Maternal First-Trimester Enterovirus Infection and Future Risk of Type 1 Diabetes in the Exposed Fetus

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Previous studies have suggested that enterovirus infections during pregnancy may increase the risk of type 1 diabetes in the offspring. Our aim was to evaluate the role of first trimester enterovirus infections in a larger cohort of pregnant women. Two series of pregnant women were analyzed as follows: 948 women (series 1) and 680 women (series 2) whose child developed clinical diabetes before the ages of 15 or 7 years, respectively. An equal number of control women with a nondiabetic child was selected. Acute enterovirus infections were diagnosed by measuring IgM class antibodies against coxsackievirus B5 (series 1) and a mixture of coxsackievirus B3, coxsackievirus A16, and echovirus 11 antigens (series 2). In series 2, all sera were also analyzed for IgG class antibodies against an enterovirus peptide antigen. In addition, 152 randomly selected case-control pairs and all IgM-positive mothers' sera were tested for enterovirus RNA (series 2). In series 1, 3.1% of case women had IgM antibodies against coxsackievirus B5 antigen compared with 4.1% of control women (NS). In series 2, 7.1% of case and 5.3% of control women had IgM against the mixture of enterovirus antigens (NS). IgG class enterovirus antibodies did not differ between the groups. Enterovirus RNA was found only in one case woman (0.3%) of the subgroup of samples and in 5.7% of 70 IgM-positive women. The results suggest that enterovirus infection during the first trimester of pregnancy is not associated with increased risk for type 1 diabetes in the child. *Diabetes* 51:2568–2571, 2002

Enteroviruses have associated with type 1 diabetes in several but not all epidemiological studies, and they can also cause diabetes in animal models (1–3). Recent prospective studies have suggested that enterovirus infections may initiate the process leading to β-cell destruction several years before clinical diabetes appears (4–9). In addition to childhood infections, some studies have suggested that fetal exposure to maternal enterovirus infections may increase the risk, resembling what has been shown to be the case in congenital rubella syndrome (10–12). Dahlquist et al. (13) analyzed sera taken at delivery from mothers whose child developed type 1 diabetes before age of 15 years. They found that the levels of IgM antibodies against coxsackievirus B3 and echovirus 6 were higher in index than in control mothers, suggesting an excess of enterovirus infections during pregnancy in index mothers (13,14). The avidity of IgG class enterovirus antibodies was also lower in index mothers as a marker of recent infections (14). The risk effect of maternal enterovirus infections was also seen in our previous study, where we found an increased frequency of enterovirus IgM during first trimester in pregnant women whose child developed diabetes before the age of 3 years (4). This risk effect was restricted to these very young children, as children whose diabetes was diagnosed at an older age (3–6 years) did not have increased frequency of maternal enterovirus infections during pregnancy.

The aim of the present study was to test whether enterovirus infection during the first trimester of pregnancy is a risk factor for type 1 diabetes in the offspring by analyzing larger series of pregnant mothers whose child subsequently manifested with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Serum samples. Since 1982 in Finland, serum samples have been taken from all pregnant women at the end of the third month of pregnancy for the national screening of infectious diseases. The leftover of these sera have been stored at −20°C at the National Public Health Institute. Two different national registries were used for the identification of children with type 1 diabetes as described below.

Series one included samples from mothers whose child manifested with diabetes before the age of 15 years during the period from 1987 to 1995. These children were first identified from a national diabetes register (15). Control mothers were randomly selected (one for each case mother) by taking the maternal sample located next to the case mother in the freezer. This guaranteed that the case and the control mothers' samples were taken within a few days apart and therefore matched very closely according to calendar time. Written permission for the use of the maternal serum was obtained from the mothers (83.8% of the mothers gave permission). Series 1 included samples from 948 pregnant women with a diabetic child (case mothers) and corresponding samples from 948 control mothers. The mean age of the index mothers was 28.0 ± 5.1, and the mean age of control mothers was 27.9 ± 5.1 years. Samples were taken from the mothers during the years of 1982–1995.

Series 2 included children developing diabetes before 7 years of age from 1983 to 1995 who were identified from a national registry based on drug reimbursement allowances of the Social Insurance Institution of Finland. The registry includes all cases developing diabetes before 30 years of age since 1965 (15). One control child without diabetes of same age and sex and living in the same municipality as the case was chosen from the Finnish Central

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CAV, coxsackievirus A; CBV, coxsackievirus B; EIA, enzyme immunoassay; EIU, enzyme immunoassay unit; RIA, radioimmunoassay.

