Reduced Frequency of HLA DRB1*03-DQB1*02 in Children with Type 1 Diabetes Associated with Enterovirus RNA

Maria E. Craig, Neville J. Howard, Martin Silink, and William D. Rawlinson

Institute of Endocrinology, The Children’s Hospital at Westmead, Sydney, Virology Research Laboratory, Virology Division, South Eastern Area Laboratory Services, Prince of Wales Hospital, Randwick, and Faculties of Science and Medicine, University of New South Wales, Kensington, New South Wales, Australia

Enteroviruses have been associated with type 1 diabetes mellitus (T1DM), but it is unclear whether there is a distinct disease subtype. Plasma and stool samples from 206 consecutively diagnosed children and 160 healthy control children were analyzed by reverse-transcription polymerase chain reaction for the RNA of 60 enteroviruses. More children with diabetes tested positive for enterovirus RNA (30% vs. 4%; odds ratio, 11.1; 95% confidence interval, 4.7–25.7; P < .001). Enterovirus 71 was detected in 25% of children; these were temporally associated with outbreaks in Southeast Asia and Australia. Fewer children with the diabetes-associated human leukocyte antigen DRB1*03-DQB1*02 genotype tested positive for enterovirus RNA (P = .02). More children presenting with severe diabetic ketoacidosis (pH, <7.1) tested positive for enterovirus RNA (P = .04). These results suggest that there is a subgroup of patients with T1DM, who are at low genetic risk, in whom enteroviruses contribute to diabetes onset.

In vitro studies of pancreatic islet cells [1] and animal models of virus-induced diabetes [2] have unequivocally demonstrated that members of the Enterovirus genus can cause insulitis and diabetes. Sequence variation in coxsackievirus (CV) type B4 (CV-B4) [3, 4] suggests that genomic variation influences diabetogenicity. Sequence homology between the 2C nonstructural protein of CV-B4 and the pancreatic β cell autoantigen GAD-65 [5] suggests that molecular mimicry may be a mechanism for enterovirus-associated diabetes. This may be HLA-restricted, in particular by HLA DR-3 [6]. However, CV-B4–induced diabetes was a consequence of virus-associated local inflammation, rather than molecular mimicry, in a transgenic murine model [7]. Furthermore, it has been shown, in the nonobese diabetic (NOD) mouse, that enterovirus infections do not initiate β cell autoreactive immunity but can accelerate the process once it has begun [8].

Studies of the association between enteroviruses and the development of type 1 diabetes mellitus (T1DM) in humans include case reports [9, 10], twin studies [11], case-control studies [12], prospective cohort studies of at-risk subjects [13], and ecological studies that have compared rates of enterovirus infection and diabetes incidence [14]. Viral culture or serological testing were used to demonstrate enterovirus infection in most of the early epidemiological studies. In many cases, serological testing was performed for a limited number of the 64 known enteroviruses, often only the CV-B
group, and it is a less sensitive measure of enterovirus infections than polymerase chain reaction (PCR) [15]. The development of nucleic-acid testing methods and direct sequencing has enabled the genotyping of isolates in more recent studies. Enterovirus RNA has been detected in children [16] and adults [17, 18] at the onset of T1DM and in children who subsequently developed T1DM [19]. However, subjects who develop diabetes in association with a viral infection may have a distinct subgroup of the disease, and they may differ from other patients with regard to genetic, autoimmune, or clinical characteristics [20]. Of the studies in which PCR has been used to detect enteroviruses in subjects at the onset of T1DM, 1 involved only 14 adult subjects [17]; in another study, among 110 children at the time of diagnosis of T1DM, neither immunologic nor genetic features were reported [16]. In the present study, the association among enterovirus infection (using reverse-transcription [RT]–PCR), enterovirus genotype (determined by sequencing), genetic predisposition to T1DM (HLA class II subtype), markers of islet cell autoimmunity (diabetes-associated antibodies), biochemical measurements (including C-peptide and pH), and clinical characteristics was investigated in a cohort of children and adolescents at the onset of T1DM.

