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Coxsackievirus B1 Is Associated With Induction of β-Cell Autoimmunity That Portends Type 1 Diabetes

The rapidly increasing incidence of type 1 diabetes implies that environmental factors are involved in the pathogenesis. Enteroviruses are among the suspected environmental triggers of the disease, and the interest in exploring the possibilities to develop vaccines against these viruses has increased. Our objective was to identify enterovirus serotypes that could be involved in the initiation of the disease process by screening neutralizing antibodies against 41 different enterovirus types in a unique longitudinal sample series from a large prospective birth-cohort study. The study participants comprised 183 case children testing persistently positive for at least two diabetes-predictive autoantibodies and 366 autoantibody-negative matched control children. Coxsackievirus B1 was associated with an increased risk of β-cell autoimmunity. This risk was strongest when infection occurred a few months before autoantibodies appeared and was attenuated by the presence of maternal antibodies against the virus. Two other coxsackieviruses, B3 and B6, were associated with a reduced risk, with an interaction pattern, suggesting immunological cross-protection against coxsackievirus B1. These results support previous observations suggesting that the group B coxsackieviruses are associated with the risk of type 1 diabetes. The clustering of the risk and protective viruses to this narrow phylogenetic lineage supports the biological plausibility of this phenomenon.

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Enteroviruses have been linked to type 1 diabetes in a number of previous studies, as reviewed previously (1,2). The recent discovery of diabetes-associated polymorphisms in the innate immune system receptor for enteroviruses (IFIH1) has further increased the interest

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in the role of enterovirus infections in the pathogenesis of the disease (3). This association has not been observed in all studies, however, and the causal relationship has remained open.

More than 100 different enterovirus serotypes have been identified, which vary in their binding to various cellular receptors and in their ability to infect different cell types and organs. Consequently, different serotypes cause a diverse spectrum of diseases. Poliomyelitis, the classical enterovirus disease, is caused by three serotypes, polioviruses 1, 2, and 3, which have a strong tropism for motoneurons in the spinal cord. This tropism is partly explained by the expression of the poliovirus receptor (CD166) on these cells. In ~1% of infected individuals, the virus spreads to the motoneurons and causes paralytic disease. Similarly, some other enteroviruses, including the six coxsackievirus B (CVB) serotypes, seem to have a tropism for human pancreatic islets in vitro (4–7) and in vivo (8–10), possibly because islet cells express the coxsackie-adenovirus receptor (CAR), which is the major receptor for CVBs (11).

The identification of the enterovirus serotypes that may induce the disease process leading to type 1 diabetes is important because it would enable further studies on the mechanisms of enterovirus-induced β-cell damage and would pave the way for the development of a preventive vaccine. The lack of this information could also explain the variable results from previous studies that have been based on assays detecting several different enterovirus types as a group (2,12). Despite the importance of this topic, large-scale systematic studies aimed at identifying diabetogenic enterovirus serotypes have not been performed. Previous reports of data from case reports and small patient series suggest that the CVB group viruses may include diabetogenic serotypes (1) but also that certain echovirus serotypes have been linked to type 1 diabetes (13).

Here, the role of enterovirus infections was studied using the birth cohort samples systematically collected in the prospective Diabetes Prediction and Prevention (DIPP) study in Finland. By screening for the presence of neutralizing antibodies directed against a panel of 41 enterovirus serotypes, we assessed the association between each individual serotype and the appearance of diabetes-predictive autoantibodies. A study of the time-relationship between infection and initiation of the autoimmune process was thus possible. This is the first large and systematic study aimed at the identification of diabetogenic enterovirus types at the time when the process appears to start.

RESEARCH DESIGN AND METHODS

Subjects

The study population was derived from the DIPP study (14). Families with children carrying an increased genetic risk for type 1 diabetes, defined by cord-blood HLA typing, were invited to participate in prospective follow-up starting from birth. Blood samples were drawn at the ages of 3, 6, 12, 18, and 24 months and once yearly thereafter. Follow-up samples were screened for islet cell antibodies (ICA), and if a child seroconverted to positivity for ICA, follow-up samples were also analyzed for autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), and the tyrosine phosphatase–related insulinoma-associated 2 molecule (IA-2A). Written consent was obtained from each family whose child took part, and the study was approved by the ethical committees of the Pirkanmaa Hospital and the Northern Ostrobothnia Hospital districts.

