differences in immune responses between different individuals and the enterovirus infection may also differ in severity and duration. If the infection spreads widely in the body, as in meningitis, the response may be longer and antibody levels rise very high (Reviewed by Tracy et al. 1995).

In Reports I and IV the screening of enterovirus infections was mostly based on a panel of EIA tests, which were designed to detect several enterovirus serotypes but not to differentiate them from each other. In previous studies particularly coxsackievirus B serotypes 4 and 5 have been connected with Type I diabetes, but other enterovirus serotypes might also be diabetogenic (Hiltunen et al. 1997, Roivainen et al. 1998, Frisk et al. 1992). In the Report I coxsackie B group infections were not detected in any of the autoantibody positive children when neutralizing antibodies were measured against coxsackievirus B reference strains (ATCC strains). This is in line with the finding that echovirus IgG but not coxsackievirus IgG was increased in autoantibody positive subjects in Report I. However, coxsackievirus B serotypes were detected in 15% of autoantibody negative children confirming their circulation in the population. This results suggest that either coxsackievirus B serotypes are not the only diabetogenic enterovirus serotypes or diabetogenic coxsackievirus B strains do not cross-neutralize ATCC reference strains in this kind of assays.

It is difficult to completely exclude the possibility that some of the enterovirus infections diagnosed may have been due to false posive findings caused e.g. by cross-reactive antibodies induced by other virus infections or contaminations in enterovirus RT-PCR. Therefore, larger series should be analysed to confirm the higher frequency of enterovirus infections in autoantibody positive children. On the other hand, all enterovirus infections were not probably detected even though a relatively wide panel of assays was used. In any case, the fact that both the case and her/his control subjects were analysed simultaneously in the same run diminish the possibility that these factors could have biased comparisons between the case and control groups.

### **Genetic regulation of enterovirus specific immune response**

HLA genotype is known to modulate the course of virus infections. A specific HLA genotype may have a stronger immune response to pathogens and regulate susceptibility to infections. Some HLA alleles are known to be associated with persistent viral infections and autoimmunity, for example individuals homozygous for HLA-DR3 are prone to chronic hepatitis B (reviewed by Tracy et al. 1995). Susceptibility to poliomyelitis is found to be polygenic, and the paralytic form of poliomyelitis has found to be HLA dependent (Wyatt 1975a, Wyatt 1975b, van Eden 1983).

In previous studies a decreased frequency of T-lymphocytes able to respond to mumps or coxsackievirus B4 was found when these antigens were presented together with HLA-DR3. This was not found for varicella-zoster or purified protein derivative of tuberculin. In contrast, an increased frequency of T-lymphocytes responding to mumps and coxsackie B4 was associated with DR4. The results were similar in type 1 diabetic and healthy individuals (Bruserud et al. 1985). Those results suggest that elements on the DR3 and DR4 molecules may control T-lymphocyte responses to mumps and coxsackie viruses.

According to Report II enterovirus antibody levels differ in subjects with different HLA-DR antigen combinations. Children having type 1 diabetes -associated risk alleles (DR3 and DR4) had higher enterovirus antibody levels than children with a protective DR2 allele. When homozygous and heterozygous children were analysed separately, the DR3-homozygous children had the highest antibody levels and DR2-homozygous children the lowest antibody levels. DR4-homozygous group and all four heterozygous groups were between these homozygotes, suggesting that homozygotes do not have generally weaker immune responses to enteroviruses. Poliovirus antibody showed the same type of HLA pattern. These antibodies are induced by parenteral IPV vaccine, thus suggesting that HLA modulate immune responses to enteroviruses rather than the course of an infection. Among high responders for CBV4 IgG and PV1 IgG (antibody levels higher than the 75 percentile of all sample analysed) most of the children had DR3- or DR4-risk allele for type 1 diabetes and only a minority had DR2-protective allele showing that immuno response to enterovirus correlates with HLA-DR mediated risk for type 1 diabetes.