SUBJECTS, MATERIALS, AND METHODS

Study design and recruitment. Using a case-control study design, 206 consecutively diagnosed children at the onset of T1DM were studied between April 1997 and September 1999 in Sydney, Australia. The study cohort represented a population-based sample from western Sydney, with a median age of 8.2 years (range, 0.7–15.7 years). Fifty-six children (27%) were aged <5 years at diagnosis. Cases of type 2 diabetes or secondary diabetes were excluded. One hundred sixty control subjects (median age, 9.4 years; range, 0.5–15.8 years) were recruited from the community. Of the control subjects, 29 had developed T1DM to date, based on ascertainment from the New South Wales (NSW) diabetes register [21]. Whole blood, serum, and fecal samples were collected from the children at diagnosis and from control subjects within 2 weeks of the patients’ diagnosis. Plasma was separated from whole blood, and all samples were frozen immediately and stored at −80°C. Demographic data, family size, ethnic background, family history of diabetes, and past and recent illnesses were recorded. Ethnicity was based on the Australian Bureau of Statistics classification [22]. Weight and height were measured on admission, and body mass index (BMI) was calculated (in kg/m²). Standard deviation scores (SDS) for BMI were based on national standards [23].

Single-step PCR. Viral RNA was extracted from 200 μL of plasma and 20% stool suspension, using a simplified guanidinium-thiocyanate method, as described elsewhere [24]. A single-step nested RT-PCR method was used to detect enterovirus RNA in clinical samples. Primers were from the highly conserved 5′ untranslated region (UTR) of the enterovirus genome: EV1 (CAA GCA CTT CTT TTT CCC CGG), EV2 (TCC TCC GGC CCC TGA ATG CG), EV3 (ATT GTC ACC ATA AGC AGC CA), and EV4 (CAC YGC ATG GCC AAT CCA A). The PCR method detected viral RNA from 60 of 64 known human enterovirus (HEV) serotypes [25], similar to findings of an earlier study that used primers EV1, EV2, and EV3 [26]. CV-A1, CV-A4, CV-A8, and CV-A13 were not tested, because isolates could not be obtained. The method did not detect samples that contained rhinovirus or hepatitis C virus.

In brief, first-round RT-PCR was performed in 1 tube (50 μL) that contained 10 μL of RNA, 0.5 μM each of primers EV1 and EV4, 0.2 mM dNTP, 10 mM dithiothreitol (DTT), Taq 10× PCR buffer, 2.0 mM MgCl₂, 1.5 U of Taq polymerase, and 4 U of avian myeloblastosis virus reverse transcriptase (Promega). First-strand cDNA was synthesized for 40 min at 42°C, then samples were subjected to 35 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s. The second-round PCR mixture (50 μL) contained 5 μL of the first-round PCR amplicon, 0.5 μM each of primers EV2 and EV3, and the same reagents and PCR cycling conditions as those for the first round, with the omission of DTT and reverse transcriptase. Reactions were performed in a GeneAmp 9600 thermal cycler (Perkin-Elmer). EV1/EV4 produced a 479-bp amplicon, and EV2/EV3 produced a 156-bp amplicon. PCR products were separated on 2% agarose gel stained with ethidium bromide and were visualized under UV light. Negative (water) and positive (enterovirus 70 cultured in LLC-MK₂ cells, diluted 1:1000) controls were included in all reactions, from the extraction step, and were run in parallel with test samples. Standard precautions were undertaken to avoid the risk of contamination and inhibition [27].

Nucleotide sequencing and phylogenetic analysis. PCR products were purified by polyethylene glycol precipitation and sequenced in both directions using an ABI 377 PRISM DNA sequencer. Nucleotide sequence analyses were performed using BLAST, Clustal W, and PileUp, as described elsewhere [24]. Phylogenetic analysis was based on parsimony, distance, and maximum-likelihood methods, using programs from the PHYLIP program [28]. Randomly resampled data sets of aligned 116-bp 5′ UTR sequences were generated for bootstrap analysis using the program SEQBOOT. Bootstrap analysis tests the significance of inferred groups within phylogenetic trees, and values indicate the frequency with which clusters occur from the multiple data sets analyzed. Bootstrap values of at least 70% show statistical support for a cluster [28]. Evolutionary distances between sequences were calculated using DNADIST. Phylogenetic trees were constructed using FITCH, and consensus trees were derived using CONSENSE. Branch lengths proportional to evolutionary distances were calculated using...
the neighbor-joining method with the programs NEIGHBOUR and PUZZLE. Phylogenetic trees were edited using TREEVIEW and RETREE. All computer programs mentioned in this paragraph were obtained at http://www.angis.org.au.

