Enterovirus RNA in Blood Is Linked to the Development of Type 1 Diabetes

Running title: Enterovirus is linked to type 1 diabetes

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**Objective:** To assess whether the detection of enterovirus RNA in blood predicts the development of clinical type 1 diabetes in a prospective birth cohort study. Further, to study the role of enteroviruses in both the initiation of the process and progression to type 1 diabetes.

**Research Design and Methods:** This is a nested case-control study where all case children (N=38) have progressed to clinical type 1 diabetes. Non-diabetic control children (N=140) were pair-wise matched for gender, date of birth, hospital district and HLA-DQ conferred genetic susceptibility to type 1 diabetes. Serum samples, drawn at 3- to 12-month intervals, were screened for enterovirus RNA using RT-PCR.

**Results:** Enterovirus RNA positive samples were more frequent among the cases than among the controls. A total of 5.1% of the samples (17/333) in the case group were enterovirus RNA positive compared to 1.9% of the samples (19/993) in the control group (P<0.01). The strongest risk for type 1 diabetes was related to enterovirus RNA positivity during the 6-month period preceding the first autoantibody positive sample (OR 7.7 [95% CI 1.9-31.5]). This risk effect was stronger in boys than in girls.

**Conclusions:** The present study supports the hypothesis that enteroviruses play a role in the pathogenesis of type 1 diabetes, especially in the initiation of the β-cell damaging process. The enterovirus-associated risk for type 1 diabetes may be stronger in boys than in girls.

Enterovirus infections are among the major candidates for environmental risk factors for type 1 diabetes. Previous studies have suggested that enterovirus epidemics associate with an increase in the incidence of type 1 diabetes and an increased frequency of enterovirus antibodies has been reported in patients with type 1 diabetes (1; 2). Several studies have detected enterovirus genome in the blood of diabetic patients, but it is unknown whether the finding reflects persistent or acute infection (3). Virus has been detected both in pancreas and in intestinal mucosa and has also shown a tropism for islets (4; 5). On some occasions coxsackievirus B (CBV) and echoviruses have even been isolated from diabetic children (6). The recent discovery that genetic polymorphism in the IFIHI1 gene (innate immune system sensor for enteroviruses) affects diabetes susceptibility, has further supported the possible role of enteroviruses (7). Experimental data support these findings as enteroviruses can cause diabetes in mice and damage β-cells in human islet cell cultures in vitro(3).

Type 1 diabetes associated autoantibodies in peripheral blood reflect initiation of the β-cell damaging processes. However, the progression towards clinical diabetes is usually slow and possible triggering infections can occur long before the presentation of clinical type 1 diabetes. Consequently, prospective follow-up series are essential for the identification of such triggers. A few prospective studies have been carried out on the possible role of enterovirus infections, but the results have been conflicting (8-11).

The aim of this study is to test risk effect of enterovirus RNA in blood for the development of type 1 diabetes in a prospective birth-cohort study. Blood samples were collected with short intervals, which made it possible to detect enterovirus RNA directly from the serum in different stages of
Enterovirus is linked to type 1 diabetes

the disease process. We have previously documented the risk effect of enteroviruses in children who developed beta-cell autoimmunity. Now the aim is to confirm these findings in children who have developed type 1 diabetes and to study the role of these viruses in both the initiation of the process and its progression to diabetes.

RESEARCH DESIGN AND METHODS

Study series. The study series included children who took part in the Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study (12). In DIPP, the families of all newborn infants at the University Hospitals of Oulu, Tampere and Turku are offered a possibility for screening of newborn infants for HLA risk genes for type 1 diabetes. Families with a child who carries increased genetic susceptibility to diabetes are invited to participate in prospective follow-up starting from birth. Blood samples are taken with 3- to 12-month intervals and regularly analyzed for type 1 diabetes-associated autoantibodies. Islet cell antibodies (ICA) have been used for the primary screening and all samples of ICA-positive children were tested for autoantibodies against insulin (IAA), glutamate decarboxylase (GADA) and the protein tyrosine phosphatase related islet antigen 2 (IA-2A). Children, who seroconverted to autoantibody positivity were observed subsequently with an interval of 3 months.

The current study is based on a nested case-control design, where the definition of the case status was based on the diagnosis of clinical type 1 diabetes. For every case child from 1 to 6 healthy autoantibody-negative control children were matched pair-wise for gender, date of birth (± 1 month), hospital district and HLA-DQ conferred genetic susceptibility to type 1 diabetes. The study population comprised a total of 38 case children (18 boys) and 140 control children (69 boys). Serum samples were collected for virus analyses from November 1994 to April 2003, and the total number of samples analyzed was 1326. Our earlier study population overlaps with the current study with 129 samples from 21 case children and 474 samples from 85 control children (11). The study was approved by the ethics committees of the participating university hospitals, and the parents gave their written informed consent to participation in the study.

Genetic and autoantibody analyses. The presence of HLA DR-DQ haplotypes associated with type 1 diabetes was determined using typing for DQB1 alleles in the first phase and informative DQA1 and DRB1 alleles in the second phase as described earlier (13). ICA, IAA, GADA, and IA-2A were analyzed as described earlier (14).

