Maternal Enterovirus Infection as a Risk Factor for Type 1 Diabetes in the Exposed Offspring

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OBJECTIVE—Maternal enterovirus infections during pregnancy have been linked to an increased risk of type 1 diabetes in the offspring. The aim of this study was to evaluate this association in a unique series of pregnant mothers whose child progressed to clinical type 1 diabetes.

RESEARCH DESIGN AND METHODS—Maternal and in utero enterovirus infections were studied in 171 offspring who presented with type 1 diabetes before the age of 11 years and in 316 control subjects matched for date and place of birth, sex, and HLA-DQ risk alleles for diabetes. Acute enterovirus infections were diagnosed by increases in enterovirus IgG and IgM in samples taken from the mother at the end of the first trimester of pregnancy and cord blood samples taken at delivery.

RESULTS—Signs of maternal enterovirus infection were observed in altogether 19.3% of the mothers of affected children and in 12.0% of the mothers of control children ($P = 0.038$). This difference was seen in different HLA risk groups and in both sexes of the offspring, and it was unrelated to the age of the child at the diagnosis of diabetes or the age of the mother at delivery.

CONCLUSIONS—These results suggest that an enterovirus infection during pregnancy is not a major risk factor for type 1 diabetes in childhood but may play a role in some susceptible subjects.

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The process leading to type 1 diabetes has been implicated to start in some cases already in utero. During pregnancy, exposures to several environmental factors may be capable of modulating the subsequent risk of type 1 diabetes in the offspring. The major candidates for such factors include toxins (e.g., nitroso compounds), dietary factors (vitamin D), and virus infections (1). Many viruses can spread to the fetus and cause severe malformations, and particularly congenital rubella and enterovirus infections have been reported to be associated with an increased risk of type 1 diabetes (2). The possible effect of congenital rubella has been attenuated by vaccinations in many countries, but the role of enteroviruses remains to be potentially important.

Postnatal enterovirus infections have been linked to autoimmune diabetes in animal and human studies (2–4), and, in some reports, maternal infections during pregnancy were also associated with an increased risk of type 1 diabetes in the offspring (5–9). These studies have been carried out, however, in relatively small patient series using variable techniques, and larger series would be needed to confirm these findings. In an earlier large-scale study, we were not able to find any clear risk effect of maternal enterovirus infections during early pregnancy (10). However, that study covered only the first trimester of pregnancy, and later stages of the pregnancy were not analyzed. The possible role of maternal enterovirus infections has also been studied as a part of ongoing prospective studies. Four of these studies observed no correlation (11–14), whereas one study showed an excess of infections during pregnancy in children who later developed type 1 diabetes-associated autoantibodies (15). Again, the number of study subjects was small in these studies, and the end point varied, as both autoantibody positivity and clinical diabetes have been used as an end point.

The aim of this study was to further evaluate the possible risk effect of maternal enterovirus infections during pregnancy in a larger series of children who have presented with clinical type 1 diabetes at a young age. The study covers the whole period of pregnancy, and the genetic susceptibility of the children is matched in the case and control groups according to their HLA-DQ genotype.

RESEARCH DESIGN AND METHODS

Study subjects

The study series included children who consented to the HLA screening from cord blood in the Type 1 Diabetes Prevention and Prediction (DIPP) Study recruiting children from the general population and running in three university hospitals in Finland (Turku, Oulu, and Tampere) (16). All of these children were screened at birth for HLA-DQ alleles that associate with an increased or decreased risk for type 1 diabetes. Children who developed

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clinical type 1 diabetes by the age of 11 years among all HLA-screened children were identified from the hospital records. The coverage of the case identification is estimated to be ~100%. The case children (N = 171; 50.3% males) were diagnosed during the years 1994–2006, and the median age at diagnosis of type 1 diabetes was 3.7 years (range 0.8–10.3 years). Two control children were selected from the same DIPP cohort and matched for the time (month and calendar year) and place (city) of birth, sex, and HLA-DQB1 genotype.

The following two samples were traced for the cases and controls: *1*) a maternal serum sample taken at the end of the first trimester of pregnancy within the national infectious screening program and stored at −20°C at the National Institute for Health and Welfare; and 2) cord blood serum sample taken at birth from all children participating in the DIPP study. All samples were taken during the years 1994–2004. The mean age of the index mothers was 29.6 ± 5.1 SD years and 29.7 ± 4.9 SD years for the control mothers. The case and control children were matched according to type 1 diabetes–associated HLA-DQB1 genotypes: one third of the children (33%) carried the high-risk genotype associated with type 1 diabetes (DQB1*02-DQA1*05/DQB1*0302), 43% carried moderate-risk genotypes (DQB1*0302/x, x≠ DQB1*02-DQA1*05, DQB1*0301, DQB1*0602 or DQB1*0603), whereas the rest (24%) had other genotypes.