Our study was a nested case-control study (Fig. 1) using the following criteria to select case and control children: Case children had turned permanently positive for two or more diabetes-predictive autoantibodies and/or progressed to clinical type 1 diabetes. Two control children were selected for each case child. They all remained nondiabetic and autoantibody-negative for at least 2 years after the earliest detection of autoantibodies in the corresponding case child and were matched for time of birth (± 1 month except in 12 children ± 2 months), sex (60% were boys), HLA-DQB1 genotype, and region. The final study cohort included 183 case and 366 control children born during the period from 1995 to 2006 and who were an average age of 31 months (range 5–122) at initial seroconversion to autoantibody positivity (Supplementary Tables 1 and 2). By the end of July 2011, 119 case children had progressed to type 1 diabetes.

HLA Genotyping

An analysis of the HLA-DQB1 genotype was performed from cord blood to identify selected alleles (DQB1*02, *03:01, *03:02, and *06:02/3) associated with susceptibility to or protection against type 1 diabetes (15). The genotyping was based on hybridization with lanthanide-labeled oligonucleotide probes detected with time-resolved fluorometry (16). Families with an infant carrying the high-risk HLA-DQB1*02/DQB1*0302 genotype or the moderate-risk DQB1*0302/x genotype (x ≠DQB1*03:02, *06:02, or *06:03) were invited for follow-up (Supplementary Table 3).

Detection of β-Cell Autoimmunity and Clinical Type 1 Diabetes

ICAs were detected by indirect immunofluorescence, and the three other autoantibodies were quantified with radiolabel-binding assays (17). We used cutoff limits for positivity of 2.5 JDRUs for ICA, 3.48 JDRUs for IAA, 5.36 JDRUs for GADA (full-length GAD65, aa 1-585, used as construct), and 0.43 JDRUs for IA-2A (the intracellular portion of the IA-2 molecule, aa 605-979, used as construct), representing the 99th percentile in more than 350 Finnish children. The ICA assay had a disease sensitivity of 100% and specificity of 98% in the fourth

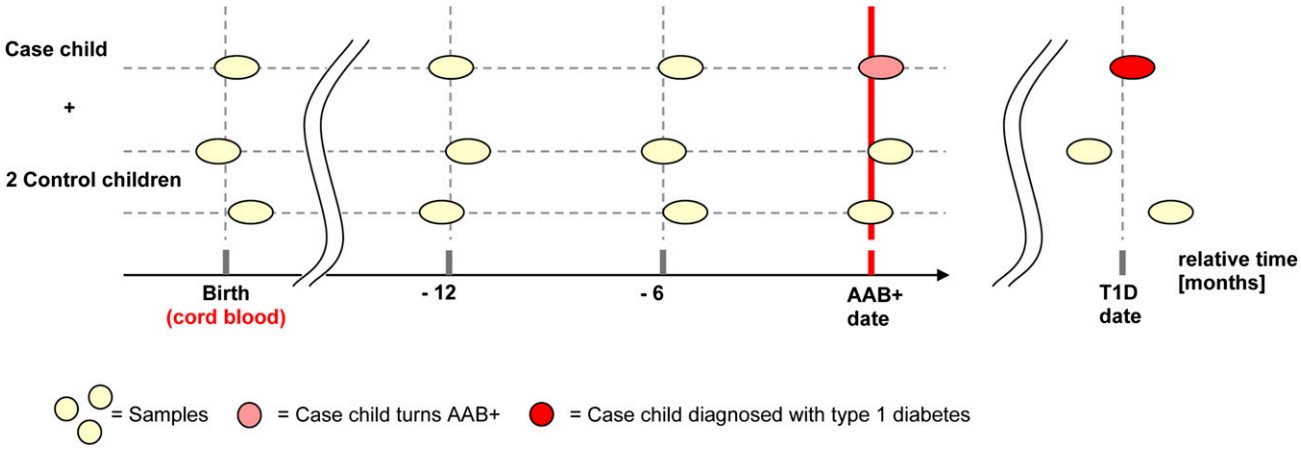

Figure 1—Study setup. The nested study consisted of 183 case/control triplets in which for each case child fulfilling the defined criteria two matched control children were selected. First, the neutralizing antibodies were analyzed in the samples where autoantibodies (AAB) were detected for the first time (AAB+ date sample) in case children and in the corresponding samples in control children (cross-sectional analysis). Next, neutralizing antibodies were screened in samples taken 6 and 12 months before AAB+ date (-12 and -6), as well as in samples taken at birth (cord blood) and at the age of 18 months, to perform longitudinal analyses for those enterovirus serotypes that were associated with the modulated diabetes risk at the cross-sectional primary screening step. However, the complete set of follow-up samples was not available from every child, which explains the small variation in the number of samples in different analyses. The information on the diagnosis of type 1 diabetes (T1D) date was used to run subcohort analyses for those triplets in which the case child progressed to type 1 diabetes.

round of the International Workshops on Standardization of the ICA assay. The disease sensitivity of the IAA assay was 58% and the specificity was 100% in the 2005 Diabetes Autoantibody Standardization Program Workshop. The same characteristics of the GADA assay were 82% and 96% and those of the IA-2A assay were 72% and 100%, respectively. The diagnosis of type 1 diabetes was based on the World Health Organization criteria.

