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Next-generation sequencing for viruses in children with rapid-onset type 1 diabetes

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Abstract

Aims/hypothesis—Viruses are candidate causative agents in the pathogenesis of autoimmune (type 1) diabetes. We hypothesised that children with a rapid onset of type 1 diabetes may have been exposed to such agents shortly before the initiation of islet autoimmunity, possibly at high dose, and thus study of these children could help identify viruses involved in the development of autoimmune diabetes.

Methods—We used next-generation sequencing to search for viruses in plasma samples and examined the history of infection and fever in children enrolled in The Environmental

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[*]Details of the TEDDY Study Group (co-authors) are provided as electronic supplementary material (ESM)

Contribution statement

AGZ, EB JK, OS, JXS, WH and BA designed this project within the TEDDY study. HSL, AGZ and EB performed statistical analyses. TB performed sequencing and viral analyses. CW, MP and HSL acquired and reviewed data. HH and MR were involved in the interpretation of results. AGZ, MR, OS, JXS, WH and ÅL are principal investigators of the TEDDY study and acquired samples and reviewed the data. AGZ, HL, EB, TB, CW and MP wrote the manuscript. All authors critically reviewed the manuscript for intellectual content and approved the final manuscript

Duality of interest

The authors confirm that there is no duality of interest associated with this manuscript.

Determinants of Diabetes in the Young (TEDDY) study who progressed to type 1 diabetes within 6 months from the appearance of islet autoimmunity, and in matched islet-autoantibody-negative controls.

Results—Viruses were not detected more frequently in plasma from rapid-onset patients than in controls during the period surrounding seroconversion. In addition, infection histories were found to be similar between children with rapid-onset diabetes and control children, although episodes of fever were reported less frequently in children with rapid-onset diabetes.

Conclusions/interpretation: These findings do not support the presence of viraemia around the time of seroconversion in young children with rapid-onset type 1 diabetes.

Keywords

GB virus C; Human herpes virus 6; Human rhinovirus C; Infections; Islet autoimmunity; Next-generation deep sequencing; Parvovirus B19; TEDDY study; Type 1 diabetes mellitus

Introduction

Type 1 diabetes is an autoimmune disorder in which disease onset is preceded by a pre-clinical period of islet autoimmunity [1]. Check points in the pre-clinical pathogenesis include the initiation of islet autoimmunity and the progression to type 1 diabetes; the rate of this process is variable [2]. Although the majority of children who develop type 1 diabetes take years to progress from islet autoimmunity to diabetes, some children have a rapid disease progression in which clinical diabetes occurs within months of the appearance of islet autoantibodies. These patients appear to have uncontrolled beta cell destruction; further investigation of this group may help to identify causative agents of autoimmune diabetes.

Viruses have been frequently, but inconclusively, implicated as candidate agents in the pathogenesis of type 1 diabetes. Although associations between type 1 diabetes and viral infections have been repeatedly reported [3-9], in particular for enterovirus infections, there are also numerous reports that fail to confirm such findings [10-12]. Exposure in children with a rapid progression to clinical diabetes may possibly take place at a higher dose or in a shorter time window before the appearance of islet autoimmunity. We therefore examined the history of infections and fever and analysed plasma samples by unbiased deep sequencing for the presence of viruses in the period around the initiation of islet autoimmunity in children who transitioned within 6 months from autoimmunity to disease in The Environmental Determinants of Diabetes in the Young (TEDDY) study [13].

Methods

TEDDY study

TEDDY is a prospective cohort study with the primary objective of identifying environmental factors associated with increased risk of islet autoimmunity and type 1 diabetes; it includes three centres in the USA (Colorado, Georgia/Florida and Washington) and three in Europe (Finland, Germany and Sweden). Infants younger than 4.5 months and carrying type-1-diabetes-associated HLA alleles (*HLA-DR*, *DQ*) were eligible to participate.

From 2004 to 2010, TEDDY screened >420,000 newborns and identified 21,589 children with high-risk *HLA-DR*/*DQ* genotypes. Of these, 8,677 (932 with first-degree family history of type 1 diabetes and 7,745 without such history) were enrolled in the prospective follow-up. Participants were seen and blood collected every 3 months up to 4 years of age, and every 6 months thereafter. Written informed consent was obtained from the parents. The study was approved by the ethical committees of the participating sites [13].