**Diabetes-associated autoantibodies.** Islet cell antibodies were detected according to a modification of an indirect immunofluorescence method, on unfixed cryostat sections of type O human pancreas [29]. Insulin autoantibodies were measured by a competitive radioimmunoassay, as described elsewhere [30]. Antibodies to GAD-65 and the protein tyrosine phosphatase were measured by radioimmunoprecipitation [31, 32].

**HLA genotyping.** HLA class II typing of the DRB1 and DQB1 alleles was performed on 190 case patients at the Tissue Typing Laboratories of the Red Cross Blood Bank, Sydney, and the Royal Melbourne Hospital. In brief, DNA was extracted from 5 mL of whole blood. Exon 2 polymorphisms were determined from PCR-amplified DNA using sequence-specific oligonucleotides, as described elsewhere [33], using methods described elsewhere [15]. Alleles were assigned on the basis of the hybridization patterns.

**Biochemical and clinical measurements.** Measurements of random C-peptide, whole-blood glucose (in mmol/L), venous pH, and urinalysis, for the detection of ketones, were performed on samples from children with newly diagnosed diabetes at initial presentation, prior to the administration of insulin. Severe diabetic ketoacidosis (DKA) was defined as pH <7.1. Random C-peptide was measured using an ultrasensitive C-peptide ELISA (Mercodia).

**Statistical analysis.** Normally distributed continuous variables were compared using a 2-sample t test, and the Mann-Whitney U test was used to compare skewed data. Cross-tabulation and χ² tests (with Yates’s continuity correction) or Fisher’s exacts tests were used to examine the relationship between categorical variables, as appropriate. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were used as measures of association. Using a cutoff of 5 years, the modifying effect of age on enterovirus PCR results in case patients and control subjects was examined using the Breslow-Day test for homogeneity. Clinical, biochemical, genetic, and immunologic markers were compared between enterovirus RNA−positive and −negative case patients using case-only analysis [34]. To assess the role of gene-environment interaction in the pathogenesis of T1DM, under the assumption of independence between genotypic and enterovirus exposure, case patients were divided into 4 groups for the analysis of HLA DQB1 subtypes: DQB1*02/*0302 (having both “high-risk” alleles), DQB1*0302/x and DQB1*02/y (homozygous or heterozygous for either of the 2 risk alleles), and DQB1x/z (subjects without either risk allele) [35]. Case patients were also analyzed for the presence or absence of the haplotype DRB1*03-DQB1*02. Multiple logistic-regression analysis was used to explore the predictors of having enterovirus RNA detected at diagnosis, after adjustment for other variables. Only the combined DRB1-DQB1 haplotype was included in the multiple logistic-regression model because of linkage disequilibrium between DRB1 and DQB1 alleles. STATA (version 6.0; StataCorp) and SPSS (version 10.0; SPSS) software were used for statistical analyses.

**RESULTS**

**Enterovirus RNA in case patients and control subjects.** Significantly more children with diabetes had enterovirus RNA detected in either plasma or stool, compared with control children: 62 (30%) of 206 versus 6 (4%) of 160 (OR, 11.1; 95% CI, 4.7–25.7; P < .001). Age was an effect modifier in this relationship when results were stratified by age <5 years (test of homogeneity of the OR: χ² = 7.0; P < .01). Among children aged <5 years, the incidence of enterovirus RNA positivity was not significantly higher in case patients than control subjects: 21 (38%) of 56 versus 4 (20%) of 20 (OR, 2.4; 95% CI, 0.7–8.4; P = .15). However, in children aged ≥5 years, the association was highly significant: 41 (27%) of 150 of case patients tested positive for enterovirus RNA versus 2 (1.4%) of 139 of control subjects (OR, 25.8; 95% CI, 5.5–121.4; P < .001). Sabin vaccine strains of poliovirus 1 (PV-1) were detected in stool specimens from 1 case patient and 1 control subject; these subjects were classified as “negative” for enterovirus RNA in all analyses.