Cells

Viruses were isolated and cultivated, and seroneutralization assays were performed using the A549, Vero, RD, and GMK cell lines. The three first cell lines were purchased from American Type Culture Collection, and GMK was acquired from the National Institute for Health and Welfare, Finland.

Neutralizing Antibodies Against Various Enterovirus Serotypes

Neutralizing antibodies were measured in serum or plasma against 44 enterovirus strains representing 41 serotypes. Most of these viruses were isolated from DIPP children and hospital patients in Finland and Sweden. All strains were plaque-purified and sequenced in their VP1 region for serotyping (18). Most of the viruses were analyzed using a standard plaque neutralization assay (19,20), whereas viruses that did not form clear plaques were analyzed using a microneutralization assay (Supplementary Table 4). All samples were screened using 1:4 and 1:16 dilutions. Inhibition was considered to be significant when the serum reduced the number of plaques more than 75% (plaque assay) or inhibited the ability of the virus to kill cells (microneutralization assay).

The identification of diabetogenic serotypes was based on a step-wise strategy (Fig. 1): First, the neutralizing antibodies were analyzed in the samples where autoantibodies were detected for the first time in case children and in the corresponding samples in control children (cross-sectional analysis). All samples showing titers of 1:4 or greater were considered positive.

In the next step, neutralizing antibodies were screened in samples from earlier time points (longitudinal analyses) for those enterovirus serotypes that were associated with diabetes risk at the cross-sectional primary screening step. These longitudinal analyses made it possible to diagnose infections by virus antibody seroconversions observed between two consecutive follow-up samples (Supplementary Fig. 1). These earlier time points included samples taken 6 and 12 months before the initial seroconversion to autoantibody positivity (Fig. 1). The mean age and age range at these time points are reported in Supplementary Table 5. Some samples were collected from the children at such a young age that they possibly contained maternal antibodies. Cord-blood samples were therefore analyzed in these children, and when the presence of maternal antibodies could bias a positive result, the sample was considered negative. In addition, cord-blood samples and samples taken at the age of 18 months were analyzed from all children for CVB1 antibodies. The following definitions were used to diagnose an acute infection: the main definition was based on “sensitive diagnostic criteria,” where transient and permanent antibody

seroconversions were both counted (if the child had serial transient seroconversions against the same virus only the first one was counted). The results were confirmed using more strict "specific diagnostic criteria," where acute infections were diagnosed by the following criteria: a seroconversion from a titer of <1:4 (seronegative) to ≥1:4 (seropositive), a titer of 1:16 in at least one of the following samples, and all subsequent samples were positive.

Statistical Analyses

The primary analysis method was conditional logistic regression using the one-to-two age, sex, HLA, and region matched case-control triplets. Data from matched case-control pairs and triplets were analyzed using Stata 8.2 software (StataCorp, College Station, TX), which allows for variable matching ratios of case subjects to control subjects. Conditional logistic regression was used to estimate the odds ratios (OR) with exact 95% confidence intervals (CI) and two-sided *P* values for univariate point estimates and multivariate modeling to assess the association between enterovirus antibodies and diabetes-predictive autoantibodies. In the first phase, a cross-sectional analysis was performed using data on the prevalence of enterovirus serotypes at the time point when the first diabetes-predictive autoantibodies were detected. The duration of exclusive and total breast-feeding was entered into a multivariate analysis to estimate adjusted ORs.

Second, to study the temporal profile of the associations detected in these cross-sectional analyses, infections occurring during all longitudinal time points before the detection of predictive autoantibodies were analyzed. The time was classified into three periods (simultaneously with the first detection of autoantibodies, 6 months before autoantibodies, and 12 months or longer before autoantibodies), and the infections were diagnosed using the sensitive and specific criteria described above.

Third, the longitudinal data were used to analyze the effect of the chronology of infections caused by different serotypes on the risk of β-cell autoimmunity.

Fourth, interactions between different serotypes were analyzed by studying the effect of different virus combinations. In addition to the raw *P* values, the *P* values that were corrected for the number of comparisons made (Bonferroni correction) are presented.