Study outcome

The study outcome is the appearance of confirmed persistent islet autoimmunity, defined as positive for at least one autoantibody (GAD65A, islet antigen-2 [IA-2A] or insulin autoantibody [IAA]) in both TEDDY core laboratories (Barbara Davis Center, Aurora, CO, USA and the University of Bristol, Bristol, UK) in two consecutive samples or in one sample in children who developed diabetes before a follow-up sample was available for autoantibody testing [14]. Families were notified of the child's autoantibody results at their next study visit. The study endpoint is the development of type 1 diabetes as defined by American Diabetes Association criteria [15].

Study participants and design

Of the TEDDY participants, 355 had islet autoimmunity, and 86 of these had progressed to type 1 diabetes by July 2011 when the current study was designed. Twenty-four of the children developed type 1 diabetes within 6 months from the appearance of islet autoimmunity and were selected for our study (Fig. 1a). Two nested case–control studies were designed.

Sequencing study

This study investigated whether viral sequences were present in plasma samples at two time points: (1) the ‘last islet-autoantibody-negative sample’; and (2) the first islet-autoantibody-positive ‘seroconversion sample’ (Fig. 1b). Fourteen of the 24 rapid-onset patients had samples with sufficient volume available at both time points. For each of these 14 patients one control child was selected. Controls were children who participated in the TEDDY study but remained negative for all three diabetes-associated islet autoantibodies and for type 1 diabetes for at least 12 months after the respective event in patients. Controls were matched by clinical site and the family history of type 1 diabetes (yes/no) if they had plasma samples at the respective time points (Table 1). Controls were randomly selected from the pool of potential controls after being matched and conditioned. Control samples used in the study were age-matched to the last islet-autoantibody-negative sample and seroconversion sample of rapid-onset patients.

Infection history study

This study investigated whether infections or fever episodes were associated with rapid progression to type 1 diabetes. All 24 rapid-onset patients, with three controls for each, were examined. Controls were selected by the same procedure as for the sequencing study. Three time periods were examined in the 96 children (Fig. 1b): (1) the autoantibody-negative period, from birth to the last islet-autoantibody-negative sample; (2) The seroconversion

period, from the last islet-autoantibody-negative sample to the first islet-autoantibody-positive sample; and (3) the progression period, from the first islet-autoantibody-positive sample to the date of type 1 diabetes diagnosis.

Nucleic acid sequencing

Total nucleic acids were extracted from 250 µl plasma (NucliSENS easyMag, Biomerieux, Marcy l'Etoile, France) and nucleic acid quantity and quality assessed (2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). Samples were subjected to reverse transcription using random octamer primers linked to an arbitrary 17-mer primer sequence and products were randomly amplified by PCR, applying the same octamer-linked 17-mer primer in conjunction with the 17-mer primer sequence without the octamer tail in a 1:9 ratio [16]. Products of >70 bp were selected by column purification (MinElute, Qiagen, Hilden, Germany) and ligated to bar-coded linkers for sequencing on the 454 Genome Sequencer FLX (454 Life Sciences, Branford, CT, USA) without fragmentation [17]. Raw sequence reads were trimmed to remove sequences derived from amplification primers and linkers, filtered to eliminate highly repetitive sequences, and assembled into contiguous sequences, which were, together with the remaining non-assembled singleton reads, compared with the non-redundant GenBank database at the nucleotide and translated amino acid levels.

Infection history data

Acute infections or illnesses experienced between study visits were collected with the corresponding dates by parents and provided to study personnel every 3 months, starting from age 6 months. Acute infections or illnesses were recorded using ICD-10 codes (www.who.int/classifications/icd/en/) and categorised as respiratory tract, gastrointestinal or other infection. Fever was documented using one of the relevant ICD-10 codes, R50, R50.9, R50.8, or R56, and was assessed when reported with or without infection.

Statistical analysis

All infections and fever reports before type 1 diabetes onset were analysed and then assessed according to the three periods of disease progression defined in the study design (Fig. 1b). Infections and fever were either counted as present/absent in each period and child irrespective of the actual number of episodes (Table 2), or the number of episodes in each period was scored and reported as the mean per child with SD (Table 2).

