

Enterovirus RNA sequences in sera of schoolchildren in the general population and their association with type 1-diabetes-associated autoantibodies

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Type 1 diabetes (T1D) is an autoimmune disease linked with genetic factors as well as with environmental triggers, such as virus infections, but the aetiology is still unclear. The authors analysed serum from autoantibody-positive ($n = 50$) and autoantibody-negative ($n = 50$) schoolchildren as well as children newly diagnosed with T1D ($n = 47$; time from diagnosis, median 5 days, interquartile range 1–12 days) for the presence and frequency of enterovirus (EV) and adenovirus sequences. The autoantibody-positive and -negative groups were part of the Karlsburg Type 1 Diabetes Risk Study of a Normal Schoolchild Population, which represents a general population without T1D first-degree relatives. There was no significant seasonality of sampling in any of the three groups investigated. EV RNA sequences were detected in 10 of 50 (20 %) autoantibody-positive children and in 17 of 47 (36 %) children newly diagnosed with T1D, but only in two of 50 (4 %) of the age- and sex-matched controls ($P < 0.05$, $P < 0.001$). Characterization of the EV amplicons by direct sequencing revealed high homology with coxsackievirus B group. For adenovirus we found no data to support an association with T1D. The data support the hypothesis that different enteroviruses may be aetiologically important as a trigger and/or accelerating factor in the process of T1D development.

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INTRODUCTION

Type 1 diabetes (T1D) results from the progressive destruction of insulin-producing pancreatic beta cells. Both genetic and environmental factors may contribute to the development of the autoimmune process that finally leads to the manifestation of T1D (Szopa *et al.*, 1993; Hyöty & Taylor, 2002; Jun & Yoon, 2004).

Viruses are one type of environmental agent suspected of having importance in the expression of the autoantigens of T1D. Associations of many viruses including rubella virus, mumps virus, cytomegalovirus and Epstein–Barr virus have been discussed in terms of the development of T1D, but the strongest evidence suggests a link with enteroviruses.

To date, many of the studies suggesting a possible association of enteroviruses with T1D have been based on serological data (Helfand *et al.*, 1995; Hyöty *et al.*, 1995; Hiltunen *et al.*, 1997; Roivainen *et al.*, 1998). Now the introduction of

molecular approaches has opened up new possibilities to evaluate the role of viral infections, and some studies have indeed confirmed the preliminary hypothesis that a link exists between enteroviral infection and development of T1D (Clements *et al.*, 1995; Andréoletti *et al.*, 1997; Nairn *et al.*, 1999; Lönnrot *et al.*, 2000a; Kawashima *et al.*, 2004). Besides, it has been shown *in vitro* that coxsackievirus B4 can replicate in pancreatic beta cells and can cause an excessive release of insulin (Titchener *et al.*, 1994). The E2 strain of human coxsackievirus B4 can induce a diabetic-like syndrome in a mouse model (Kang *et al.*, 1994). Determinants of virulence have been identified in the 5' untranslated region (5'-NTR) and the capsid proteins VP1, VP2 and VP4 (Ramsingh *et al.*, 1992).

However, there are also studies that do not support the hypothesis that enterovirus (EV) infections are a risk factor for T1D. In a prospective study within a cohort of genetically high risk children no evidence was found that EV infections are associated with the process leading to beta-cell autoimmunity (Graves *et al.*, 2003). In a group of newly diag-

Abbreviations: ADV, adenovirus; EV, enterovirus; IQR, interquartile range; 5'-NTR, 5' untranslated region; T1D, type 1 diabetes.

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nosed T1D children in Finland no EV RNA was detectable in contrast to a group of prediabetic children with an EV RNA prevalence of 22 % (Lönnrot *et al.*, 2000b).

Adenoviruses are also widespread and cause several clinical syndromes. Persistent or latent adenovirus (ADV) infection can occur in lymphocytes (Matsuse *et al.*, 1992; Mentel *et al.*, 1997). In studies to determine whether there is an association between virus infection and beta-cell autoimmunity ADV were rarely involved and analysis was mostly based on antibody detection (Lönnrot *et al.*, 2000a; Salminen *et al.*, 2003; Sadeharju *et al.*, 2003).

