Enterovirus Infection as a Risk Factor for β-Cell Autoimmunity in a Prospectively Observed Birth Cohort

The Finnish Diabetes Prediction and Prevention Study

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Previous studies suggest that enterovirus infections may initiate and accelerate β-cell damage years before the clinical manifestation of type 1 diabetes. We have now analyzed the role of enterovirus infections in the initiation of autoimmunity in children who have tested positive for diabetes-associated autoantibodies in a prospective study starting at birth (the Finnish Diabetes Prediction and Prevention Study). The frequency of enterovirus infections was studied using both serology and testing for the presence of enterovirus RNA in the sera of 21 children who developed and retained autoantibodies and in 104 control subjects chosen from the same study cohort and matched for the time of birth, sex, and HLA alleles determining genetic diabetes susceptibility. Sample intervals were taken as basic units of follow-up, to which the observed number of infections was adjusted. Enterovirus infections were detected in 26% of sample intervals in the case subjects and in 18% of the sample intervals in the control children (*P* = 0.03). A temporal relationship between enterovirus infections and the induction of autoimmunity was found; enterovirus infections were detected in 57% of the case subjects during a 6-month follow-up period preceding the first appearance of autoantibodies compared with 31% of the matched control children in the same age-group (odds ratio 3.7, 95% CI 1.2–11.4). The frequency of adenovirus infections did not differ between the patient and control groups. Our data imply that enterovirus infections are associated with the development of β-cell autoimmunity and provide evidence for the role of enteroviruses in the initiation of β-cell destruction. *Diabetes* 49:1314–1318, 2000

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CVB4, Coxsackievirus B4; DIPP, Diabetes Prediction and Prevention; EIA, enzyme immunoassay; EIU, enzyme immune units; EV11, echovirus 11; GADA, GAD antibodies; IA-2A, IA-2 protein antibodies; IAA, insulin autoantibodies; ICA, islet cell antibodies; JDFU, Juvenile Diabetes Foundation units; OR, odds ratio; PBS, phosphate-buffered saline; RIA, radioimmunoassay; RT-PCR, reverse transcriptase–polymerase chain reaction; RU, relative units.

The etiology of type 1 diabetes comprises both genetic and environmental components. Enterovirus infections have long been suspected as potential environmental factors playing a role in the pathogenesis of type 1 diabetes (1,2). Recent prospective studies, based on enterovirus serology (3–5) and detection of enterovirus RNA in sera of prediabetic children (6), suggest that enterovirus infections may initiate and accelerate the β-cell–damaging process years before the clinical manifestation of type 1 diabetes. Enterovirus RNA has also been detected more frequently in patients with newly diagnosed type 1 diabetes than in healthy control subjects (7–9).

Clear signs of β-cell damage often appear months or years before the manifestation of clinical diabetes (10), suggesting that prospective studies starting before the appearance of autoantibodies may be helpful in studying the etiology of the disease. In addition, early recognition of the individuals with ongoing β-cell damage may make it possibile to delay or halt β-cell destruction. The Finnish Diabetes Prediction and Prevention (DIPP) Study is a prospective population-based birth-cohort study in which Finnish children with increased genetic risk for type 1 diabetes are studied for the appearance of diabetes-associated autoantibodies at 3- to 12-month intervals from birth. We have now studied the frequency of enterovirus infections in 21 DIPP cohort children who developed autoantibodies during follow-up and in their control subjects who were matched for the time of birth, sex, and HLA risk alleles. For comparison, adenovirus infections were analyzed in the same children. Short sample intervals during the follow-up, together with a large panel of sensitive virological assays, create an optimal setting for the evaluation of the role of these infections within the framework of the present study design.

RESEARCH DESIGN AND METHODS

Subjects. The case subjects and control subjects were participants of the DIPP Study, which was initiated in Finland in 1994. In this trial, all babies born at the University Hospitals of Oulu, Tampere, and Turku were screened with parental permission for type 1 diabetes–associated HLA-DQB1 alleles. The HLA-DQB1 alleles associated with type 1 diabetes risk or protection were determined from cord blood samples as previously described (11).