**Comparison of enterovirus RNA-positive and −negative cases.** A univariate analysis of cases, stratified by enterovirus PCR result at diagnosis, is shown in table 1. Case patients with high C-peptide levels (above the 90th percentile) were significantly more likely to be negative for enterovirus RNA: 18 (13%) of 144 versus 2 (3%) of 60 (P = .04). Case patients with enterovirus RNA levels were more likely to present with severe DKA: 8 (16%) of 15 versus 7 (6%) of 108 (P = .03). No differences were found for age, sex, blood glucose level, presence of ketones, or BMI SDS at diagnosis for those who tested positive versus negative for enterovirus RNA. The presence of enterovirus RNA was not influenced by a history of infection during the preceding 3 months, duration of symptoms of hyperglycemia prior to diagnosis (defined as polyuria, polydipsia, weight loss, or lethargy), a first-degree relative with T1DM, number of siblings, household size, or socioeconomic status (data not shown). Ninety-five percent of case patients were born in Australia. Some 69% of case patients were white; the remainder were of European, Southeast Asian, Middle Eastern, South Pacific, and other ethnic origins. PCR results were not associated with ethnicity (table 1).

Seven children (3.4%) had no diabetes-associated autoantibodies detected at diagnosis; all were aged >5 years. Among children aged >5 years, there was some indication that those with negative antibodies were more likely to be RNA-positive:

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Table 1. Characteristics of children with diabetes at diagnosis, stratified by enterovirus polymerase chain reaction results

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Enterovirus RNA positive</th>
<th>Enterovirus RNA negative</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. (%) patients</td>
<td>62 (30)</td>
<td>144 (70)</td>
<td></td>
</tr>
<tr>
<td>No. (%) male</td>
<td>25 (40)</td>
<td>54 (38)</td>
<td>NS</td>
</tr>
<tr>
<td>Age, median years (range)</td>
<td>8.2 (3.4–10.7)</td>
<td>8.0 (5.2–11.3)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) with C-peptide in 90th percentile</td>
<td>2 (3)</td>
<td>18 (13)</td>
<td>.04</td>
</tr>
<tr>
<td>No. (%) with severe DKA</td>
<td>8 (16)</td>
<td>7 (6)</td>
<td>.04</td>
</tr>
<tr>
<td>Blood glucose level, median mmol/L (range)</td>
<td>22.4 (20.2–32.8)</td>
<td>26.7 (19.4–34.9)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) with ketosis</td>
<td>56 (90)</td>
<td>125 (87)</td>
<td>NS</td>
</tr>
<tr>
<td>Body mass index SDS, median (IQR)</td>
<td>−0.34 (−1.59–0.43)</td>
<td>−0.46 (−1.28–0.29)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) white</td>
<td>39 (63)</td>
<td>101 (71)</td>
<td>NS</td>
</tr>
<tr>
<td>No. (%) negative for ICA, IAA, GAD-65, and IA-2</td>
<td>4 (7)</td>
<td>3 (2)</td>
<td>NS</td>
</tr>
</tbody>
</table>

NOTE. DKA, diabetic ketoacidosis; IAA, insulin autoantibodies; IA-2, tyrosine phosphatase; ICA, islet cell antibodies; IQR, interquartile range; NS, not significant; SDS, standard deviation score.

* Defined as pH < 7.1 (mmol/L).

** Defined as > 370 pmol/L.

4 (10%) of 41 versus 3 (3%) of 109; however, this did not reach statistical significance (P = .09). There was no significant association between the number of diabetes-associated antibodies or any of the 4 individual autoantibodies and presence or absence of enterovirus RNA.

The median number of cases of diabetes diagnosed per month was 7 (range, 2–15). There was a significant seasonal variation in the proportion of enterovirus RNA–positive cases during the study period (χ² = 18.6; P = .04). The highest rate was found during winter 1998 (16 [57%] of 28 case patients were enterovirus RNA positive), and the lowest rate was found during winter 1999 (2 [11%] of 19 case patients were positive).