The aim of our study was to assess if there is a link between EV and ADV infection and T1D in a general population without first-degree diabetic relatives. Sera taken from schoolchildren in the prediabetic stage, as defined by autoantibody status, and during the onset of T1D were evaluated for nucleic acid sequences by PCR.

Methods

Subjects. The samples were derived from the Karlsburg Type 1 Diabetes Risk Study of a Normal Schoolchild Population, which represents a normal cross-section of the population in the eastern part of Mecklenburg-Vorpommern, Germany, with no first-degree relatives with T1D (Strebelow *et al.*, 1999; Schlosser *et al.*, 2002). The parents/carers of all the children provided written informed consent and the study was approved by the ethical committee of the Ernst Moritz Arndt University Greifswald and by the Ministry of Culture and Education of Mecklenburg-Vorpommern.

Subjects were randomly selected, and serum samples were stored at -20°C until assayed. The present study is based on three groups: I, 50 samples from prediabetic children (28 male/22 female), positive for autoantibodies against glutamate decarboxylase GAD65, protein tyrosinophosphatase IA-2c, insulin and/or islet cytoplasmic antigens in the study mentioned above, who had a median age of 12 years [interquartile range (IQR) 10–14 years]; II, 50 samples from antibody-negative control children (24 male/26 female) from the same study, who had a median age of 14 years (IQR 12–16 years); III, 47 samples from children with newly diagnosed T1D (24 male/23 female), who had a median age of 13 years (IQR 11–15 years). Samples from patients in group III were taken soon after the diagnosis of diabetes (median 5 days, IQR 1–12 days) and were obtained from the Center of Diabetes and Metabolic Disorders, Karlsburg. There was no significant seasonality of sampling in any of the three groups investigated.

Detection of EV RNA. RNA extraction was performed using a QIAamp viral RNA mini kit (Qiagen) according to the manufacturer's protocol.

The screening PCR was carried out by nested RT-PCR using primer pairs derived from the highly conserved 5'-NTR, CX3 (5'-CGGTGGCTGCGTTGGCGCC-3' position 354–373) and CX10 (5'-ATTGTGACCATAAGCAGCCA-3' position 599–580), and CX8 (5'-AAACACGGACACCCAAAGTA-3' position 563–544) and CX9 (5'-GGCCCTGAATGCGGCTAAT-3' position 451–470) (Severini *et al.*, 1993), and was performed using the QIAGEN one-step RT-PCR kit (Qiagen). Reverse transcription was carried out for 30 min at 50°C followed by an initial PCR activation step at 95°C for 15 min and then 40 cycles of 40 s at 94°C , 1 min at 53°C and 1 min at 72°C . The nested PCR consisted of 5 min at 94°C , followed by 30 cycles of 40 s at 94°C , 1 min at 55°C and 1 min at 72°C , and a final extension at 72°C for 7 min. Amplification was performed using 30 pmol of each primer with a sensitivity of 20 copies per reaction. The specificity of the assay was analysed by reactions with

nucleic acids from herpes simplex virus, varicella-zoster virus, cytomegalovirus, adenovirus, Epstein–Barr virus, parvovirus B19, respiratory syncytial virus, and influenza A and B viruses; no amplification was observed.

The real-time PCR was performed with primers and probe also derived from the 5'-NTR, EV2 (5'-CCCCTGAATGCGGCTAATC-3' position 451–469), EV-CSF-R (5'-ATTGTCACCATAAGCAGCCA-3' position 596–577) and EV3 (5'-6-FAM-CGGAACCGACT ACTTTGGGTGTC CGT-3'-TAMRA position 532–557) (Corless *et al.*, 2002). Primers and probe were synthesized by TIB MOLBIOL.