The infants carrying the HLA-DQB1*02/*0302 or the *0302/x genotype (x referring to other alleles than *02, *0301, or *0602) were then observed from

birth with an interval of 3–6 months during the first 2 years of life and subsequently with an interval of 6–12 months. The follow-up samples were screened for islet cell antibodies (ICA). All samples from children who test positive for ICA are then also screened for glutamic acid decarboxylase antibodies (GADA), insulin autoantibodies (IAA), and tyrosine phosphatase-related IA-2 protein antibodies (IA-2A).

A total of 21 children (10 boys and 11 girls), who were identified as the first subjects in the DIPP cohort with continuous signs of β-cell damage, were the case subjects in this study. Type 1 diabetes has subsequently been diagnosed in 5 of them. The last follow-up sample from the remaining 16 children was positive for ICA in all of the cases (mean level 69.5 Juvenile Diabetes Foundation units [JDFU]), GADA in 13 cases (mean level 115.5 relative units [RU]), IAA in 13 cases (mean level 23.1 RU), and IA-2A in 7 cases (mean level 38.4 RU). The last follow-up sample from these 16 cases was thus positive for 4 autoantibodies in 6 cases, 3 autoantibodies in 7 cases, 2 autoantibodies in 1 case, and 1 autoantibody in 2 children (both have been constantly ICA positive for at least 2 years).

The cases were born between November 1994 and June 1997 and have been followed from birth. The mean follow-up time was 20 months (range 9–29 months). Samples were drawn at birth (cord blood) and subsequently at 3- to 6-month intervals. Altogether, 20 cord blood samples and 125 follow-up serum samples from the case children were analyzed. Nine of the patients had the HLA-DQB1*02/*0302 genotype, and 12 of the patients had the HLA-DQB1*0302/x genotype. Of the 21 case children, 4 had a father with type 1 diabetes, whereas all other first-degree relatives of the case children were nondiabetic.

Of the control children, 3–6 (mean 5) matched for the time of birth, sex, and HLA-DQB1 alleles, were chosen from the DIPP cohort for each case. The control group comprised 104 children from whom 98 cord blood samples and 567 follow-up serum samples were analyzed. The control subjects were followed from birth according to the same protocol as the case subjects. Their mean follow-up time was 20 months (range 9–31 months). All control children remained 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.

Entero- and adenovirus antibody analyses. IgG and IgA class antibodies against purified Coxackievirus B4 (CVB4), purified echovirus 11 (EV11), and a synthetic enterovirus peptide antigen (sequence KEVPALTAVETGAT-C derived from an immunodominant region of capsid protein VP1 [12], which is a common epitope for several enteroviruses [13]) were measured using enzyme immunoassay (EIA) as described (3,14). The purified CVB4 and EV11 were incubated at 56°C for 15 min to expose antigenic determinants common for various enterovirus serotypes. IgG and IgA class antibodies against adenovirus hexon protein were measured in EIA as a control (15).

Microtiter plates (Nunc Immunoplate; Nunc, Glostrup, Denmark) were coated by the antigens in 1.0 μg/ml (CVB4 and EV11), 1.8 μg/ml (Adeno), and 2.5 μg/ml (bovine serum albumin-conjugated peptide) concentration in carbonate buffer (pH 9.4). Serum samples were analyzed in 1/100 (IgA), 1/500 (EV11 IgG) and 1/2000 (other IgG assays) dilution in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin and 0.05% Tween 20. Binding of antibodies was documented using peroxidase-conjugated antihuman IgG and IgA (P214 and P216, respectively; Dako, Copenhagen, Denmark).

All serum samples of each child were analyzed in the same assay. Results of the EIA tests were expressed in enzyme immune units (EIU), which show the relative antibody reactivity of the sample compared with positive and negative reference sera included in each assay.