The study was approved by the Ethical Committees of the University Hospitals of Oulu, Tampere, and Turku. The parents of the children gave written informed consent and permission to use the samples analyzed in the current study. Laboratory personnel were blinded regarding the case-control status of each sample, and the samples of the index and matched control mother were analyzed coded in the same test run.

Antibody analyses

IgM class enterovirus antibodies were measured against a mixture of three enterovirus antigens (coxsackievirus B3 [CBV3], coxsackievirus A16 [CAV16], and echovirus 11 [EV11]) by a capture enzyme immunoassay (EIA) as previously described (10). Sera were incubated for 90 min at 37°C in 1/100 dilution in PBS plus 1% BSA plus 0.05% Tween 20 in microtiter wells coated with a monoclonal antibody to human μ-chain (Medix Biochemica, Kauniainen, Finland). After washings, a mixture of three enterovirus antigens (10 μg/mL of each) were incubated for 60 min at 37°C. Antigens were heat-treated for 15 min at 56°C to expose epitopes that are cross-reactive between different enterovirus serotypes. Bound antigen was detected by a corresponding mixture of biotinylated detection antibodies (10 μg/mL, 60 min at 37°C) produced by immunizing rabbits with purified heat-treated CBV3, CAV16, and EV11. Finally, streptavidin-horseradish peroxidase conjugate (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD) was added, and the color reaction of the OPD substrate (3 mg/ml, *o*-phenylenediamine dihydrochloride) was measured at 492 nm. Samples were considered IgM positive when the absorbance exceeded three times the absorbance of the negative control serum. All IgM-positive samples were reanalyzed using each of the three enterovirus antigens separately and a mock-infected control antigen to confirm the specificity of antibody binding. In addition, IgM-positive samples were analyzed for the presence of IgM class Epstein-Barr virus (EBV) antibodies (Enzygnost*Anti-EBV/IgM, Dade Behring, Marburg, Germany) to assess the specificity of the IgM responses.

IgG class antibodies were analyzed using an indirect EIA as previously described (10). Antigens used were purified CBV4 and a synthetic peptide carrying an antigen epitope of the VP1 protein common for enterovirus serotypes and is recognized by a majority of circulating antibodies of enterovirus infected humans as described earlier (17–20). CBV4 was first heat-treated (30 min at 56°C) to expose antigenic epitopes, which are cross-reactive between various enterovirus serotypes. Microtiter plates (Nunc Immunoplate; Nunc, Glostrup, Roskilde, Denmark) were coated with the antigen at 2.5 (BSA-conjugated peptide) and 2.4 μg/mL (CBV4) concentrations in 10 mmol/L carbonate buffer (pH 9.4). Serum samples were analyzed at a 1/1,000 (peptide antigen) and 1/2,000 (CBV4 antigen) dilution in PBS supplemented with 1% BSA and 0.05% Tween 20. Binding of specific antibodies was detected using peroxidase-conjugated anti-human IgG (P214; Dako, Copenhagen, Denmark) as the second layer. Antibody levels were expressed in EIA units (EIU) indicating the relative antibody activity of the sample compared with known positive and negative reference sera. A twofold or greater increase in the antibody levels against the antigens and exceeding the cutoff levels for seropositivity (15 EIU) was considered significant, indicating an infection between the two subsequent samples. These antibody assays have been validated in our previous studies by analyzing sera from children with confirmed enterovirus infections. They show high specificity (~100%), and their sensitivity depends on the virus serotype causing the infection and timing of sample drawn (typically 50–70% in this kind of study). The performance of these assays has also been documented by other groups (21). Samples from cases and controls and also different time points of pregnancy were analyzed in the same run.

Statistical analyses

The frequency of enterovirus infections was compared between case and control subjects with conditional logistic regression models to enable matching of one to two controls per case. Unconditional logistic regression was used to assess the association between sex of the offspring and enterovirus infection during pregnancy. Results are presented as odds ratios and their 95% CI. Differences in levels of antibodies were assessed with Mann-Whitney U test and paired t test. The software packages used were STATA version 8.2 (Stata Corporation, College Station, TX) and SPSS version 14.0 (SPSS Inc., Chicago, IL). A P value ≤0.05 was considered statistically significant.

RESULTS—Altogether, 974 samples were analyzed. Both cord blood and maternal samples from the first trimester were available from 171 cases and 316 controls, resulting in 145 (87%) case-control triplets (one index with two matched controls), whereas 26 cases had one matched control.