Enterovirus RT-PCR. RNA was extracted from 140μl of serum or plasma using QIAamp viral RNA kit (Qiagen, Hilden, Germany). Screening for enterovirus RNA was done by RT-PCR followed by hybridization of PCR amplicons using enterovirus specific probe. The detection limit for the method is less than 0.015 fg of RNA, which is equivalent to less than 4 copies of enteroviral RNA genome (15). All RNA positive samples were retested twice and at least two positive out of three tests were interpreted as a positive sample.

Statistical analyses. The significance of difference in the number of enterovirus infections between case and control children was tested using conditional logistic regression analysis (STATA statistical software, College Station, TX). To adjust for more frequent sample collection from autoantibody positive children than control children conditional logistic regression analysis was calculated as proportions (%) of positive samples. Effect of HLA type for EV infections was evaluated using Mann Whitney test.
RESULTS
Enterovirus RNA was detected in 2.7% of serum samples (36/1326). In case children a total of 5.1% of the samples (17/333) were enterovirus RNA positive compared to 1.9% (19/993) in control children (P<0.01; Table 1).

The frequency of enterovirus RNA positivity was further analyzed during different stages of the preclinical disease process. The frequency of enterovirus RNA in the case children peaked during the 6-month period before the appearance of the first autoantibody when 15.2% of the samples from the case children and 3.3% of the samples from the control children were positive (OR=7.7 [95% CI 1.9-31.5], P<0.004). The lowest frequency of enterovirus RNA was observed during the time period from birth to 6 months before the autoantibody positivity when 2.4% of the samples were positive among the case children and 0.7% in the control group (OR=3.1 [95% CI 0.4-22.4], P<0.27). After autoantibody seroconversion, 3.9% vs 2.2% of the samples were enterovirus RNA positive, respectively (Table 1 and Fig. 1).

The risk effect of enterovirus RNA was stronger among boys than in girls (Table 1). This was similarly seen in infections occurring before and after autoantibody seroconversion. The highest risk was related to infections which occurred in boys during the 6-month period prior to the first autoantibody-positive sample (OR=18.2 [95% CI 2.0-164.5] in cases vs. OR=3.1 [95% IC 0.4-21.8, P<0.01], in controls, respectively) (Table 1).

The age of the child had an influence on the frequency of enterovirus RNA in serum. In children who were younger than 6 months of age only 1.0% of the samples were enterovirus RNA positive compared to 3.5% of samples in 6-18-month-old children and 5.0% of the samples in children aged 18-24 months. At the age of 2 years the frequency of virus-positive samples decreased to 4.3% and further to 2.0% in children older than 2 years. Case children had the first enterovirus RNA positivity earlier than control children (median 10 months vs. 16 months, respectively) (Fig. 2a). The age when autoantibodies were first detected varied from 4 months to 3 years and 5 months (median 12 months) (Fig 2b.).

In three case children more than one follow-up sample was enterovirus RNA positive (two positive samples in one child and three positive samples in two children). None of these samples were consecutive and there were virus-negative samples in between the positive ones. Different virus genotypes were present in repeatedly positive samples in each of these children.

More samples were enterovirus RNA positive in case children with high risk HLA DR3-DQ2/DR4-DQ8 genotype than in children who carried moderate risk genotypes with the DR4-DQ8 haplotype (6.8% vs. 2.2%, P<0.002).

DISCUSSION
In the present study enterovirus RNA was detected in serum long before the diagnosis of clinical type 1 diabetes. The frequency of virus peaked during the 6-month window that preceded the first appearance of diabetes-associated autoantibodies. This temporal relationship has also been observed in our previous studies suggesting that enterovirus infections may play a role in the initiation of the β-cell damaging process (8; 11). Enterovirus RNA was also more common in the case than control children after the initial autoantibody seroconversion but was not detected in samples taken close to the presentation of diabetes. The absence of enterovirus RNA at the diagnosis of diabetes is in contrast to the majority of the retrospective case-control studies where altogether an average of 31 % of the patients and 6 % of the control subjects have been
Enterovirus is linked to type 1 diabetes

positive for enterovirus RNA (3). The controversy might be due to methodological differences, e.g. the sensitivity of the PCR applied or the type of samples collected (16). On the other hand, it may also reflect a true difference in enterovirus epidemiology. In fact, we have previously observed that enterovirus infections are less frequent in Finland compared to many other countries (17; 18). In any case, the present study emphasizes the importance of those infections which occur during the early stages of the diabetogenic pathway possibly playing a role in the initiation of the β-cell damaging process rather than the later stages of the process.

As enterovirus viremia usually lasts for a maximum of 2 weeks, the number of enterovirus episodes is largely underestimated if samples are collected at longer intervals. Accordingly, we can estimate the true number of enterovirus RNA positive episodes (N of positive samples / detection period covered by the collected samples * total follow-up time) which would be 154 episodes in case and 254 in control children (mean 3.7 vs. 1.6 episodes per child). However, it is also possible that the difference between the case and control children reflects prolonged enterovirus episodes in the case group.