RESULTS

Seroprevalences of CVB1, CVB3, and CVB6 Show a Cross-Sectional Association With the Risk to Develop Autoantibodies

Neutralizing antibodies were initially screened against 41 enterovirus serotypes in the first sample positive for diabetes-predictive autoantibodies. The conditional logistic regression analyses showed that CVB1 antibodies were more frequent in the case children than in the control children (59.0% vs. 50.1%; OR 1.5 [95% CI 1.0–2.2]; $P = 0.04$) suggesting that an infection with this enterovirus is associated with an increased risk of β-cell autoimmunity (Table 1). The statistical significance disappears when the *P* value is multiplied by the number of tested serotypes ($N = 41$). The high seroprevalence of CVB1 in the control children (50.1%) indicates that this enterovirus is a common serotype in the population studied. Only one case and one control child were negative for all 41 tested enterovirus serotypes (the median number of positive serotypes was 9 in both groups).

Neutralizing antibodies to two closely related serotypes, CVB3 and CVB6, were less frequent in case children than in control children, indicating a strong protective association for CVB3 (5.8% vs. 12.8%; OR 0.4 [95% CI 0.2–0.8]; $P = 0.01$) and a weaker protective association for CVB6 (26.6% vs. 35.3%; OR 0.6 [95% CI 0.4–1.0]; $P = 0.04$) (Table 1). As above, the statistical significance disappears when these *P* values are multiplied by the number of tested serotypes. However, the fact that the protective serotypes were the closest genetically to CVB1 (Fig. 2) and no protective association was seen for more distant strains among the 41 analyzed, suggests that these findings reflect a true biological phenomenon. In fact, they support the plausible hypothesis that some immunological cross-protection exists between these closely related enterovirus types. The analysis of potential interactions between CVB1 and the other CVB serotypes indicated a clear risk effect when the child had experienced CVB1 alone without these protective serotypes (OR 2.5 [95% CI 1.4–4.7]; $P = 0.003$), whereas children infected by both CVB1 and one or more of the protective serotypes were not at risk (Table 2 and Supplementary Table 6).

The risk association of CVB1 and the protective association of CVB3 and CVB6 was also seen in the subcohort of 119 children who progressed to clinical type 1 diabetes (OR for CVB1 was 1.8 [95% CI 1.1–2.9]; $P = 0.025$), both among boys and girls and in different age groups (data not shown). The effects of CVB1 and CVB3 remained significant after adjustment for the duration of breast-feeding and the number of older siblings, whereas the effect of CVB6 became nonsignificant (a clear trend was observed also for CVB6; Supplementary Table 7).

The CVB1 Risk Association was Confirmed in Longitudinal Analyses Before the Appearance of the First Autoantibodies in Case Children

The timing of infection with CVB1 was further assessed in a longitudinal analysis by detecting seroconversions in the neutralizing antibodies between consecutive follow-up samples collected before the first autoantibody-positive sample. The results showed an increased risk of autoantibody positivity when a CVB1 infection preceded the autoantibody appearance (Table 3). This association was

Table 1—ORs for the association between neutralizing antibodies to 44 enteroviruses (41 serotypes) and signs of progressive β-cell autoimmunity (positivity for two or more diabetes-predictive autoantibodies) in 183 case and 366 matched control children