In Report I children had more enterovirus infections if they had HLA-DQB1\*0302/X or \*0302/02 risk alleles for type 1 diabetes compared to children with HLA-DQB1\*02/X risk allele, which represents the lowest risk of these three risk types for developing type 1 diabetes. Thus in this

study, too, it was seen that HLA affects the frequency of enterovirus infections or then that they are more easily detectable in risk genotype subjects.

According to these results, the subjects should be matched for HLA genotype when enterovirus infections are analysed using serological assays. Children with HLA risk alleles for type 1 diabetes have higher antibody response to enteroviruses but may also have more enterovirus infections. This phenomenon may also play a role in the pathogenesis of type 1 diabetes. A stronger immune response may e.g. lead to more severe tissue pathology in infected organs in subjects with HLA –risk alleles for type 1 diabetes. Sometimes it can be difficult to match for HLA genotype because the frequency of HLA risk alleles is rather low in unaffected subjects. In Reports I, III and IV we matched children for diabetes associated HLA genotype, when analysing enterovirus antibody levels or the frequency of enterovirus infections. The fact that more enterovirus infections were diagnosed in case children than in HLA matched control children suggests that enterovirus infections are associated with the pathogenesis of type 1 diabetes.

## **Enterovirus infections as risk factors for beta-cell autoimmunity**

### ***Maternal enterovirus infections***

Enterovirus infections are also common in adults, not only in children. Twenty-three per cent of mothers in Report I and 15 % of mothers in Report IV had an enterovirus infection during pregnancy. In Report III it was found that mothers had quite high antibody levels against enterovirus antigens suggesting frequent exposure to enteroviruses. Previously it has been observed that enterovirus antibody levels have decreased in pregnant women in Finland in recent decades. This suggests that the frequency of enterovirus infections has decreased at the same time as the incidence of type 1 diabetes has increased (Viskari et al. 2000). This may have a biological significance making the infants more susceptible for enterovirus infections as maternal IgG antibodies are transferred to the child transplacentally and protect the child for a few months after the birth and may partly explain the increasing incidence of type 1 diabetes.

Mothers of children with diabetes associated autoantibodies and the mothers of matched control children had almost equal enterovirus antibody levels during pregnancy (Report III) suggesting that enterovirus infections during pregnancy may not influence the risk of type 1 diabetes.

This is in line with some previous studies, where no excess of enterovirus infections was found in mothers of children with type 1 diabetes (Table 5). On the other hand, there are also studies indicating that enterovirus infections during pregnancy may be a risk factor for type 1 diabetes in the child (Table 5). There is also a case report where a child was born with neonatal insulin-deficient diabetes and her mother had echovirus 6 infection during pregnancy (Otonkoski et al. 2000). In one recent study the blood spot samples from 2-4 days old children who developed later type 1 diabetes were analysed, and more enteroviral RNA positive samples were detected in these children than in control children who did not develop type 1 diabetes (Dalhquist et al. 2004). This excess of infections may reflect the role of enterovirus infections acquired in utero or immediately after birth. Also in Report I mothers of the children with diabetes associated autoantibodies had more infections during pregnancy than their control mothers. Thus, the role of maternal enterovirus infections during pregnancy remains a matter of controversy and more extensive studies are needed to resolve this question.

During pregnancy mothers' enterovirus antibody levels were lower in samples taken at delivery compared to samples taken at the end of first trimester (Report IV). The decrease in antibody concentration is probably due to haemodilution occurring during pregnancy (Ailus 1994). It is known that the plasma volume increases by 30-50% during pregnancy (Lund and Donovan 1976). After pregnancy enterovirus antibody levels reverted to the earlier stage in three months as expected. Some of the mothers had an enterovirus infection during pregnancy and their antibody levels increased from the sample taken at the beginning of pregnancy to the sample taken at delivery.

Children of mothers who had particularly high enterovirus CBV4 antibody levels at delivery had fewer enterovirus infections before the age of one year than the other children (Report IV). This suggests that mother's high antibody levels protects children from infections. Mothers of children with diabetes-associated autoantibodies had lower enterovirus antibody levels. This may reflect defective protection of the child against enterovirus infections and increase susceptibility to enterovirus induced beta-cell autoimmunity.