IgM and IgA class enterovirus antibodies were measured against a mixture of 3 enterovirus antigens (Coxsackievirus B3, Coxsackievirus A16, and EV11) using a capture EIA method, which is a modification of our previously used capture radioimmunoassay (RIA) (3,5). Monoclonal anti–human IgM or IgA antibody–sensitized microtiter 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 a comparable mixture of biotinylated detection antibodies was added (10 μg/ml for each antibody, 60 min at 37°C). These detection antibodies were produced by immunizing rabbits by purified heat-treated Coxsackievirus B3, Coxsackievirus A16, and EV11, respectively. 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 color reaction of the orto-phenylenediamine-dihydrochloride substrate (30 min at 37°C) was recorded at 492 nm. The samples giving absorbance values that exceeded the cut-off level for antibody positivity were reanalyzed using mock-infected cell lysate as antigen and were considered negative if this reactivity exceeded 50% of that obtained with the virus antigen. The results obtained by these EIA methods were comparable with those obtained by the capture RIA (3) when samples from patients with confirmed enterovirus infections were analyzed.

Detection of enterovirus RNA. RNA was extracted according to the manufacturer's protocol (QIAamp viral RNA kit; Qiagen, Hilden, Germany) from 140 μl of the serum samples. The samples had been stored at –70°C, and they were thawed for the first time for RNA extraction. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed as described (16) using a primer pair from the highly conserved 5′ noncoding region of enteroviruses. Separate rooms were used for each step of the RT-PCR assay, and positive and negative controls were included in each assay. The RT-PCR amplicons were hybridized with a europium-labeled enterovirus-specific oligonucleotide probe and a liquid-phase assay on microtiter plate format (16). Europium fluorescence was measured using time-resolved detection. The sample signal was compared with the mean background fluorescence of the negative controls. A 5-fold or greater signal-to-background ratio was considered positive, indicating a current enterovirus infection. All samples positive in the assay were confirmed to be positive by repeated RT-PCR and subsequent hybridization assay.

Diagnostic criteria for entero- and adenovirus infections. During the follow-up, a 2-fold or greater increase in the antibody level against an antigen, observed between 2 consecutive samples and exceeding the cut-off level of seropositivity (15 EIU), was considered to be significant and indicated an infection. Likewise, in the capture IgA and IgM assays, a 2-fold or greater increase in the antibody level exceeding the cut-off level of seropositivity was considered to indicate an infection. In the capture assays, the cut-off level of seropositivity was determined to be 3 times the level obtained with conjugate controls, which were included in each assay. Presence of enterovirus RNA was taken as a marker of current infection.

During pregnancy, a 2-fold or greater increase in the antibody level between the maternal sample taken at the end of the first trimester and the child's cord blood sample was considered to indicate an infection. A maternal IgM class antibody level exceeding the previously described cut-off level was considered to indicate an infection during the first months of pregnancy. In addition, the presence of enterovirus RNA in the maternal sample or the cord blood sample was taken as a marker of enterovirus infection during the first months of pregnancy or just before birth, respectively.

Evaluation of the amount of viral exposure. The occurrence of infections was studied during 3 periods of follow-up, namely during the entire follow-up of children, during a 6-month period just before the first detection of autoantibodies, and during pregnancy. The number of infections was adjusted to either the number of children (the 6-month period and intrauterine period) or the number of follow-up sample intervals in each child (frequency of infections during the entire follow-up). The latter was used to eliminate the possible confounding effect of minor variations in the frequency of sampling, because the frequency of sampling is correlated with the probability of detecting viremia and/or significant increase in antibody level. Sample intervals were taken as basic units of follow-up.

Autoantibody analyses. ICA, GADA, and protein tyrosine phosphatase-related IA-2 molecule (IA-2A) were analyzed as described (17,18). IAA levels were measured with a recently described microassay (19). The detection limit of ICA was 2.5 JDFU. The cut-off limits for positivity for IAA, GADA, and IA-2A were set at the 99th percentile (1.56 RU for IAA, 5.35 RU for GADA, and 0.43 RU for IA-2A) in more than 370 nondiabetic Finnish children. All samples resulting in an antibody level between the 97.5th and the 99.5th percentiles were reanalyzed to confirm antibody negativity or positivity.

Statistical analyses. Possible differences in the infection frequencies between the case subjects and the control subjects were tested using the 2-tailed paired Student's *t* test. Differences in the occurrence of infections between the case and control children during the 6-month period before the first detection of autoantibodies and the difference in the occurrence of in utero infections between the case and control children was assessed using the Mantel-Haenszel odds ratio (multiple and varying number of control subjects per case) using Stata statistical software. The differences in the number of infections among case subjects before seroconversion to autoantibody positivity versus the other sample intervals during the follow-up were tested using the χ^2 test or Fisher's exact test.