	NAB prevalence			
Virus	% Case	% Control	OR (95% CI)	*P* value
CVA4	28.7	31.7	0.9 (0.6–1.3)	0.46
CVA5	15.0	15.0	1.0 (0.6–1.9)	0.96
CVA6	17.1	14.7	1.2 (0.7–2.1)	0.44
CVA10	69.8	61.8	1.4 (0.8–2.5)	0.27
CVA16	12.5	16.6	0.7 (0.4–1.2)	0.23
EV71	8.4	7.9	1.1 (0.5–2.4)	0.89
CVA9	7.5	8.4	0.9 (0.4–1.8)	0.72
CVB1	**59.0**	**50.1**	**1.5 (1.0–2.2)**	**0.04**
CVB2	46.6	48.8	0.9 (0.6–1.3)	0.61
CVB3	**5.8**	**12.8**	**0.4 (0.2–0.8)**	**0.01**
CVB4-wt*	5.2	8.1	0.6 (0.2–1.3)	0.19
CVB4-rs#	5.2	7.2	0.7 (0.3–1.5)	0.34
CVB5	7.5	7.8	0.9 (0.4–1.9)	0.81
CVB6	**26.6**	**35.3**	**0.6 (0.4–1.0)**	**0.04**
E1	25.9	26.2	0.6 (0.3–1.3)	0.19
E2	9.1	9.9	1.0 (0.5–1.9)	0.95
E3-wt	5.2	4.1	1.3 (0.6–3.2)	0.54
E3-rs	43.8	39.9	1.2 (0.8–1.8)	0.33
E4	1.2	1.5	0.8 (0.2–4.1)	0.79
E5	36.0	37.2	0.9 (0.6–1.5)	0.73
E6	8.6	7.5	1.2 (0.6–2.3)	0.65
E7	18.4	17.9	1.0 (0.6–1.7)	0.90
E9	7.6	9.8	0.7 (0.3–1.5)	0.34
E11	32.4	36.3	0.8 (0.5–1.2)	0.33
E12	36.8	31.7	1.3 (0.9–2.0)	0.22
E13	2.3	4.1	0.5 (0.2–1.7)	0.30
E14	7.6	5.6	1.3 (0.6–2.9)	0.43
E15	8.7	13.0	0.6 (0.3–1.2)	0.13
E17	5.8	7.8	0.7 (0.3–1.5)	0.31
E18	3.5	3.8	0.9(0.3–2.5)	0.87
E19	9.6	13.1	0.7 (0.3–1.5)	0.34
E20	6.4	5.2	1.3 (0.6–2.8)	0.50
E21	28	32.4	0.8 (0.5–1.2)	0.29
E25	6.4	4.3	1.5 (0.7–3.2)	0.34
E26	1.7	3.5	0.5 (0.1–1.8)	0.27
E27	5.8	6.5	0.9 (0.4–1.9)	0.75
E29	9.3	7.4	1.3 (0.6–2.8)	0.45
E30-wt-1	98.0	96.8	0.8 (0.2–3.8)	0.79
E30-wt-2	72.3	76.1	0.8 (0.5–1.3)	0.38
E32	43.9	43.2	1.0 (0.6–1.6)	0.97
E33	81.9	80.7	1.2 (0.7–1.8)	0.56
EV74	60.1	59.6	1.0 (0.7–1.5)	0.97
EV78	2.3	4.4	0.5 (0.2–1.6)	0.25
EV94	5.3	5.0	1.1 (0.7–3.1)	0.93

CVA, coxsackievirus A; E, echovirus; EV, enterovirus; and NAB, neutralizing antibody. % case represents the antibody prevalence in case children and % control represents the prevalence in control children. Data in bold type are statistically significant. *wt, wild-type strain. #rs, reference strain.