**Table 5.**

*Studies evaluating possible effect of maternal enterovirus infections during pregnancy on the risk of type 1 diabetes in the child.*

*Ev inf = Enterovirus infections in cases and in controls.*

*EV IgA, IgG, IgM ab = IgA, IgG and IgM class antibodies to enterovirus antigens.*

*PCR = Detection of enterovirus RNA using PCR assay. (modified from Hyöty 2004)*

| <b>Samples taken</b>                | <b>Ev inf in cases</b> | <b>Ev inf in controls</b> | <b>End point</b>  | <b>Methods</b>           | <b>Result</b> | <b>Reference</b>         |
|-------------------------------------|------------------------|---------------------------|-------------------|--------------------------|---------------|--------------------------|
| 1 <sup>st</sup> trimester           | 9 / 96                 | 1 / 96                    | Clinical diabetes | EV IgA, IgG, IgM ab      | +             | Hyöty et al. 1995        |
| Delivery                            | 38 / 55                | 17 / 55                   | Clinical diabetes | EV IgM ab                | +             | Dahlquist et al. 1995    |
| 1 <sup>st</sup> trimester           | 6 / 85                 | 1 / 172                   | Clinical diabetes | EV IgM ab +PCR           | +             | Dahlquist et al. 1999    |
| 1 <sup>st</sup> trimester, delivery | 2 / 21                 | 17 / 104                  | Autoantibodies    | EV IgA, IgG, IgM ab +PCR | -             | Lönnrot et al. 2000      |
| Delivery                            | 2 / 28, 1 / 16         | 7 / 51, 15 / 110          | Autoantibodies    | EV IgG, IgM ab           | -             | Fuchtenbusch et al. 2001 |
| 1 <sup>st</sup> trimester           | 29 / 948, 48 / 680     | 39 / 948, 36 / 680        | Clinical diabetes | EV IgG, IgM ab           | -             | Viskari et al. 2002      |
| 1 <sup>st</sup> trimester, delivery | 6 / 41                 | 29 / 196                  | Autoantibodies    | EV IgA, IgG ab +PCR      | -             | Salminen et al. 2003     |

### ***Childhood infections***

According to the results presented in Reports I and III, children with diabetes-associated autoantibodies have higher enterovirus antibody levels and more enterovirus infections than their matched control children. Higher enterovirus antibody levels suggest that these children have had either more enterovirus infections or their humoral immune response is stronger to these viruses. In Report III this humoral immune response to poliovirus 1 did not differ between the case and control children. As exposure to polioviruses is nowadays limited to inactivated vaccine in Finland, the results indicate that the case children had had more enterovirus infections rather than stronger



immune response to enterovirus antigens. This is also supported by the fact that case and control children were matched for gender and HLA-DQB1 alleles, both of which could have an effect on immune responsiveness to enteroviruses. It has been also speculated, that children who develop type 1 diabetes may have a defect in a humoral immune responsiveness which could make them susceptible to enterovirus infections, but the regulation of high responsiveness against enterovirus antigens has found to be normal, not disturbed, in autoantibody –positive children (Heino et al. 2001). This also suggests that having higher enterovirus antibody levels, these children in Reports I and III have had more enterovirus infections. Adenovirus antibody levels were also comparable in case and control children, suggesting that these case children are not more susceptible to virus infections in general. Accordingly, this excess of enterovirus infections in children who develop signs of beta-cell autoimmunity suggests that they are risk factors for type 1 diabetes.

In these two studies (DIPP and TRIGR), the results show that this excess of infections is observed mainly before the first appearance of the autoantibodies, i.e. before the autoimmune process starts (Report I, Lönnrot et al. 2000, Salminen et al. 2003). This kind of temporal relationship further supports a causal relationship.

Males are known to have more complications from enterovirus infections than females (Morens and Pallansch 1995). In Report III the difference in enterovirus antibody levels between the case and control children was seen in males but not in females. In Report I males had more enterovirus infections than females in general. These results are in line with previous findings suggesting that males are more susceptible to enterovirus induced beta-cell damage (Hiltunen et al. 1997, Lönnrot et al. 2000). Interestingly, in many countries the incidence of type 1 diabetes is also higher in boys than in girls (Tuomilehto et al. 1999). In a recent report the seasonality observed in type 1 diabetes diagnoses was more marked in boys than in girls (Weets et al. 2004), which fits in with the assumption that enterovirus infections may be a risk factor particularly in boys.