Conditional logistic regression adjusted for maternal age at delivery was used to obtain the estimate of OR for the factor of interest. A p value <0.05 was considered significant. All reported p values are two-sided without adjustment for multiple testing. All statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA).

Results

Sequencing study

Unbiased sequencing identified viruses in six (11%) of 56 plasma samples (electronic supplementary material [ESM] Table 1). Samples from four (14%) of the 28 children

analysed (one patient, three controls) were positive for virus. The positive patient sample contained a picornavirus, human rhinovirus C (HRV-C), identified in the last islet-autoantibody-negative sample. Rhinoviruses are commonly associated with upper respiratory tract infection and otitis media, and not known to occur in blood. However, the species rhinovirus C differs from species rhinovirus A and B [18], and has been reported in blood with a peak of viremia two days after the onset of symptoms [19]. Fever was not reported for this patient during the autoantibody-negative period, but three respiratory infections were reported, with the last one recorded 1 day before sampling. Three different viruses were detected in the autoantibody-negative controls. Human parvovirus B19 (B19V), the causative agent of fifth disease (erythema infectiosum, 'slapped cheek syndrome' [20]) was identified in the paired control of the virus-positive case, with fever reported 30 days before sampling; no rash was recorded for this child. In the second positive control child, both samples tested contained human herpesvirus 6 (HHV-6), which can establish latent infection. Two species of HHV-6 are recognised: HHV-6B is considered the causative agent of sixth disease (exanthema subitum or roseola infantum [21]), while little is known about the pathogenicity of the less frequently diagnosed HHV-6A. The sequence identified in this child was distantly related to HHV-6A. The third control child was positive in both plasma samples for GB virus C (GBV-C), which is known to establish persistent infections consistent with detection over a 3 month time period [22].

Infection history study

All rapid-onset patients and 70 of 72 controls reported at least one infection prior to the onset of type 1 diabetes (Table 1). The proportions of patients and controls with infections reported during the islet-autoantibody-negative (75% vs 74%), seroconversion (71% vs 72%) and progression periods (71% vs 67%) were similar. Moreover, the number of infections per child during each of these periods was similar between patients and controls (Table 2). Respiratory infections were the most commonly reported infections in both patients and controls.

In contrast to the reported infections, the frequency of reported fever episodes differed between patients and controls (Table 1). Overall, fever was reported less frequently in patients than in controls. Although the difference was seen during the seroconversion (p=0.030) and progression periods (p=0.033), it was not present during the autoantibody-negative period.

Discussion

Infection during infancy is a candidate trigger of islet autoimmunity and type 1 diabetes [3-5]. In TEDDY participants who progressed rapidly to diabetes, unbiased sequencing capable of detecting any possible agent failed to detect viraemia around the time of seroconversion more frequently than in controls. Rapid progression to diabetes was also not associated with reported infectious episodes. We had hypothesised that rapidly progressing patients would provide a best-case scenario for detecting potentially associated viruses in blood, because of the fulminant presentation of diabetes. However, viraemia was detected in only one of 14 patients. These findings cannot exclude the possibility that a causative virus

is acquired before the age of 6 months and absent or only present for a brief time in plasma during the months leading to the appearance of islet autoantibodies and progression to diabetes. Although the sensitivity of the sequencing approach may be limited in detecting a low-grade persistent infection, next-generation sequencing techniques have been successful in identifying viral causes of disease in several scenarios [23-26]. In TEDDY, consistent detection of the same virus was seen for two consecutive samples from two children, suggesting that this method can reproducibly detect viraemia in plasma samples. Retrospective quantification of identified viruses in the analysed plasma samples by specific real-time PCR indicated target loads down to a range of 200-1000 molecules/250 μl plasma, a detection limit suitable to detect active systemic infection.

A limitation of our study was the relatively low number of cases studied and the length of the sampling interval which, at 3 months, may have been too wide to identify all systemic infections. Viraemia can be as short as a few days, and additional samples collected during fever episodes would have been desirable. However, ad hoc sampling aside from the scheduled TEDDY visits was not part of the TEDDY study protocol. Although infections were reported frequently in our children, the analysis of infection history data did not support a prominent role of infection; infection frequencies were similar in patients and controls during all three observation periods and for all three infection categories.