Total RNA was reverse transcribed using TaqMan reverse transcription reagents (Applied Biosystems) with 2.5 μM random hexamers for 10 min at 25°C , 30 min at 48°C and 5 min at 95°C . Amplification was performed using 100 pmol of each primer and 30 pmol of the probe. The cycle profile was 2 min at 50°C and 10 min at 95°C , followed by 50 cycles of 15 s at 95°C and 1 min at 60°C with iCycler IQ Real-Time Detection System version 3.0 (Bio-Rad). The sensitivity of the assay was around 80 copies per reaction using serial dilutions of a cloned EV transcript.

The sequence reaction was based on amplimers with primer sets derived from the 5'-NTR, P1 (5'-CGGTACCTTTGTGCGCCTGT-3' position 63–82) and P4 (5'-TTAGGATTAGCCGCATTCAG-3' position 475–457), and P6 (5'-GCACCTCTGTTACCCC-3' position 168–183) and P9 (5'-TCAATAGACTCTTCGCAC-3' position 433–416) (Nairn & Clements, 1999). PCR products were purified with a PCR purification kit (Qiagen) and sequenced by use of 40 pmol of primers P6 and P9 with the BigDye Terminator Cycle sequencing kit (Applied Biosystems). Cycle sequencing consisted of 25 cycles of 94°C for 10 s, 55°C for 5 s and 60°C for 4 min. Analysis of the products was carried out on an ABI PRISM 310 automatic sequencer (Perkin Elmer). Sequence alignment was performed using the Genetics Computer Group (GCG) program package.

Detection of ADV DNA. DNA was extracted from 100 μl of the serum samples using the QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's protocol.

Primer sets were derived from the hexon region, ADH1 (5'-GCCGCGATGGTCTTACATGCACATC-3' position 18 858–18 883) and ADH2 (5'-CAGCAGCCGCGGATGTCAAAGT-3' position 19 136–19 158), and ADH3 (5'-CCACCGAGACGTAATTCAGCC-3' position 18 938–18 958) and ADH4 (5'-CACACGGTTGTACCCCA CAGC-3' position 19 096–19 116) (Allard *et al.*, 1992). PCR amplification conditions were 5 min at 94°C , followed by 30 cycles of 45 s at 94°C , 90 s at 55°C and 90 s at 72°C , and a final extension step at 72°C for 10 min. Amplification was performed using 0.08 μM of each primer used in the first PCR and 0.16 μM for primer sets used in the nested PCR. The assay detects 1 pg of ADV DNA isolated from ADV2-infected HeLa cells. Amplified DNA was visualized on 2.5 % ethidium-bromide-stained agarose gels.

Real-time PCR was performed with primers and probe located in the same region, AD-F (5'-CCGCGAGTGGTCTTACATGCA-3' position 22–41), AD-R (5'-AAACTTGTATTTC AGGCTGAAGTACGT-3' position 109–135) and AD-P (5'-6-FAM-TGCACCAGCCCGGG GCTCAGTACTCCGA-3'-TAMRA position 61–89) (Heim *et al.*, 2003, modified) and the cycle program was 2 min at 50°C and 10 min at 95°C , followed by 50 cycles of 15 s at 95°C and 1 min at 60°C . The assay is able to detect 10 pg of ADV DNA.

Sequence analysis was based on material amplified with primer sets derived from the hexon region, HX 5-1 (5'-AAGATGGC-CACCCCTCGATGATGCCGAGT-3' position 1–31) and HX 3-1 (5'-CACTTATGTGGTGGCGTTGCCGCGGAGAACGG-3' position 2806–2835), and HX 5-3 (5'-CACATCGCCGGACAGGATGCTTCG GAGTA-3' position 40–68) and HX 3-4 (5'-GTGTTGTGAGC CATGGGGAAGAAGGTGGC-3' position 1825–1854). Primers S28

(5'-ACCCACGATGTGACCAC-3' position 157–173) and S52 (5'-CCCATGTTGCCAGTGCTGTTGTARTACA-3' position 986–1013) were used for the sequence reaction (Takeuchi *et al.*, 1999). The concentration of each primer was 0.2 μ M. The primer sets concentration used in the sequencing reaction was 3.2 μ M. The conditions were the same as described for EV sequencing above.