Both these studies, DIPP and TRIGR, are prospective studies. This is the best approach to analyse risk factors for type 1 diabetes in human studies, because the time preceding the onset of the disease is important. The time before the first appearance of autoantibodies is particularly interesting as the factors initiating the process should operate during that period. Their detection may be impossible from samples taken at the manifestation of clinical disease, when markers of infections may have disappeared.

Case and control children in DIPP and TRIGR were matched differently, in DIPP for gender,

HLA alleles and the date of birth and in TRIGR for gender, HLA alleles and the intervention group. The result was still the same. It is difficult to determine what these matching criteria should be to avoid overmatching, but according to these results the gender and HLA alleles should at least be matched when enterovirus infections are analysed.

In Report I the risk effect of enterovirus infections was compared to that of adenovirus infections. Adenovirus infections were diagnosed using only a single test (IgG and IgA class antibodies against adenovirus hexon antigen) while enterovirus infections were diagnosed using a much wider panel of assays. Therefore, the sensitivities of these methods to detect enterovirus and adenovirus infections were probably not identical and in this respect the results were not directly comparable. In the present study autoantibody positive children had had more enterovirus infections than autoantibody –negative children. This kind of phenomenon has also been found in previous prospective studies suggesting that enterovirus infections can increase the risk of beta-cell damage (Hyöty et al. 1995, Hiltunen et al. 1997, Lönnrot et al. 2000, Salminen et al. 2003). However, there are also controversial findings from other studies (Fuchtenbuch et al. 2001, Graves et al. 2003) and the pathogenetic significance of enterovirus infections needs to be confirmed. The reason for these differences is not known. The methods used in these studies have been quite different leading to varying sensitivity to diagnose enterovirus infections. For example, both the panel of virus assays and the length of sample intervals have an influence on the results in these kinds of prospective studies.

### **Interactions between breast milk, infant formula and enterovirus infections**

In Finland children are fed from birth with breast milk or milk formula. Infant's milk formulas are made industrially using cow's milk as the main raw material. Cow's milk proteins are alien to the infant's immunosystem and induce immune response. Thus, cow's milk proteins activate a gut associated immune system (GALT) which may have an effect on immune response and protection against enterovirus infections. The primary replication site of enteroviruses is GALT, thus enabling local interaction between these agents. Such interactions may be important in the pathogenesis of type 1 diabetes as both cow's milk proteins and enterovirus infections are among the main candidates for environmental triggers of type 1 diabetes.

In the TRIGR study children were divided into two groups having either a casein hydrolysate formula or regular cow's milk based formula as described earlier (Åkerblom et al. 1999). In Report I

the frequency of enterovirus infections did not differ between those two groups. However, this pilot study was too small to detect such an effect reliably and it is important to analyse enterovirus infections in these two differently nutritioned children groups to identify possible interactions between cow's milk and enteroviruses in larger series.

IAA are mostly the first appeared antibodies in diabetes-associated autoantibody positive children (Ziegler et al. 1999) and so immunization to insulin has been suggested to be the primary event in the process leading to type 1 diabetes. Cow's milk contains bovine insulin and it has been shown to cause the first immunization to insulin (Vaarala et al. 1998). It is also suggested that the immune responsiveness to oral antigens could be modified by early enterovirus infections. In fact in one study enterovirus infections during the first months of life were associated with stronger immune response to bovine insulin (Vaarala et al. 2002). It is possible that enterovirus infections may either increase the absorption of bovine insulin molecules or enhance intestinal immunity and thus modify the response to dietary insulin. Thus these interactions should be studied later in larger studies.