Population Registry. The unique identification codes of biological mothers of case and control children were identified from the Population Registry and cross-linked with the serum bank registry. Altogether, 680 case and 680 control mothers were included in the study. The mean age ± SD of the case mothers was 28.3 ± 5.0 years, and that of the control mothers was 28.1 ± 5.3 years. The samples were taken during the years 1983–1994 in different parts of Finland. The monthly distribution of the samples was approximately the same in case and control women. The samples were analyzed anonymously according to the approval given by the Data Protection Office of the Ministry of Justice.

There was an overlap among the case mothers of the two series (68.5% of the case mothers of series 2 were also included in series 1). Control mothers did not overlap between the two series, and they were selected using different criteria. In both studies, laboratory personnel were blinded regarding the case-control status of each sample.

Antibody analyses

IgM-radioimmunoassay. IgM class antibodies to heat-treated coxsackievirus B5 were analyzed by using a heavy-chain capture-based radioimmunoassay (RIA) as previously described (4,16). In brief, 1/100 dilution of serum was added to duplicate wells on microtiter plates coated with a monoclonal antibody to human μ-chain (Medix Biochemica, Kauniainen, Finland). After washings with PBS containing 0.1% Tween 20, 30,000 cpm metabolically 35S-radiolabelled coxsackievirus B5 (purified as described in ref. 16) was added and incubated for 1 h at 37°C. After three washings, 150 μl scintillator (Optiphase Supermix; Wallac, Turku, Finland) was added, and the bound virus label was counted in a Wallac MicroBeta scintillation counter. The count minus the background was used to indicate the relative antibody concentration. Sera were scored antibody positive if the mean counts per minute of two wells exceeded the mean counts per minute of negative control +2 SD.

IgM-enzyme immunoassay. IgM-class enterovirus antibodies were measured against a mixture of three enterovirus antigens (coxsackievirus B3, coxsackievirus A16, and echovirus 11) by using a capture enzyme inmmunoassay (EIA) as previously described (8). This method is based on the same monoclonal anti-human IgM antibody–coated microtiter plates as the IgM-RIA described above (Medix Biochemica, Kauniainen, Finland). Sera were incubated for 90 min at 37°C in 1/100 dilution in PBS + 1% BSA + 0.05% Tween 20. After washing, a mixture of three enterovirus antigens (10 μg/ml each) were incubated for 60 min at 37°C. Antigens were heat-treated for 15 min at 56°C to expose epitopes, which are cross-reactive between different enterovirus serotypes. Bound antigen was detected by a corresponding mixture of biotinylated detection antibodies (10 μg/ml for 60 min at 37°C) produced by immunizing rabbits by purified heat-treated coxsackievirus B3, coxsackievirus A16, and echovirus 11, respectively. Finally, streptavidin-horseradish peroxidase conjugate (Bethesda Research Laboratories Life Technologies, Gaithersburg, MD) was added, and the color reaction of the OPD substrate was recorded at 492 nm. All IgM-positive sera were reanalyzed using the mock-infected control antigen and each of the three virus antigens separately to study the specificity of IgM responses. The sample was taken as IgM negative if the reactivity against the control antigen exceeded 50% of that observed against the virus antigen.

IgG-EIA. IgG-class antibodies were analyzed against a synthetic peptide carrying an antigen epitope of VP1 protein using an indirect EIA as previously described and two serum dilutions (1/500 and 1/5,000) (16). The epitope consists of conserved residues common for all known enterovirus serotypes, and it is recognized by a majority of serum antibodies of enterovirus-infected humans (17–20). Antibody levels were expressed in enzyme immunoassay units (EIUs) indicating the relative antibody activity of the sample compared with known positive and negative reference sera. The cutoff level for seronegativity was 15 EIU, deduced from the distribution of antibody levels in acute infections and in background population.

Detection of enterovirus RNA. The 152 randomly selected index mothers and 152 control mothers were tested for the presence of enterovirus RNA in serum using an RT-PCR assay and a liquid-phased hybridization–based detection system of enterovirus-specific sequences as described (21). In addition, all samples that were positive for echovirus IgM in EIA (40 index and 30 control mothers in series 2) were tested in the same way. This RT-PCR assay amplifies all enteroviruses and is a highly sensitive method for the detection of enterovirus RNA (described in detail in ref. 21).

Statistical analyses. Univariate analyses were done using McNemar's test (antibody positivity) or paired *t* test (mother's age). Multivariate analyses of the risk of Coxsackie positivity were done using conditional logistic regression for a 1:1 matched case-control design. We used *P* values of 0.05 or less to imply statistical significance (22).