Antibody analysis. Antibodies of IgG and IgM classes were measured using a commercial kit (Virotech) according to the manufacturer's protocol.

Statistical analysis. The level of significance between individual groups studied was assessed by explorative two-sided chi-squared statistics with Yates correction or Fisher's exact test as appropriate. All the statistical analysis and calculation of percentiles were performed using the Statistical Package for Social Sciences, version 11.0 (SPSS). A two-tailed *P* value of 0.05 or less was considered to indicate statistical significance.

RESULTS

EV sequences were detected by nested PCR in 10 of 50 (20 %) ($P < 0.05$) children in the prediabetic phase, as defined by autoantibody positivity. In the group of children with newly diagnosed T1D, 17 of 47 (36 %) were positive for EV RNA ($P < 0.001$). In contrast, only two out of 50 samples (4 %) of the matched controls were found to be positive for EV RNA sequences (Table 1, Fig. 1).

The specificity of positive screening (nested) PCR results was confirmed by real-time PCR based on the TaqMan principle as well as by nucleic acid sequencing of the nested PCR amplicons. Five of the positive samples were also positive by the less-sensitive TaqMan PCR, with results close to the detection limit of the latter assay.

Sequencing was feasible with seven amplicons from the prediabetic group. Sequences of the amplicons were aligned with EV sequences in GenBank. Four amplicons from the prediabetic group had a high degree of homology to CVB 4 (96 %), two had high homology to CVB 2 (94–95 %) and a single one had high homology to CVB 6 (99 %; AY373076,

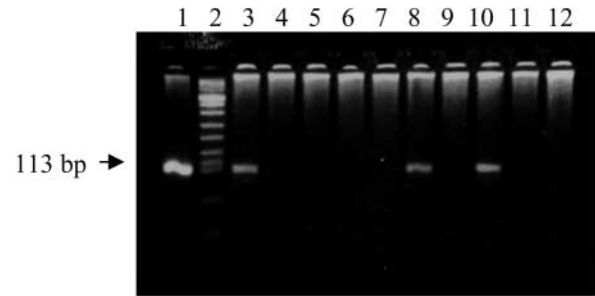


Fig. 1. Nested RT-PCR products of nine samples of newly diagnosed T1D children. An EV-specific fragment of 113 bp is visible in lanes 3, 8 and 10. Lane 1 corresponds to the positive control and lane 12 to the negative control.

AY373053, AF114384). Sequence data of eight amplicons from the newly diagnosed T1D group showed a high similarity to CVB 4 (96 %; AY373076).

EV-specific antibody status determined by ELISA is shown in Table 1. Within the group of children in the prediabetic stage, 26 of 50 (52 %) had IgG antibodies against EV. Anti-EV IgG was detected in 21 of the 47 children with newly diagnosed T1D (44.7 %), and in one case accompanied by IgM. However, EV IgG antibodies were also detected in 27 of 50 (54 %) of the control children.

The relationship between EV-RNA-positive samples and EV-specific antibodies in the prediabetic and newly diagnosed children is demonstrated in Table 2. Antibodies were detected in six of 10 (60 %) EV-RNA-positive samples from the prediabetic group and in 11 of 17 (64.7 %) EV-RNA-positive samples from the newly diagnosed T1D children (Table 2).

ADV DNA was detected in 10 of 50 (20 %) samples from prediabetic children and in three of 47 (6.3 %) samples from newly diagnosed T1D children in comparison with six of 50 (12 %) of the matched-control group (Table 1). All samples positive for ADV DNA were also positive for ADV-specific antibodies as determined by IgG ELISA.