One study has previously been published on the possible effect of breast-feeding on childhood enterovirus infections. In that study absence of breast-feeding was found to be associated with increased risk of enterovirus infections during infancy (Jenista et al. 1984). In Report IV short exclusive breast-feeding increased the risk of enterovirus infections during the first year of life. The protective effect of breast-feeding was connected to enterovirus antibodies in breast milk, which seemed to be more important than transplacentally acquired antibodies in the protection against enterovirus infections. The primary replication of enteroviruses takes place in gut mucosa, thus antibodies in breast milk can act directly neutralizing the virus in the intestine and preventing its subsequent spread to the blood (viremia).

Some viruses, like HIV (Black 1996) and cytomegalovirus (Vochem et al. 1998), can be transmitted via breast milk, but there are no previous studies about possible presence of enteroviruses in breast milk. In the present study enterovirus RNA was not found in any of the breast milk samples (Report IV). This suggests that enteroviruses are not excreted into breast milk and breast milk is not an important transmission route for enterovirus infection.

Mothers with type 1 diabetes had higher enterovirus antibody levels in breast milk than unaffected mothers. Their children also had fewer enterovirus infections and experienced the first infections later than children of non-diabetic mothers. The reason for the higher enterovirus antibody levels in mothers with type 1 diabetes is not known but could be connected to higher frequency of

HLA risk allele for Type I diabetes in these mothers. These alleles have been shown to be associated with stronger immune response to enteroviruses as described in Report II. The prevalence of type 1 diabetes in parents of newly-diagnosed diabetic children is higher in the fathers than in mothers (Tuomilehto et al. 1992, Warram et al. 1991). The higher maternal enterovirus antibodies in mothers with type 1 diabetes may protect the child against neonatal enterovirus infection and could thus contribute to lower risk of type 1 diabetes in children of diabetic mothers than diabetic fathers. In addition, the mothers with type 1 diabetes may have experienced the diabetogenic enterovirus infection and give protection specifically to these serotypes.

### **Future Aspects**

This study supports previous findings suggesting that enterovirus infections are a risk factor for beta-cell autoimmunity in genetically susceptible individuals. The mechanisms of this process are not known, but according to animal and in vitro models the virus may infect the islets and induce an autoimmune process by activating dendritic cells to present beta-cell autoantigens ("bystander activation"). If enteroviruses are a true risk factor for type 1 diabetes this would open possibilities to prevent the disease by vaccine or antiviral treatments. In theory, the development of such a vaccine against these enteroviruses should be possible, because vaccines have been developed against polio viruses, which are also enteroviruses. There are more than 60 enteroviruses, and the diabetogenic strains or diabetogenic serotypes should be identified to be included in such vaccine. In addition, more data is needed about the mechanisms to make the vaccine safe. Large prospective studies are therefore needed. These multicentre studies are also important to establish the other potential risk factors in the pathogenesis of type 1 diabetes. Environmental factors may also differ somewhat in different parts of world, and international comparisons are therefore important.

In most studies potential risk factors for type 1 diabetes have been studied independently from each other, but as mentioned earlier, there may be interactions between two or more factors in the progression of beta-cell damage. Certain factors may potentiate each other's effect while others may have inhibitory interactions, as shown for enterovirus infections and infant feeding in the present study. These kinds of interactions are rather poorly characterized so far and require more research in the future.

## CONCLUSIONS

The following conclusions can be drawn from the data presented in present study:

1. Diabetes-associated risk HLA-DR alleles are associated with a strong humoral immune responsiveness and protective alleles with a weak immune responsiveness to enterovirus antigens. This may have a pathogenic significance in virus-induced beta-cell damage. For example, children with high risk HLA alleles could develop stronger immune mediated tissue pathology in response to the infection.
2. As both HLA and gender have an influence on enterovirus antibody levels, these factors should be matched in case-control studies evaluating the role of enteroviruses in type 1 diabetes.
3. Children with diabetes associated autoantibodies have more enterovirus infections than their control subjects and they have these infections earlier. This suggests that enteroviruses may play a role in the pathogenesis of type 1 diabetes.
4. Mother's high enterovirus antibody levels and breast-feeding protect the child from enterovirus infections during the first year of life. Enterovirus antibodies in breast milk may be more important in this protection than transplacentally acquired maternal antibodies.

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