Significant negative association between enterovirus RNA and HLA DRB1*03-DQB1*02. Case patients in whom enterovirus RNA was detected were less likely to have the haplotype HLA DRB1*03-DQB1*02 (table 2). The odds of having enterovirus RNA at diagnosis were also lower in subjects with at least 1 copy of either of these alleles. Heterozygosity for DQB1*02/*0302 or HLA DRB1*03/*04 was not statistically significantly associated with enterovirus RNA positivity, but there was some indication that the odds of enterovirus infection being present at the time of diagnosis were lower for children who were heterozygous for DRB1*03/DQB1*02 (P = .09). The z/z genotype (the absence of either DQB1*02 or *0302) was associated with increased odds of enterovirus RNA positivity. There was no significant difference in the frequency of HLA DRB1*03-DQB1*02 between those who were RNA positive and negative; however, ethnicity was an effect modifier for the association between DRB1*03-DQB1*02 and enterovirus RNA (test of homogeneity of the OR, P < .01). The OR for enterovirus RNA in nonwhites with this haplotype was 0.10 (95% CI, 0.03–0.33), and that in whites was 0.87 (95% CI, 0.40–1.88). In multiple logistic-regression analysis, DRB1*03-DQB1*02 and severe DKA at diagnosis remained significant explanatory variables in the model (adjusted OR for DRB1*03-DQB1*02, 0.44; 95% CI, 0.23–0.85; adjusted OR for pH < 7.1, 3.31; 95% CI, 1.06–10.38). A C-peptide level greater than the 90th percentile was no longer significant (P = .09) in this model.

Molecular epidemiology of enteroviruses. Fourteen enterovirus genotypes were detected in plasma or stool samples from children with diabetes. Genotyping was based on the alignment of published enterovirus 5′ UTR sequences and sequences of isolates previously serotyped from Australia. In 6 cases, sequences were incomplete, and the virus could not be genotyped. Most (n = 37) belonged to HEV groups A and B [36]; EV-71 (n = 16; 25.4%), CV-B1 (n = 14; 22.2%), CV-B3 (n = 7; 11.1%), and echovirus 30 were detected most frequently. There were significantly more isolates of EV-71 detected during the second half of the study period; 3 of 19 EV-71 isolates were detected in the 15 months before June 1998, compared with 13 (81%) 16 of EV-71 isolates detected in the 15 months after July 1998 (P = .01, Fisher’s exact test). No CV-B4 subtypes were detected. Sequence homology (compared with published sequences) was 87%–100%. In 21 children with diabetes (34% of enterovirus RNA–positive cases, 10% of all cases), enterovirus RNA was detected in both stool and plasma samples. The same enterovirus sequence was detected in stool and plasma samples from these children. No cases of mixed infection with different enterovirus genotypes were found [37].

A phylogenetic analysis of sequenced isolates from the present study, with GenBank accession numbers, is shown in figure 1. Representative sequences from the 5 major enterovirus species (HEV-A, -B, -C, -D, and PV) have been included. The isolates clustered into 2 main groups; the first cluster contained
PV-1, CV-A11, CV-A20, and EV-70 (which belong to HEV-C, HEV-D, and PV species), and the second group contained the remaining enteroviruses (HEV-A and HEV-B). The 2 groups were well supported, with a bootstrap value of 94%, indicating distinct clusters. The second cluster did not contain any well-supported subgroups.

The nucleotide sequences of EV-71 isolates from the study were aligned with published sequences from outbreaks of hand, foot, and mouth disease and EV-71 infections in Southeast Asia that occurred during 1997–1998 [38–40] (data not shown). Sequence homology between EV-71 isolates was 81%–99%. One isolate from an 11-year-old girl who developed T1DM in January 1999 differed from the Taiwanese isolates by $3\text{ nt}$ [41]. The isolates clustered into 2 main groups, which was strongly supported with a bootstrap value of 100%. All of the Southeast Asian ($n = 27$) and most of the Australian ($n = 14$) isolates formed one group, whereas a small number of Australian isolates formed a second cluster ($n = 3$). The isolates did not form 3 or 5 distinct genotypes, as has been described elsewhere [41, 42] in a phylogenetic analysis of the VP1 region.