Altogether, 19.3% of the mothers of diabetic children and 12.0% of the mothers of control children had signs of an acute enterovirus infection when the whole period of pregnancy was analyzed (P = 0.038, Table 1). In the samples from the first trimester, IgM antibodies were observed in 8.8 and 4.1% of the mothers of case and control children, respectively (P = 0.048). When samples were tested separately against the three different antigens, 3.5 versus 0.9% were positive for CBV3, 3.5 versus 1.9% for EV11, and 5.8 versus 2.8% for CAV16 in cases and controls, respectively (not significant). No IgM antibodies were observed in any of the cord blood samples. The enterovirus IgM-positive samples were

Table 1—*Detection of acute enterovirus infection during pregnancy in the case and control children*

	Case	Control	*P* value	Odds ratio	95% CI
n	171	316			
Maternal IgM* (first trimester)	15 (8.8%)	13 (4.1%)	0.048	2.17	1.01–4.68
Fetal IgM* (cord blood)	0	0	NS		
IgG†	18 (10.5%)	27 (8.5%)	NS	1.5	0.76–2.95
All tests	33 (19.3%)	38 (12.0%)	0.038	1.74	1.03–2.94

*Mixture of Coxsackie B3 (CBV3), Echo 11 (EV11), and Coxsackie A 16 (CAV16) antigens. †Twofold or greater titer increase between samples taken at the end of the first trimester and cord blood (either against CBV4 or EV-antigen).

not positive for EBV IgM or showed reactivity against the mock-infected control antigen.

A significant increase in IgG antibody levels (against CBV4 or EV peptide antigen or both) occurred between the first trimester sample and the cord blood sample in 10.5% of the mothers of type 1 diabetic children and in 8.5% of the mothers of the control children, respectively (not significant). The levels of IgG enterovirus antibodies did not differ between case and control children either at the end of the first trimester or in cord blood: the median IgG antibody level against CBV4 antigen was 21 EIU and 19 EIU in the first trimester samples and 21 EIU and 23 EIU in cord blood for case and control subjects, respectively. The proportion of seropositive mothers was 75% in case mothers and 69% in control mothers against the enterovirus peptide antigen (not significant) and 65 and 57% for purified CBV4 antigen, respectively (not significant).

The difference between case and control mothers was not related to the HLA-DQ genotype of the child: 14.3 versus 9.5% of case and control mothers whose child carried the high-risk HLA genotype had infection during the whole period of pregnancy compared with 21.9 versus 13.2% of mothers whose child had lower-risk HLA genotypes. This difference was also observed in mothers of both female and male offspring, even though the overall frequency of enterovirus infections was significantly higher in the mothers of female than male offspring: 18.2% of mothers with female offspring had signs of enterovirus infection (either IgM or IgG titer rise) during pregnancy compared with 11.0% of mothers with male offspring ($P = 0.023$; Table 2). When analyzed separately, IgM positivity was more frequent in first-trimester samples when the offspring was female (7.9%) than male (3.7%) ($P = 0.045$; Table 2). No significant difference was observed in the frequency of IgG titer rise, nor was there any difference in enterovirus IgG antibody levels in mothers with female offspring compared with mothers with male offspring: the median IgG antibody level against CBV4 antigen was 19 EIU and 22 EIU in the first-trimester samples and 19 EIU and 22 EIU in cord blood for male and female offspring, respectively.

The frequency of enterovirus infections or levels of enterovirus antibodies was not associated with the age of the mother at delivery or the age of the child at the diagnosis of type 1 diabetes. The observed enterovirus infections were distributed throughout the whole study period.

Table 2—*Detection of acute enterovirus infection during pregnancy according to the sex of the offspring*

	Male	Female	*P* value	Odds ratio	95% CI
n	246	241			
Maternal IgM* (first trimester)	9 (3.7%)	19 (7.9%)	0.045	2.25	1.00–5.09
Fetal IgM* (cord blood)	0	0	NS		
IgG†	18 (7.3%)	25 (10.4%)	NS	1.47	0.78–2.76
All tests	27 (11.0%)	44 (18.2%)	0.023	1.81	1.08–3.04

*Mixture of Coxsackie B3 (CBV3), Echo 11 (EV11), and Coxsackie A 16 (CAV16) antigens. †Twofold or greater titer increase between samples taken at the end of the first trimester and cord blood (either against CBV4 or EV-antigen).

CONCLUSIONS—The role of intrauterine exposure to maternal enterovirus infection during pregnancy in the pathogenesis of type 1 diabetes has been studied with variable results (2). Previous surveys have been based on relatively small series and have varied for the virus assays and the end point used (clinical diabetes versus autoantibody positivity in the child). In the current study, we wanted to apply the strongest possible end point to minimize the variation due to individual differences in the progression of β-cell damage. Therefore, we included only children who had developed clinical type 1 diabetes and whose disease started at an early age.