The lower frequency of fever reported for patients than for controls is a potentially important finding that needs to be substantiated in a larger study. If confirmed, it suggests a protective effect of fever as a marker of more vigorous infection defence and effective virus elimination.

In conclusion, next-generation sequencing of the plasma virome of patients rapidly developing autoimmune type 1 diabetes in early childhood did not provide evidence for viraemia around the time of seroconversion in these children.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ICD-10	International Classification of Disease (ICD)-10
TEDDY	The Environmental Determinants of Diabetes in the Young

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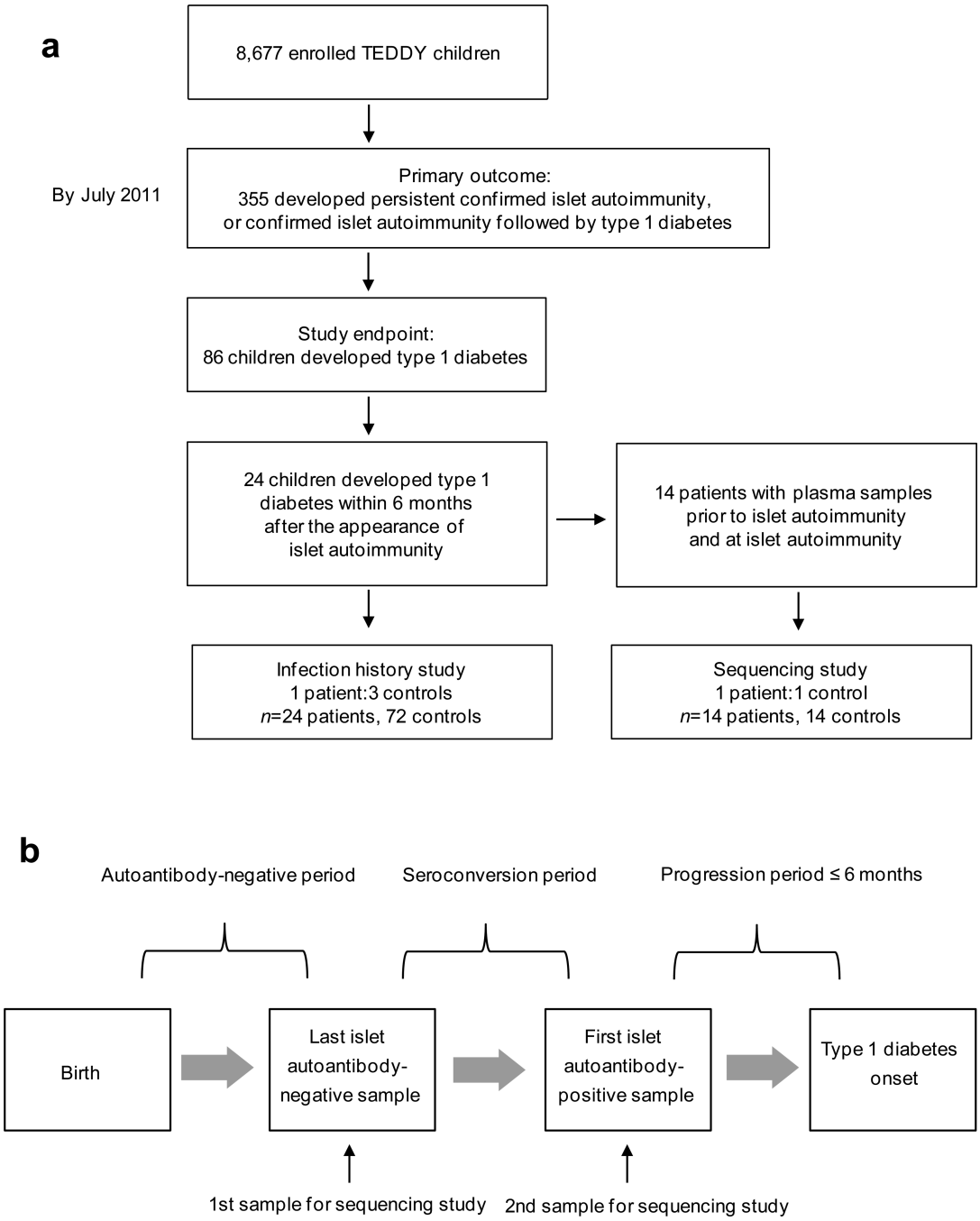

Fig. 1.