RESULTS

Enterovirus infections during the entire follow-up of children. Enterovirus infections were diagnosed during the entire observation period in 26% (33 of 125) of the sample intervals in the case subjects and in 18% (103 of 567) of

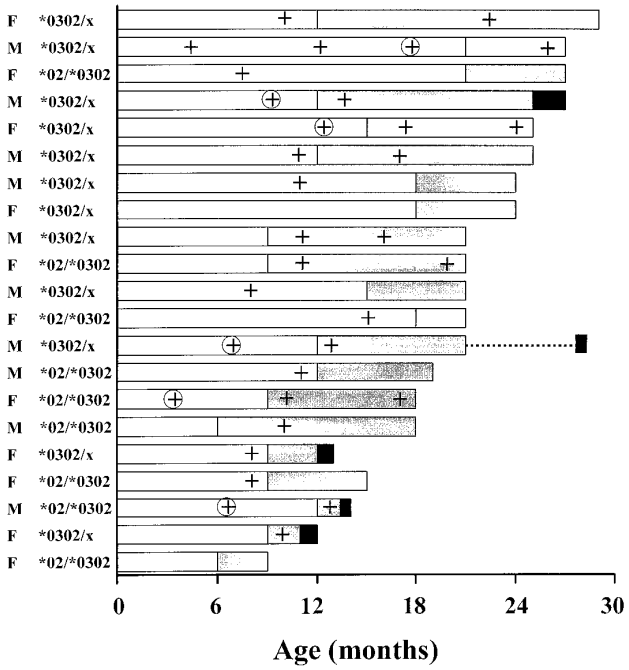

FIG. 1. The sex (M/F) and the HLA-DQB1 alleles of each case child (*n* = 21) are shown on the left. The horizontal bars indicate the length of the follow-up starting at birth. All case children were initially negative for diabetes-associated autoantibodies (□) but developed permanent autoantibody positivity (□). Five case children have progressed to clinical type 1 diabetes (■). Enterovirus infections occurring during the follow-up are marked with +, and ⊕ indicates infections in which enterovirus RNA was detected.