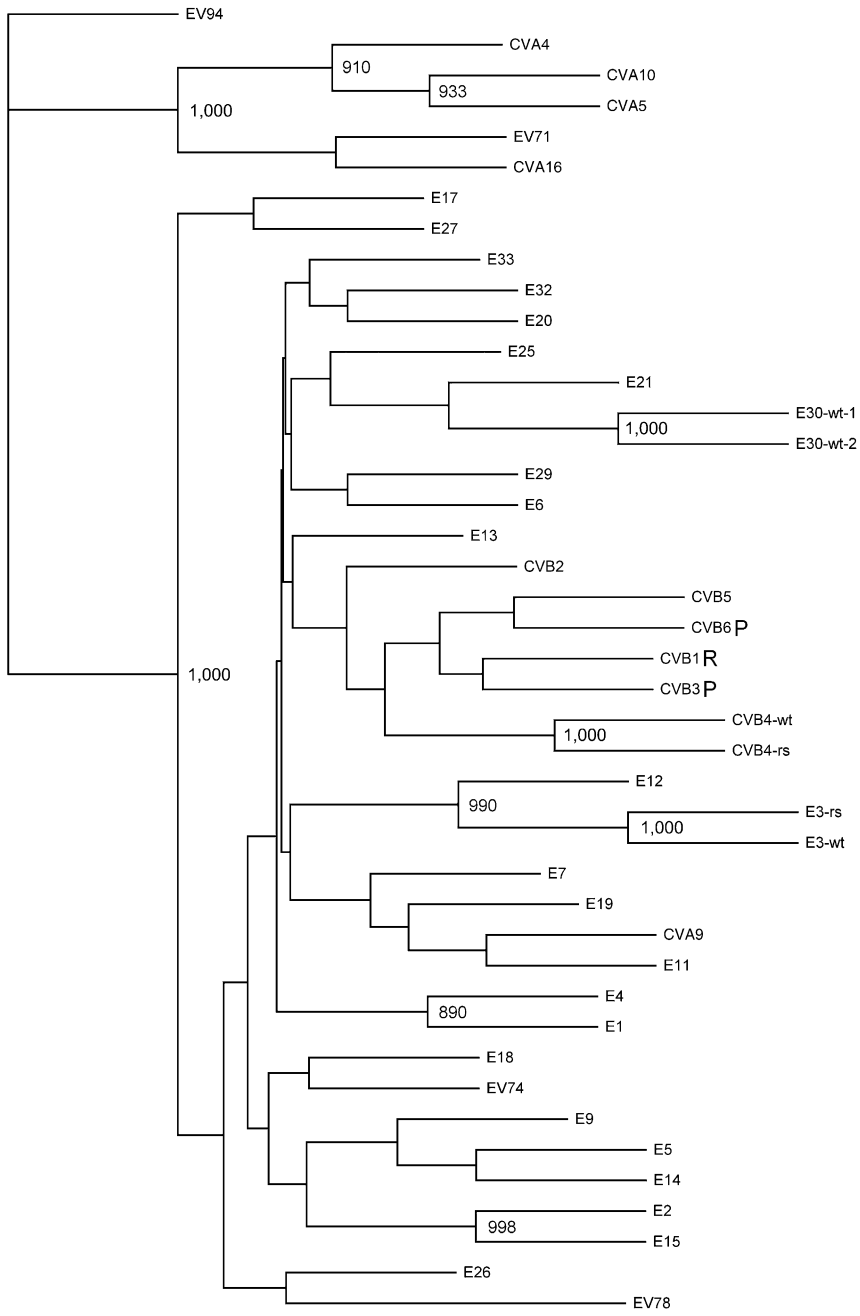

Figure 2—Consensus phylogenetic tree of the 44 virus strains based on 104 amino acids of the VP1 region. The part of VP1 region of all 44 viruses was sequenced, and the obtained sequences were blasted against the National Center for Biotechnology Information non-redundant nucleotide database. Phylogenetic analysis was done using the PHYLIP: Phylogeny Inference Package, version 3.69 program (Joe Felsenstein, 1993, University of Washington, Seattle, WA). The phylogenetic tree was constructed using the Protdist program with the parameters of the Kimura 2 model, and the amino acid matrix was processed with the Kitsch program. The consensus tree was treated with the Consense program. This analysis implies a close genetic relationship of the three CVB viruses that were associated with β-cell autoimmunity. The bootstrap confidence levels were analyzed with 1,000 pseudoreplicate data sets, and bootstrap levels higher than 70% were plotted onto the tree. CVA, coxsackievirus A; E, echovirus; EV, enterovirus; P, protective CVB3 and CVB6 strains; R, risk-associated CVB1 strain.

strongest when CVB1 infections preceded the first autoantibody-positive sample by a few months and was observed using both the sensitive and strict infection criteria. The association was also seen in the subgroup of children who progressed to clinical type 1 diabetes.

Chronological Order of CVB Infections

When the longitudinal data were analyzed to study the effect of the order of infections with CVB1 and the protective CVB serotypes, some trends suggesting a potential order effect were observed. When CVB1 was the first infecting serotype to occur, the children were at risk

Table 2—Association of different combinations of risk- and protective-type CVB infections with the risk of β-cell autoimmunity as defined by virus antibody positivity at the time of autoantibody seroconversion (cross-sectional analysis among 180 case and 360 matched control children)

Antibodies against risk serotype	Antibodies against protective serotypes	OR (95% CI)	*P* value
CVB1 neg	CVB3 or CVB6 pos	1 (Reference)	
CVB1 neg	CVB3 and CVB6 neg	1.6 (0.9–3.1)	0.12
CVB1 pos	CVB3 or CVB6 pos	1.5 (0.8–2.9)	0.20
CVB1 pos	CVB3 and CVB6 neg	**2.5 (1.4–4.7)**	**0.003**

Data in bold type are statistically significant. The reference group comprises children with the lowest predicted risk being seropositive for the protective serotypes but not for CVB1.

for developing autoantibodies, whereas when CVB3 or CVB6 infection occurred first, the risk of developing autoantibodies was lower (Supplementary Table 8). This again supports the conclusion that infection by CVB3 or CVB6 provides some immunological protection from the diabetogenic effect of CVB1.

Maternal Antibodies Modulate the Risk Effect of CVB1

The cord-blood samples and samples taken at the age of 18 months were analyzed to explore whether protective maternal CVB1 antibodies in cord blood can modulate the risk association of CVB1 infections in young infants. The risk association was strongest in the group who experienced CVB1 without maternal CVB1 antibodies (OR 2.6 [95% CI 1.1–5.9]; $P = 0.02$) (Table 4).