Table 1. Determination of the presence of EV RNA, EV antibodies and ADV DNA in serum samples from prediabetic and newly diagnosed T1D children

Data presented as no. (%).

| Group | No. of children | Positive test result | | | |
|------------------------------|-----------------|----------------------|-----------|-----------|-----------|
| | | Screening RT-PCR | IgG ELISA | IgM ELISA | ADV DNA |
| Prediabetic children | 50 | 10 (20.0)* | 26 (52.0) | 0 (0) | 10 (20.0) |
| Newly diagnosed T1D children | 47 | 17 (36.0)† | 21 (44.7) | 1 (2.1) | 3 (6.3) |
| Control | 50 | 2 (4.0) | 27 (54.0) | 0 (0) | 6 (12.0) |

* $P < 0.05$ vs control.

† $P < 0.001$ vs control (χ^2 test).

Table 2. Relationship between EV-RNA-positive samples and EV-specific antibodies in prediabetic and newly diagnosed T1D children

| Group | EV RNA positive (no.) | ELISA IgG | |
|------------------------------|-----------------------|----------------|--------------|
| | | Positive (no.) | Positive (%) |
| Prediabetic children | 10 | 6 | 60.0 |
| Newly diagnosed T1D children | 17 | 11 | 64.7 |
| Control | 2 | 2 | 100 |

DISCUSSION

The present data indicate a significantly higher frequency of EV RNA in type 1 diabetic children at the onset of disease as well as in prediabetic children, as defined by autoantibody analysis, than in children of a control group. These results are in agreement with other studies including prediabetic children, and support the hypothesis that different enteroviruses may be associated in the initiation of beta-cell destruction (Lönnrot *et al.*, 2000a; Salminen *et al.*, 2003). Our results are also in accordance with those of groups that have shown an increased frequency of EV infections in children with T1D (Clements *et al.*, 1995; Nairn *et al.*, 1999; Kawashima *et al.*, 2004). It is interesting that there is also evidence for a role for EV in adult T1D (Andréoletti *et al.*, 1997).

The difference between our study and previously reported studies is that the material analysed here was derived from schoolchildren defined as being at risk due to the presence of at least one of the diabetes-associated autoantibodies. These children were identified by screening a normal cross-section of the population who had no first-degree relatives with T1D (Strebelow *et al.*, 1999; Schlosser *et al.*, 2002).

We extended our investigation to adenoviruses that use the coxsackievirus/adenovirus receptor, such as coxsackievirus B, a receptor molecule that is known to be a member of the immunoglobulin superfamily (Yanagawa *et al.*, 2004). Similarly to previous studies based on measuring ADV-specific antibody titres, we did not find a link between ADV and T1D. The frequencies of ADV sequences in autoantibody-positive at-risk subjects and in children with newly diagnosed T1D did not differ significantly from the control group.

There are studies that have shown that several serotypes of EV may be associated with T1D. Results based on a neutralization assay including children from the prediabetic phase and at onset of T1D suggest the involvement of not only coxsackievirus B4 but also other coxsackievirus B serotypes as well as A9 (Roivainen *et al.*, 1998). Using a semi-nested version of RT-PCR with primers located in the 5'-NTR, EV sequences that had a high homology to CVB 3 and CVB 4 were detected in adults with newly diagnosed T1D (Andréoletti *et al.*, 1997). We also used sequence alignment to confirm our screening results over the same nucleotide region and found the highest degree of sequence homology to CVB 2, CVB 4 and CVB 6.

Recent studies have shown that, for molecular typing, sequences of the VP1 region correlate more with serotype classification (Oberste *et al.*, 1999, 2004a, b; Thoelen *et al.*, 2003). In general these studies are based on isolated strains. However, in the case of limited material direct sequencing of amplicons of the 5'-NTR is useful to get information about the relationship between involved strains.

The importance of environmental factors like EV infection has been also supported by experimental infections of mice. All six serotypes of coxsackievirus B can be made diabetogenic by repeated passages in mouse pancreas. Molecular characterization revealed limited genetic changes located not only in the capsid protein involved in virus-cell interaction but also in the non-structural proteins. This would mean that mutations in the non-structural genes may also affect virulence (Al-Hello *et al.*, 2005).

In conclusion these results based on a normal schoolchild population without T1D first-degree relatives support the role of EV infections in the initiation of T1D and the necessity of establishing concepts for prevention and/or treatment of EV infections.

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