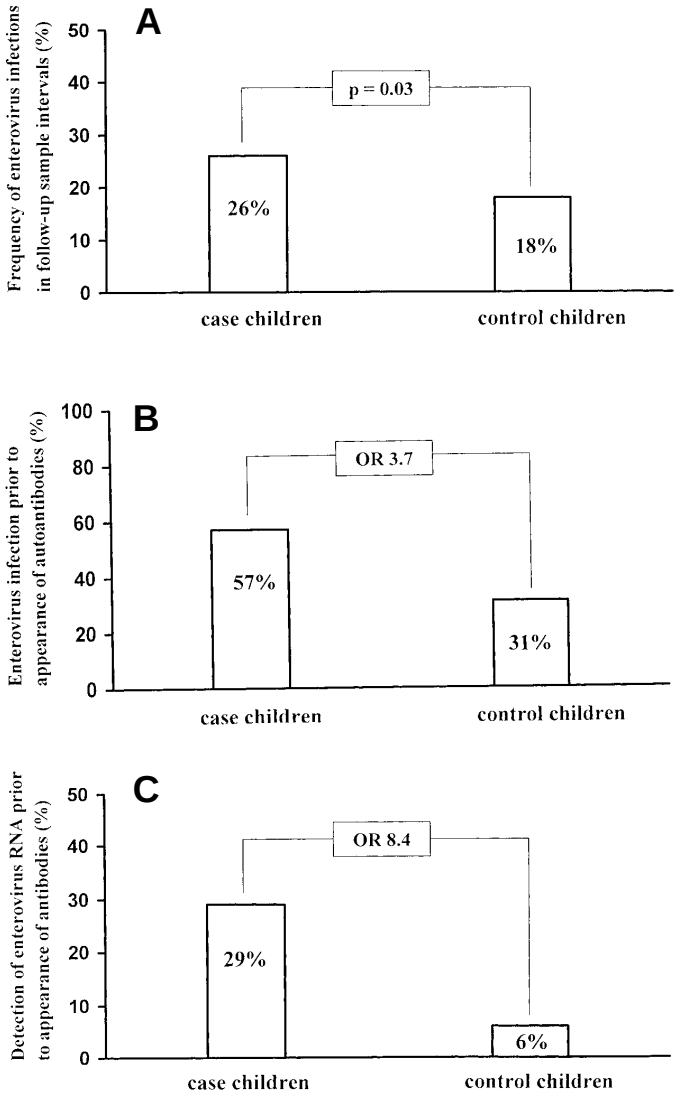

FIG. 2. *A*: Frequency of enterovirus infections in the sample intervals of the entire follow-up period of the case and control children. *B*: Occurrence of enterovirus infections in the case studies and control subjects during a 6-month observation period before the appearance of the first autoantibodies in the case children. *C*: Enterovirus RNA detection in the case subjects and control subjects during a 6-month observation period before the appearance of the first autoantibodies in the case children.

those in the control children (P = 0.03) (Fig. 1 and 2*A*). Enterovirus RNA was detected in 4% of the samples from the case subjects and in 3% of the samples from the control children (P = 0.8). Sex had no effect on the frequency of enterovirus infections among case subjects (P = 0.5) or control subjects (P = 0.8), but the difference in the frequency of enterovirus infections between the case and control groups was more marked among boys (28 vs. 18%, P = 0.02) than among girls (23 vs. 18%, P = 0.3). Only 2 of 33 (6%) and 9 of 103 (9%) enterovirus infections were diagnosed before the age of 6 months in the case subjects and control subjects, respectively. Enterovirus infections occurred as frequently in children with the HLA-DQB1*02/*0302 genotype as in children with the *0302/x genotype (P = 0.5 for the difference among the case subjects, P = 0.3 for the difference among the control subjects).

Enterovirus infections 0–6 months before the first detection of autoantibodies. A 6-month follow-up period preceding the appearance of autoantibodies in the case subjects was analyzed separately to evaluate possible temporal relationship between infections and the appearance of autoantibodies. A matching 6-month period of each control child was observed for comparison. Enterovirus infections were detected in 12 of the 21 case children (57%) and in 32 of the 104 control children (31%) (odds ratio [OR] 3.7, 95% CI 1.2–11.4) (Fig. 2*B*). The difference was even more marked when the occurrence of enterovirus RNA was compared between case and control children during this 6-month period (RNA was detected in 29 of the case subjects vs. 6% of the control subjects,OR 8.4, 95% CI 1.7–40.2) (Fig. 2*C*).

The 6-month follow-up period included 1 or 2 sample intervals per each child. Among the case children, enterovirus infections were detected in 36% of the sample intervals during this 6-month period compared with 23% of all sample intervals during the rest of the follow-up (P = 0.2). Also, enterovirus RNA was detected more frequently during this 6-month period than during the rest of the follow-up of case children (18 vs. 0% of samples, respectively, P = 0.0002). Among the control children, the frequency of enterovirus infections did not differ between the sample intervals of the 6-month period and the rest of the follow-up (enterovirus infections: 20 vs. 17%, P = 0.5; detection of enterovirus RNA: 4 vs. 4%, P = 0.9).

Enterovirus infections during pregnancy. The occurrence of enterovirus infections during pregnancy was evaluated using the maternal serum samples as well as the cord blood samples.

Two mothers of the 21 case children (10%) and 17 mothers of the 104 control children (16%) had had an enterovirus infection during pregnancy (OR = 0.5, 95% CI 0.1–2.5).

Adenovirus infections. Adenovirus infection was detected in 7% of the sample intervals in both the case subjects and the control subjects (P = 0.9). Neither sex nor HLA-DQB1 genotypes had an effect on the frequency of adenovirus infections. The frequency of adenovirus infections did not differ between the case subjects and the control subjects during the 6-month period before the case subjects' seroconversion to autoantibody positivity (P = 0.9). None of the 21 case children's mothers had had an adenovirus infection during pregnancy and only 3 mothers of the 104 control children had experienced an adenovirus infection during pregnancy (P = 0.5).