One possible explanation for the variable results of previous studies is provided by the differences in the selection of controls. For example, all earlier studies that have used clinical type 1 diabetes as the end point have not matched the affected children and control subjects for HLA-conferred susceptibility to the disease. This may be a confounding factor, as HLA risk alleles might influence both enterovirus-induced pathology and the host's immune responsiveness to enterovirus antigens (22,23). Therefore, we carefully matched the study subjects for date of birth, sex, place of birth, and HLA-DQ genotype. Furthermore, in most previous studies, samples have been available either from the first trimester of pregnancy or from birth, and accordingly, it has been difficult to analyze infections during the whole period of pregnancy. In the current study, we were able to use samples from early pregnancy and time of birth (cord blood), which made it possible to measure not only IgM responses at a certain time point but also increases in IgG antibodies between the first trimester and cord blood samples.

The group of enteroviruses comprises >100 serotypes. Thus, the assays used in the current study for enterovirus-specific IgM or IgG class antibodies were made cross-reactive on purpose with the intention to detect also immune responses induced by enterovirus serotypes other than those included in the antigen preparations. This is reflected, for example, in IgM responses against different antigens of the cocktail antigen panel, as 4 of the 19 CAV16 IgM-positive samples were also positive for CBV3 IgM. IgG levels in the maternal circulation at delivery have been shown to correlate with those in cord blood, and thus, a diagnostic increase in IgG class antibodies reflects maternal

infection during the second or third trimester of pregnancy (15). Enterovirus infections were diagnosed as an increase in IgG levels in 9.2% of the mothers, but no detectable IgM was observed in the cord blood samples, even though the fetus is known to be competent of producing IgM class antibodies already in utero (24,25). This may reflect the lack of fetal infection in these cases. In contrast, it is acknowledged that a proportion of the enterovirus infections was probably missed because it is practically impossible to cover all enterovirus types even by this kind of a widely cross-reactive antigen panel. Moreover, the individual response to antigens may vary in persistence, level, and type of response. This is particularly true for cord blood samples, as newborn infants have more monotypic responses than older children who have already experienced several enterovirus infections and generate so-called anamnestic responses. However, the observed differences in the antibody responses are considered meaningful, as the analyses were made with a case-control study design exactly in the same manner in both groups.

Mothers of female offspring had markers of more frequent enterovirus infections during pregnancy than mothers of male offspring irrespectively of their diabetes status. Logistic regression analyses indicated that it was not due to confounding factors such as age of the mother. This phenomenon has not been previously reported and may have been observed just by chance in our study. In contrast, it is possible that the fetus may have sex-specific hormonal influences on the maternal immune system, and hormones secreted by the female fetus may lead to stronger maternal immune responses than those of male fetus (26,27). In fact, previous studies have suggested that maternal asthma may worsen during pregnancy when carrying a female fetus compared with a male fetus. It has been implicated that a female fetus alters maternal asthma during pregnancy by upregulating maternal inflammatory pathways (28). A similar phenomenon may also contribute to a stronger immune response to enteroviruses.

If maternal enterovirus infections had been a major risk factor for type 1 diabetes, we would have expected a clear excess of infections in the mothers of case children. When the results from IgM and IgG assays were combined, the case mothers showed markers of enterovirus infection more frequently than control mothers. More frequent IgM responses were also seen in the samples from the first trimester among the case mothers than among the control mothers. The higher frequency of infections in the case than in the control group was not associated with the HLA genotype. Altogether, the results suggest that enterovirus infections during pregnancy are not a major risk factor for type 1 diabetes in the offspring. However, it seems that they may play a role in a subgroup of patients, as indicated by somewhat more frequent infections in the mothers of children affected by type 1 diabetes. This is in line with our previous study in which the frequency of enterovirus-specific IgM did not significantly differ between pregnant women whose offspring later developed type 1 diabetes and control mothers (10). However, the fact that a slightly increased frequency of enterovirus infections was observed in mothers of children with type 1 diabetes in both these studies, together with other publications showing similar findings (5–9,11,29), indicates that the role of maternal enterovirus infections during pregnancy cannot be excluded in a subgroup of type 1 diabetic patients.

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H.V. supervised the laboratory, analyzed the data, and wrote the manuscript in collaboration with all of the authors. M.K. participated in the study design and is a member of the Steering Committee of the DIPP study. S.T. contributed to the design of the study. H.Hu. was responsible for the statistical analysis of the data. R.V. and O.S. were responsible for the clinical follow-up of DIPP participants in Oulu and Turku, respectively, and are members of the Steering Committee of the DIPP study. J.I. was responsible for the HLA genotyping and is a member of the Steering Committee of the DIPP study. H.-M.S. contributed to study design. H.Hy. was responsible for the overall study design and is a member of the Steering Committee of the DIPP study. H.V., M.K., S.T., H.Hu., R.V., J.I., O.S., H.-M.S., and H.Hy. contributed to the revision of the manuscript. H.Hy. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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