Our previous studies among DIPP children suggest that the detection of the first diabetes-associated autoantibody and enterovirus RNA in stools show similar seasonal variation (19). In the present study, the same seasonal pattern was seen in the detection of enterovirus RNA in serum (frequent in the autumn and winter). In addition, viral RNA was most common in serum at the age when autoantibodies were most frequently induced. Autoantibodies became detectable soon after detection of enterovirus RNA in the serum. In mouse models this time interval has also been short (20).

It has been previously shown that children are protected against enterovirus infections by maternal antibodies and that this protection is at least partly mediated by antibodies in breast milk. In Finland, children are breastfed for an average of 8 months (range 0.15-23 months) (21). This may explain partly the low frequency of viral RNA observed in less than 6-month-old infants. Viral RNA was most frequent in 12-18-months-old children suggesting that there is a susceptibility period at that age.

Enterovirus persistence has been shown to play a role in chronic cardiomyopathies and it may also be involved in type 1 diabetes (5; 22; 23). In the present study no signs of persistent systemic infection were seen - enterovirus genomes which were detected from repeatedly positive children represented different viral genotypes. However, in persisting infections the virus replication can occur at a very low level, and the virus may not be detectable in peripheral blood even though it may be present in the pancreas or other organs (4; 23-25).

The risk effect of enterovirus RNA on autoantibody development was stronger among boys than girls. Boys are also known to be more susceptible to general complications of enterovirus infections. The higher number of enterovirus RNA positive samples in children with the DR3-DQ2/DR4-DQ8 genotype merits further investigation to find out if this genotype is particularly susceptible to diabetogenic effect enteroviruses.

In conclusion, the present study supports the hypothesis that enteroviruses play a role in the pathogenesis of type 1 diabetes. The presence of virus in serum was shown to be a risk factor for the development of β-cell specific autoimmunity which progress to clinical diabetes, especially among boys.
Author Contributions. Olli Simell, Mikael Knip, Jorma Ilonen, Riitta Veijola and Heikki Hyöty are members of the Steering Committee of the DIPP study. Heini Huhtala was responsible for the statistical analysis of the data, Jorma Ilonen for the HLA genotyping, Mikael Knip for the auto-antibody analysis, Heikki Hyöty for overall study design. Mika Martiskainen and Sisko Tauriainen contributed to the design of the study and writing of the manuscript. Sami Oikarinen supervised laboratory and data analysis and wrote the manuscript in collaboration with all authors.

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Enterovirus is linked to type 1 diabetes.

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Enterovirus is linked to type 1 diabetes

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Table 1. Risk effect of the detection of enterovirus RNA in serum for later development of clinical type 1 diabetes. The effect is calculated as odd ratios (OR) and 95% confidence intervals (CI). The abbreviations as in figure 1.

| Time period                          | OR   | 95% CI  | p<   |
|--------------------------------------|------|---------|------|
| All                                  |      |         |      |
| Birth-before Aab                     | 6.2  | 1.8-21  | 0.004|
| Birth-before 6 month period prior Aab| 3.1  | 0.4-22.4| 0.27 |
| 6 month period prior Aab             | 7.7  | 1.9-31.5| 0.004|
| Boys                                 |      |         |      |
| Birth-before Aab                     | 18.8 | 2.2-163.7| 0.008|
| Birth-before 6 month period prior Aab| 3.9  | 0.2-63.3| 0.34 |
| 6 month period prior Aab             | 18.2 | 2.0-164.5| 0.01 |
| Girls                                |      |         |      |
| Birth-before Aab                     | 2.6  | 0.5-12.3| 0.24 |
| Birth-before 6 month period prior Aab| 2.4  | 0.2-39.7| 0.53 |
| 6 month period prior Aab             | 3.1  | 0.4-21.8| 0.26 |

FIGURE LEGENDS

Figure 1. Enterovirus RNA positivity in serum samples during different stages of the diabetic disease process. Birth-T1D = time from birth to diagnosis of type 1 diabetes. Birth - before 6 month period prior AAb = time from birth to 6 months before the seroconversion to positivity for the first autoantibody. 6 month period prior AAb = 6-months time window before autoantibody seroconversion. AAb-T1D = period from autoantibody seroconversion to diagnosis of type 1 diabetes. Panel A presents the whole cohort, B boys and C girls. Black bars = case children and white bars = control children. The differences between case and control children were tested using conditional logistic regression analysis (P-values shown).

Figure 2. Age of the first detection of enterovirus RNA (A) and autoantibodies (B) in serum. Black bars = case children and white bars = control children.
Enterovirus is linked to type 1 diabetes

Figure 1

A

B

C
Figure 2

A

Proportion of EV+ samples (%)

6 12 18 24 30 36 36≤

Age (months)

B

N of AAb+ subject

6 12 18 24 30 36 36≤

Age (months)

Enterovirus is linked to type 1 diabetes