DISCUSSION

The frequency of enterovirus infections during the entire follow-up period was higher in the children who presented with signs of β-cell autoimmunity than in their control subjects matched for time of birth, sex, and the HLA-DQB alleles (P = 0.03). The case and control children had similar frequencies of adenovirus infections, implying that the children who developed autoantibodies are not more susceptible to viral infections in general.

Specifically, enterovirus infections were more frequent in the case children during the 6-month period preceding the appearance of the first diabetes-associated autoantibodies than in the matched time interval for the control children (OR 3.7, 95% CI 1.2–11.4). The difference between case subjects and control subjects was even more pronounced for enterovirus RNA detection during this interval (OR 8.4, 95% CI 1.7–40.2). In addition, positive enterovirus RNA findings in case children were significantly clustered around this 6-month period. This temporal association between enterovirus infections and the onset of β-cell autoimmunity, a phenomenon that has also been implicated by our previous studies (5,6), is consistent with the hypothesis that enteroviruses may be one of the factors initiating autoimmunity. The relationship between enterovirus RNA in serum and the appearance of autoantibodies is particularly intriguing because RNA in serum is a marker of viremia and viremia would allow the spreading of the virus to various target organs, including the pancreas.

The HLA genes are potentially important regulators of the immune responses directed against enteroviruses because T-cell responses against enterovirus antigens differ between children with different HLA alleles (21,22). Thus, the HLA-defined genetic background is a possible confounding factor when immune responses against enteroviruses are studied. It is also possible that the same HLA alleles confer increased susceptibility to both enterovirus infections and type 1 diabetes. In our study, the possible confounding effect of the HLA-DQB1 alleles was eliminated by the matching of HLA-DQB1 alleles between the case and control children, and yet the frequency of enterovirus infections differed between the case subjects and control subjects.

Enteroviruses are among the most complex groups of viruses when considering serological diagnosis because the enterovirus genus comprises >60 different serotypes. Several serological assays were used in our study to detect antibodies against as many enterovirus serotypes as possible. Nevertheless, it is likely that not all enterovirus infections were detected by these assays. For infections caused by Coxsackie B serotypes, the sensitivity of the serological assays is between 90 and 100%, but in the case of infections caused by Coxsackie A and echovirus serotypes, the sensitivity is not higher than 75%. An RT-PCR was also used to further improve the diagnostic sensitivity. This RT-PCR assay detects the RNA of all enteroviruses because the amplified sequence is common to all known enteroviruses (16). However, all enterovirus infections do not necessarily cause viremia, and the assay result is also dependent on the moment of sampling because the viremic period usually lasts only a few weeks at most. Therefore, it is evident that only a fraction of enterovirus infections was detected by the RT-PCR assay. Despite all these problems regarding enterovirus diagnosis, the setting for the diagnosis of enterovirus infections was exceptionally good in this study because of the relatively short sample intervals and the complementary qualities of the serological assays and the RT-PCR assay.

It is not possible, at the present stage, to conclude whether the observed enterovirus infections represent specific serotypes or genetic cluster(s) of enteroviruses, because neither the RT-PCR assay nor the serological assays used are specific for any serotypes or genetic clusters. Previously, Coxsackie B virus serotypes have most often been associated with type 1 diabetes, but also other serotypes may be diabetogenic (23–25).

In this study, the occurrence of enterovirus infections during pregnancy did not differ between the case and control groups. This observation is in contrast with previous reports suggesting that enterovirus infections during pregnancy increase the offspring's risk to develop type 1 diabetes (3,4,26). An explanation for this could be that different end points were used in these studies (i.e., autoantibody positivity in this study and clinical diabetes in the previous studies). Larger study series are obviously needed to assess whether enterovirus infection during pregnancy truly affects the risk of diabetes in the offspring.

The rapidly increasing incidence of type 1 diabetes in the Western world calls for action, and vaccination against enteroviruses has been proposed as one possible way to decrease the incidence. Our findings support the hypothesis that enterovirus infections are indeed a risk factor for the development of β-cell autoimmunity, but as the mechanism of enterovirus-induced autoimmunity is still unclear, more detailed studies are needed before enterovirus vaccinations may be used as a feasible approach in the prevention of type 1 diabetes.

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