TABLE 1
Proportion of IgM-positive mothers according to the age of the child at the diagnosis of diabetes

Index child's age at diagnosis (years)	Series 1	Series 2	
	CBV5-IgM	Antigen mixture–IgM (ECHO11, CBV3, CAV16)	ECHO11-IgM
0–2			
Case mothers	3.0	5.8	5.4
Control mothers	3.6	5.0	2.9
3–6			
Case mothers	2.7	7.7	6.6
Control mothers	2.0	5.5	4.4
0–6			
Case mothers	3.0	7.1	6.2
Control mothers	2.4	5.3	3.7
0–15			
Case mothers	3.1		
Control mothers	4.1		

Data are %. The differences between case and control mothers were not statistically significant.

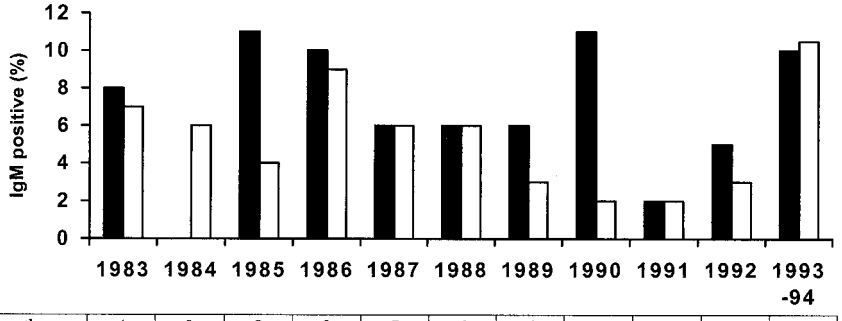
RESULTS

In series 1, altogether 3.1% of the 948 mothers with a child with diabetes had IgM class antibodies against heat-treated coxsackievirus B5 at the end of the third month of pregnancy. The corresponding prevalence in the control mothers was 4.1% (NS).

In series 2, altogether 7.1% of case mothers had IgM class antibodies against the mixture of coxsackievirus B3, coxsackievirus Ag (CAV)-9, and echovirus 11 antigens compared with 5.3% of control mothers (NS). When IgM-positive cases of series 2 were reanalyzed for IgM against each of the three enterovirus antigens separately, echovirus-binding IgM tended to be more frequent in case than in control mothers (6.2 vs. 3.7%; $P < 0.05$), but this difference did not remain statistically significant when adjusted for the number of comparisons ($P = 0.15$ after Bonferroni's correction). Coxsackievirus B3–binding IgM was found in 2.6% of case mothers and in 2.6% of control mothers, and CAV16-binding IgM was found in 0.7% of case and 1.3% of control mothers, respectively, indicating no differences between the groups.

In our previous study, maternal enterovirus infections during pregnancy were associated with manifestation of diabetes in children aged <3 years. However, in the present study no difference was found in this particular age group—3.0% of case women and 3.6% of control women were positive for coxsackie B5 IgM (series 1) and 5.8 vs. 5.0% for IgM against the mixture of coxsackievirus B3, CAV16, and echovirus 11 antigens (series 2) (Table 1). Positive enterovirus IgM was not associated with the sex of the child or the age of the mother, and IgM-positive samples were distributed over the study period. The difference between case and control mothers was the strongest in the years 1985 and 1990 (Fig. 1).

IgG-class enterovirus antibodies did not differ between the case and control groups. The median IgG levels were 44 EIU (range 0–160) and 45 EIU (0–156), and the proportion of seropositives was 83 and 84%, respectively. IgG levels were significantly higher in the 84 IgM-positive mothers than in the 1,276 IgM-negative mothers (median values 71 vs. 44 EIU, respectively; $P < 0.001$).



	1983	1984	1985	1986	1987	1988	1989	1990	1991	1992	1993-94
■ Case mother	4	0	8	9	5	6	6	5	1	1	2
□ Control mother	4	1	3	8	5	7	3	1	1	1	2

FIG. 1. Proportion of mothers positive for IgM (>15 EIU) against the enterovirus antigen mixture (echovirus 11, coxsackievirus B3, and coxsackievirus A16 antigens) in the years of 1983–1994 (series 2). Table indicates the number of IgM-positive samples each year in case and control mothers.

Enterovirus RNA was found in serum by RT-PCR in one case mother (0.7%) in a subgroup of 152 case and 152 control mothers randomly selected from series 2. In addition, all IgM-positive mothers in series 2 were tested for the presence of enterovirus RNA in serum. Only 2 of the 40 echovirus IgM-positive case mothers (5%) and 2 of the 30 echovirus IgM-positive control mothers (6.7%) were positive for enterovirus RNA.