### DISCUSSION

Enterovirus RNA was detected in plasma and stool samples from 30% of children at the onset of T1DM, significantly more often than in healthy control children (4%). These results implicate enterovirus infection as a late trigger in the multistep process of diabetes pathogenesis in some children. RT-PCR was used for the diagnosis of enterovirus infection, using primers that detect the majority human enterovirus subtypes, unlike the method of many previous studies, which relied on serological evidence of enterovirus infection. Children with HLA DRB1*03-DQB1*02, which has been associated with susceptibility to T1DM [43], were significantly less likely to have enterovirus RNA detected at diagnosis. Together, these results support the putative role for enterovirus infection as an etiologic factor in children with T1DM, particularly in those without the typical genetic predisposition, including children of nonwhite origin.

It is not known how long the enterovirus RNA was present in children prior to their presentation with symptomatic hyperglycemia, and it is not possible to determine this from a case-control study of children at the onset of diabetes. Enterovirus RNA can persist for months in the serum of patients with other illnesses, such as chronic fatigue syndrome [44] and immunodeficiency [45], and a persistence of CV-B4 has been demonstrated in a murine model of T1DM [46]. In a prospective study of children at genetic risk for T1DM, a significant association was found between the development of diabetes-associated antibodies and a preceding enterovirus infection [19]. However, in one child who had multiple enterovirus infections, phylogenetic analysis of sequences demonstrated that genetically distinct enteroviruses were detected at different time points, and no enterovirus RNA was detected in samples taken between the RNA-positive samples, which suggests that chronic infection was unlikely. The absence of an association between the presence of enterovirus RNA and a history of recent or more-frequent infections in the children studied by us suggests that enterovirus infection was a recent trigger for their diabetes.

The rate of enterovirus RNA positivity in children with diabetes was significantly higher than that in healthy control children sampled at the same time. There are few data demonstrating rates of enterovirus infections in the general population, particularly in asymptomatic children and adolescents. Young children have frequent viral infections, and 50%–80% of children have an enterovirus infection by age 9 months [47]. Detection rates for enterovirus RNA in nasopharyngeal aspirates have been reported to be 3.5%–17% [48, 49] in patients with respiratory illnesses, but detection rates were 0%–6% in serum or whole blood in asymptomatic children and adults [13, 16, 18]. The lack of statistically significant difference in enterovirus RNA positivity in children aged <5 years may be related to the higher background rate of enterovirus infections in younger children and the small sample size of children aged <5 years in the present study. How-

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**Table 2. HLA DRB1 and DQB1 genotypes in children with diabetes, stratified by enterovirus polymerase chain reaction results.**

<table>
<thead>
<tr>
<th>HLA genotype</th>
<th>Enterovirus RNA positive, % ($n = 58$)</th>
<th>Enterovirus RNA negative, % ($n = 132$)</th>
<th>OR (95% CI)</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DQB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>*02/*0302</td>
<td>28</td>
<td>39</td>
<td>&gt;1</td>
<td></td>
</tr>
<tr>
<td>*0302/x</td>
<td>26</td>
<td>20</td>
<td>&gt;1</td>
<td></td>
</tr>
<tr>
<td>*02/y</td>
<td>26</td>
<td>33</td>
<td>&gt;1</td>
<td></td>
</tr>
<tr>
<td>z/z</td>
<td>19</td>
<td>9</td>
<td>2.52 (1.02–6.22)</td>
<td>.04</td>
</tr>
<tr>
<td>DRB1<em>03-DQB1</em>02</td>
<td>44</td>
<td>63</td>
<td>0.46 (0.24–0.87)</td>
<td>.02</td>
</tr>
</tbody>
</table>

**NOTE.** CI, confidence interval; OR, odds ratio.
Figure 1. Dendogram of the phylogenetic relationship among enterovirus (EV) isolates from the study. The GenBank accession nos. of isolates are shown, and samples from control subjects are marked (asterisks). Published sequences from the 5 human EV species are included. Branch lengths are proportional to evolutionary distances. The isolates clustered into 2 main groups, which were well supported, with a bootstrap value of 94%.

However, the highly significant difference in rates of enterovirus infection in children aged ≥5 years (who would be more likely to have immunity to many enteroviruses by this age) supports the importance of enterovirus infection as a potential trigger for diabetes in older children, as was shown in a study of IgM responses to the CV-B group [50]. Furthermore, frequencies of DRB1*03 have been reported to decline in children aged ≥6 years who have T1DM [33].