(**a**) Flow chart of the study population for the infection history and sequencing studies, and (**b**) flow chart of the time points investigated in the infection history and sequencing study

Table 1

Infection and fever reported in the infection history study

Infection status	Patient n (%)	Control n (%)	OR (95% CI)	*p* value
Birth to type 1 diabetes				
Any infection	24 (100)	70 (97)		
Fever	17 (71)	62 (86)	0.22 (0.06, 0.88)	0.032
Fever without infectious illness	9 (38)	22 (31)	1.21 (0.44, 3.30)	0.715
Fever with any infectious illness	14 (58)	56 (78)	0.36 (0.12, 1.10)	0.072
Respiratory tract	24 (100)	68 (94)		
Gastrointestinal tract	11(46)	29 (40)	1.55 (0.50, 4.76)	0.447
Other	5 (21)	13 (18)	1.40 (0.36, 5.48)	0.630
Autoantibody-negative period				
Any infection	18 (75)	53 (74)	0.94 (0.20, 4.52)	0.940
Fever	13 (54)	38 (53)	1.10 (0.31, 3.85)	0.882
Fever without infectious illness	6 (25)	8 (11)	1.95 (0.59, 6.50)	0.275
Fever with any infectious illness	9 (38)	33 (46)	0.76 (0.22, 2.60)	0.658
Respiratory tract	18 (75)	49 (68)	1.89 (0.40, 8.89)	0.420
Gastrointestinal tract	5 (21)	16 (22)	0.96 (0.27, 3.44)	0.944
Other	2 (8)	8 (11)	0.57 (0.08, 3.87)	0.564
Seroconversion period				
Any infection	17 (71)	52 (72)	0.95 (0.31, 2.86)	0.922
Fever	4 (17)	30 (42)	0.27 (0.08, 0.88)	0.030
Fever without infectious illness	2 (8)	11 (15)	0.47 (0.09, 2.56)	0.380
Fever with any infectious illness	3 (13)	26 (36)	0.23 (0.06, 0.88)	0.032
Respiratory tract	15 (63)	49 (68)	0.71 (0.24, 2.15)	0.543
Gastrointestinal tract	3 (13)	10 (14)	1.25 (0.27, 5.84)	0.776
Other	1 (4)	4 (6)	1.11 (0.10, 11.86)	0.931
Progression period				
Any infection	17 (71)	48 (67)	1.34 (0.30, 6.08)	0.706
Fever	5 (21)	30 (42)	0.14 (0.02, 0.85)	0.033
Fever without infectious illness	2 (8)	8 (11)	0.76 (0.14, 4.13)	0.752
Fever with any infectious illness	5 (21)	25 (35)	0.30 (0.07, 1.40)	0.126
Respiratory tract	15 (63)	43 (60)	1.06 (0.31, 3.60)	0.921
Gastrointestinal tract	6 (25)	10 (14)	2.64 (0.70, 9.95)	0.153
Other	2 (8)	3 (4)	2.65 (0.33, 21.11)	0.358

Table 2

Number of infections and fever reports per case or control in infection history study

	Patient mean (SD)	Control mean (SD)	OR (95 % CI)	*p* value
Autoantibody-negative period				
Any infection	3.5 (3.9)	2.8 (3.6)	1.22 (0.93, 1.60)	0.150
Fever	1.0 (1.2)	1.1 (1.5)	0.97 (0.62, 1.53)	0.901
Seroconversion period				
Any infection	1.7 (2.0)	2.0 (1.9)	0.93 (0.69, 1.25)	0.615
Fever	0.3 (0.8)	0.8 (1.3)	0.61 (0.33, 1.12)	0.108
Progression period				
Any infection	1.6 (1.5)	1.7 (2.1)	0.95 (0.66, 1.37)	0.782
Fever	0.4 (0.9)	0.7 (0.9)	0.57 (0.26, 1.21)	0.143