DISCUSSION

This case-control study nested in the DIPP birth cohort is the first systematic study aimed at identifying enterovirus subtypes possibly associated with the induction of β-cell autoantibodies. The study has several unique strengths. First, it is based on the analysis of neutralizing antibodies, which is the most reliable way to diagnose prior infection caused by a given enterovirus serotype. Second, it covers a large number of different serotypes ($n = 41$), most of which represent wild-type strains circulating in the background population. Third, it was performed in a prospective birth-cohort study including a longitudinal sample series starting from cord blood, which allowed the timing of the infections to be determined in relation to the time when autoantibodies first appeared. Fourth, the case and control subjects were matched for the most relevant potential confounders such as HLA-defined diabetes risk, sex, time of birth, age at sampling, and the area of residence. Finally, the results provided by the cross-sectional and longitudinal analyses using different infection criteria were coherent.

We believe that the finding that the three serotypes identified are closely related phylogenetically (Fig. 2) is very significant. Indeed, if the signals detected in this study were due to arbitrary random noise in the methods, it would be unlikely that they would cluster together phylogenetically. Close clustering, on the other hand, is precisely what would be expected for serotypes that could be causative or protective, based on the highly plausible hypothesis of some degree of immunological cross-protection, as discussed subsequently.

The outcome reported here is consistent with the diabetogenic role of enteroviruses postulated in the literature and with predictions that can be made in searching for diabetogenic viruses. Prospective studies have shown

Table 3—The risk for β-cell autoimmunity associated with CVB1 infections according to the time when these infections were diagnosed in 183 cases and 366 matched control children

	Sensitive diagnostic criteria		Specific diagnostic criteria	
Timing of CVB1 infection*	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Whole nested case-control series				
No infection	1 (Reference)		1 (Reference)	
12 months or longer before autoantibodies	1.3 (0.8–2.3)	0.33	1.0 (0.5–2.2)	0.93
6 months before autoantibodies	**2.0 (1.1–3.6)**	**0.03**	1.9 (0.7–5.2)	0.23
Simultaneously with autoantibodies	1.5 (0.9–2.4)	0.11	**2.1 (1.0–4.4)**	**0.04**
Case children who progressed to type 1 diabetes and their control children				
No infection	1 (Reference)		1 (Reference)	
12 months or longer before autoantibodies	1.0 (0.4–2.2)	0.91	0.7 (0.2–2.0)	0.48
6 months before autoantibodies	**2.0 (1.0–4.2)**	**0.05**	1.8 (0.6–5.0)	0.27
Simultaneously with autoantibodies	1.6 (0.89–2.9)	0.11	**2.5 (1.1–5.6)**	**0.03**

The diabetes subgroup included 119 case children who progressed to clinical type 1 diabetes and their 239 matched control children. The sensitive and specific diagnostic criteria analyses were performed as defined in the RESEARCH DESIGN AND METHODS. Data in bold type are statistically significant. *Average time in relation to autoantibody seroconversion.

Table 4—The risk of β-cell autoimmunity in children according to their exposure to CVB1 by the age of 18 months (CVB1 seropositive at that age) and presence of protective CVB1 antibodies in cord blood among 127 case and 254 matched control children

CVB1 seropositivity		Observed risk of β-cell autoimmunity		
Cord blood	18 months	OR (95% CI)	*P* value	Expected risk of β-cell autoimmunity***
Pos*	Neg**	1 (Reference)		Lowest
Neg	Neg**	1.6 (0.7–3.9)	0.28	Low
Pos*	Pos	2.1 (0.8–5.6)	0.12	High
Neg	Pos	**2.6 (1.1–5.9)**	**0.02**	Highest

Data in bold type are statistically significant. *Only antibody titers 16 or higher were considered positive in cord blood because low antibody levels disappear rapidly from the child's circulation. **Negative antibody result does not exclude early CVB1 infection due to possible transient antibody responses in these very young infants. ***Expected risk refers to theoretical risk predicted on the basis of CVB1 seropositivity in cord blood (maternal antibodies) and at the age of 18 months.

that the autoimmune process usually begins at an early age (<3 years) (20,21) and that autoantibodies appear annually in "epidemic" peaks (20). Consequently, the causative agent is probably frequent in the background population circulating continuously in very young children. The epidemiology of CVB1 fits with these predictions. CVB1 has been one of the most frequent enteroviruses isolated in recent years in the U.S. (22,23) as well as in Korea (24), India (25), Tunisia (26), Western Germany (27), and Finland (28). It can cause severe systemic infections in young infants (29,30) and infects human pancreatic islets in vitro, being one of the most cytolytic enterovirus serotype in this model (7). In fact, insulitis and islet cell damage have been described in infants who have died of CVB1 infection (31). Certain CVB1 strains induce also persistent infections in mice that lead to chronic inflammatory myopathy (32). One can estimate from the generated data that less than 5% of CVB1-infected children go on to develop type 1 diabetes. This fits with the low attack rate typical for enterovirus diseases; for example, in the beginning of the 20th century, almost the entire population became infected by polioviruses but less than 1% developed motor neuron damage and paralysis. This implies also that the ORs obtained from serological screening studies remain relatively modest, even though CVB1 infection may explain most of the cases.