Significantly fewer enterovirus RNA–positive case patients had HLA DRB1*03-DQB1*02, compared with enterovirus RNA–negative case patients, and children with the z/z genotype were significantly more likely to be enterovirus RNA positive (table 2). These results imply a significant gene-environment interaction in the pathogenesis of T1DM [34]. In the cohort studied here, the reduced frequency of HLA DRB1*03-DQB1*02 in the enterovirus RNA–positive case patients may be due to the significant association in nonwhite children. Moderate or high-risk HLA DR alleles were found in only 2 of 6 enterovirus RNA–positive adults with T1DM in a French study [18]. A lack of association between HLA genotype and enterovirus infection.
in children with β cell autoimmunity or diabetes was reported in Finnish and white children [13, 51, 52]. In contrast, HLA-DRB1*03 was found more commonly in CV-B IgM–positive case patients [50], although serological testing is a less sensitive measure of infection than PCR [15]. The differences between these studies may be explained by the varying effect of HLA genes on the predisposition toward T1DM among different populations, the presence or absence of other disease-modifying genes, and nongenetic factors such as infection, because the predisposition toward T1DM is likely to be both multigenic and multifactorial [53]. In contrast, there may be other triggers of autoimmunity, such as rotavirus [54], in children at greater genetic risk of T1DM.

A case-control study of children at the time of presentation with diabetes cannot fully address the mechanisms of diabetes pathogenesis or the role of enteroviruses throughout the entire disease process. However, children in whom enterovirus RNA was detected at diagnosis may represent a subgroup of this complex disease. The timing of enterovirus infection, after the accumulation of autoreactive T cells, has been demonstrated to accelerate the development of T1DM in the NOD mouse, via a bystander effect [8]. Children who are at a lower genetic risk of the classic progressive form of autoimmune diabetes may have a more aggressive subtype of diabetes that is triggered by an enterovirus. In these children, diabetes may be the result of an acute cytolitic infection of the β cell rather than a chronic autoimmune process, although autoantibodies will be found in many of these children as markers of acute β cell destruction. In support of this hypothesis are the higher rate of severe DKA at presentation in the enterovirus RNA–positive group and a trend for more enterovirus RNA–positive case patients to be antibody negative, all of whom were aged >5 years. Alternatively, because enterovirus RNA was detected in subjects with both high- and low-risk HLA alleles, it is possible that enterovirus infections may have initiated β cell destruction in patients with low-risk HLA alleles and accelerated the process in patients with high-risk HLA alleles [17, 55].

The 14 enterovirus genotypes detected in the children with T1DM are likely to reflect subtypes present in the community during the study period, rather than a few “diabetogenic” subtypes (such as CV-B4). Case reports of EV-71 in NSW [56] and an outbreak of EV-71 in western Australia [57] indicated that EV-71 was commonly found in Australia during the study period. Indeed, EV-71 was the most commonly detected enterovirus (25.4%) from children with diabetes in our study, and the majority of EV-71 isolates (81%) were detected during the latter half of the study period, in parallel with Australian and Southeast Asian outbreaks. This supports the proposal that multiple enterovirus subtypes can be associated with the development of diabetes [58]; however, a frequent association between EV-71 and T1DM has not been reported. Our results may indicate that enterovirus infection per se, rather than a limited number of subtypes, is associated with the development of diabetes. Genetic determinants of diabetogenicity [3] and virulence [4] have been identified throughout the genome of CV-B4, but the relationship between sequence variation and T1DM for most enteroviruses is not known.

T1DM is a heterogeneous disease, and new subtypes of the disease are being identified. In a large cohort of children and adolescents, we have demonstrated that multiple enterovirus subtypes are found frequently in plasma and stool samples of children at the time of diagnosis of T1DM. We have also demonstrated a more aggressive enterovirus-induced subgroup of autoimmune T1DM that is associated with reduced genetic predisposition to diabetes in nonwhite children. This subtype differs from the recently described “novel” nonautoimmune fulminant subtype of T1DM described in Japanese adults [20]. Further characterization of the subgroups of T1DM is necessary, to enable appropriate prevention strategies such as immunization or immunomodulation to be implemented. Finally, because we have shown that a wide range of enteroviruses may trigger T1DM, there is a need for the further characterization of the genomic determinants of diabetogenicity in human enteroviruses.

Acknowledgments

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