DISCUSSION

Intrauterine infections may cause malformations and functional defects in affected organs (23), and they may also play a role in the induction of the β-cell damage leading to type 1 diabetes. Congenital rubella is a well-known example of intrauterine virus infection, which can also result in type 1 diabetes (10–12). Intrauterine exposure to maternal enterovirus infection has recently been reported to be associated with the pathogenesis of type 1 diabetes (4,13,14,24). Two of these studies comprised samples from early pregnancy (4,24) and were able to evaluate the role of infections occurring during the first trimester of pregnancy. The two others were based on samples taken at delivery (13,14), when infections occurring during later stages of pregnancy could also be documented. The association proposed in these relatively small series could not be confirmed in the present study, in which we analyzed first trimester sera from a large number of pregnant mothers of type 1 diabetic children.

In the present study, two separate approaches were used. Series 1 included mothers whose child developed diabetes before the age of 15 years. Maternal samples were analyzed using the same virus antibody assay, which indicated the risk effect during the first trimester in our previous study (4). Series 2 included mothers whose child developed diabetes before the age of 7 years, and a wider battery of virological methods was used (both antibody assays and detection of viral RNA by RT-PCR). However, there was an overlap among the case mothers of the two series (68.5% of the case mother of series 2 were included also in series 1). Control mothers did not overlap between the two series, and they were selected using different criteria.

Enterovirus IgM was found in a relatively small proportion of case mothers in both series, showing no difference in control mothers. IgG levels to enterovirus antigen were higher in IgM-positive than in IgM-negative mothers. Several factors may influence the persisting levels of enterovirus-specific IgG in adult humans, but the observed elevated levels, together with IgM, are consistent with the view that these mothers had had a concurrent or recent enterovirus infection. Despite the observed trend for slightly higher frequency of IgM class antibodies against echovirus 11 in case mothers than in control mothers, our current results suggest that enterovirus infection during early pregnancy is not a major risk factor for type 1 diabetes. It is difficult to say if this trend could reflect a role of enterovirus infections in a small subgroup of patients, as suggested in earlier studies (4,13,14,24). However, it is quite clear that even if maternal enterovirus infections were involved in some diabetes cases, the proportion of all cases of diabetes would be very small. In our previous study the risk effect of intrauterine enterovirus exposure was observed in a subgroup of very young children whose diabetes was diagnosed before the age of 3 years (4), whereas in the present study such an exposure was not higher in children diagnosed with type 1 diabetes at a very young age than that in children diagnosed at an older age. We do not know the reason(s) for the discrepancy between the current results and those of our previous report, but it seems likely that the small number of case and control subjects available in the initial study has caused the reported association by chance.

Assays for enterovirus-specific IgM used in this study and in most previous publications on the topic are cross-reactive for a purpose, i.e., they are also intended to detect IgM responses induced by enterovirus serotypes other than those included in the antigen preparations. However, it is very difficult to know how well they cover all of the many enterovirus serotypes in this sense. It is possible that part of enterovirus infections are missed because of the deficient coverage. The more recently developed RT-PCR assays for enteroviral RNA are based on genomic sequences that are fully conserved among the known enterovirus serotypes and are, thus, expected to detect all serotypes and strains much more uniformly. Indeed, regarding newly diagnosed type 1 diabetes, several recent studies have been consistent in demonstrating enterovirus RNA, specifically in blood specimens from the patients at diagnosis of diabetes and several months previous, in contrast to much more variable results in previous IgM surveys in newly diagnosed diabetic patients and control subjects (8,9,25–28). We found enterovirus RNA in the IgM-positive mothers significantly more often than in the IgM-negative samples, suggesting that part of the IgM-positive samples represented genuine acute enterovirus infections. However, analysis of a randomly selected sub-

set of all sera revealed that the apparent overall prevalence of RNA positivity among the mothers was very low. The viremic phase in acute enterovirus infections is considered to last for a much shorter time than the subsequent IgM response. The long storage of the sera at −20°C may have additionally reduced the amount of intact virus RNA. Consequently, a systematic screening for enterovirus RNA in the entire material was not carried out.

Altogether, the results suggest that first trimester maternal enterovirus infection is not a major risk factor for type 1 diabetes in the offspring. This study covered only the first trimester of pregnancy, and further studies are needed to find out whether infections occurring later during the pregnancy have an effect on the risk of type 1 diabetes.

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