Surprisingly, the current study revealed that infections by two other CVBs, CVB3 and CVB6, were associated with a decreased risk of β-cell autoimmunity. A possible protective effect of CVB3 has actually been reported in a smaller study where patients with newly diagnosed type 1 diabetes were found to be less frequently positive for neutralizing antibodies against this serotype than control subjects (26). This phenomenon could be explained by immunological cross-protection induced by CVB3 and CVB6 against the diabetogenic effect of CVB1. Such cross-protection, most likely due to cell-mediated immunity, has been reported in other virus diseases, such as among different rotavirus, papillomavirus, and poliovirus types (33–37). Cross-protection is also supported by the increased CVB1-related risk in children who were infected by CVB1 but none of the protective serotypes. Prevention of lethal CVB1 infection by a prior CVB3 infection has also been observed in a mouse model, fitting nicely with the findings in the current study (38). In addition to cross-protection, other mechanisms related to the induction of β-cell tolerance may mediate the protective effect of viruses against type 1 diabetes as described in NOD mice (39,40). In both cases, the close relationship between the protective and the diabetogenic serotypes suggests a particular impact of the CVB group enteroviruses on the risk of diabetes. Because CVBs are the only enteroviruses to use CAR, it can be hypothesized that they share some specific characteristics in terms of antigenicity and/or tropism.

Despite its virtues, the current study also has limitations. The first relates to the population studied being exclusively from Finland and covering a relatively limited 10-year period. Consequently, we cannot exclude a timing effect of CVB1 infections or a strain-specific effect of this serotype. A timing effect could also explain the low prevalence of the CVB4 serotype, which has been linked to type 1 diabetes in previous studies. Accordingly, confirming these findings in other populations will be important. The statistical power of the current study allowed the identification of viruses with major risk effects, whereas viruses with weaker effects might have been missed. Adding new datasets would also help to assess further the combined effect of the three identified CVB serotypes. The virus strains used in the neutralization assay represent the most common enterovirus serotypes (22,23) but do not include all serotypes known today (many of them are also difficult to cultivate and to produce cytopathic effect in vitro). Therefore, we cannot exclude the possibility that other risk or protective serotypes might have been missed.

The current findings have aspects that fit with causality: First, the CVB1-related risk effect showed logical time relationship: it preceded the initiation of the autoimmune process. In addition, CVB1 infections peaked a few months before autoantibodies first appeared, which overlaps with the previously observed peak in the frequency of enterovirus RNA in serum (41), fitting with the rapid induction of islet autoantibodies in enterovirus-infected mice (42). Second, the accumulation of risk and protective viruses to a small subgroup of phylogenically close enteroviruses supports the

biological relevance of our findings. Third, the discovery of protective viruses fits with immunological cross-protection attenuating infections caused by closely related viruses. Fourth, the observation that maternal CVB1 antibodies modulated the risk effect of CVB1 supports biological plausibility because maternal antibodies protect the child against enterovirus infections (43,44). Finally, we observed a similar risk effect of CVB1 in another study where neutralizing antibodies were analyzed in patients with newly diagnosed type 1 diabetes and control subjects in five European countries (41).

In summary, the results are in line with the previous literature suggesting a link between enterovirus infections and type 1 diabetes. The identification of CVB1 as a potentially diabetogenic virus type is a new discovery that offers possibilities to explore the mechanisms of enterovirus-induced diabetes and may also open the door for the development of an enterovirus vaccine against the disease. Further studies are needed to confirm these findings in other populations. The identification of serotypes with opposite effects on type 1 diabetes implies that serotype-specific methods should be used in such studies.

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Duality of Interest. H.Hy. and M.K. are minor (<5%) shareholders and members of the board of Vactech Ltd., which develops vaccines against picornaviruses. No other potential conflicts of interest relevant to this article were reported.

Author Contributions. V.L., J.W.A., M.K., and H.Hy. were part of the steering group that designed the study. O.S., J.I., R.V., M.K., and H.Hy. participated in the recruitment of children to the DIPP study. O.H.L., H.Ho., O.P., S.O., M.M.H., T.R., R.H., M.K., and H.Hy. participated in the virus analyses. S.M.V. was responsible for the dietary data (breast-feeding). H.Hu., P.A., and J.L. analyzed data. O.H.L. and H.Ho. wrote the initial draft of the manuscript. All authors contributed to the data interpretation, to the preparation of the manuscript, and to the final version of the manuscript. H.Hy. and H.Ho. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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