Human Enterovirus RNA in Monthly Fecal Samples and Islet Autoimmunity in Norwegian Children With High Genetic Risk for Type 1 Diabetes

The MIDIA study

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OBJECTIVE — To test whether the frequency of human enterovirus RNA in fecal samples collected monthly from early infancy was associated with development of multiple islet autoantibodies in children with the highest risk HLA genotype.

RESEARCH DESIGN AND METHODS — Individuals carrying the HLA *DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02* genotype were identified at birth and followed with monthly stool samples from age 3 to 35 months. Blood samples taken at age 3, 6, 9, and 12 months and then annually were tested for autoantibodies to insulin, GAD 65 and IA-2. Among 911 children, 27 developed positivity for two or more islet autoantibodies in two or more consecutive samples (case subjects). Two control subjects per case subject were matched by follow-up time, date of birth, and county of residence. Stool samples were analyzed for enterovirus with a semiquantitative real-time RT-PCR.

RESULTS — The frequency of human enterovirus RNA in stool samples from case subjects before seroconversion (43 of 339, 12.7%) did not differ from the frequency in control subjects (94 of 692, 13.6%) ($P = 0.97$). Results remained essentially unchanged after adjustment for potential confounders, restriction to various time windows before seroconversion, or infections in the 1st year of life or after inclusion of samples collected after seroconversion. There was no difference in the average quantity of enterovirus RNA or in the frequency of repeatedly positive samples. The estimated relative risk for islet autoimmunity per enterovirus RNA–positive sample during follow-up (nested case-control analysis) was 1.12 (95% CI 0.66–1.91).

CONCLUSIONS — There was no support for the hypothesis that fecal shedding of enteroviral RNA is a major predictor of advanced islet autoimmunity.

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Human enteroviruses have been considered as possible environmental triggers or accelerators of islet autoimmunity leading to type 1 diabetes (1,2). They have been observed more frequently in patients in whom type 1 diabetes was recently diagnosed compared with control subjects, and there is also evidence of the virus in the pancreata of subjects who died shortly after disease onset (3). However, an interpretation of virus occurrence at or shortly after diagnosis is difficult with respect to type 1 diabetes pathogenesis, because the autoimmune process starts months to years before its clinical manifestation.

Potential causal relations between infections and development of islet autoimmunity are best assessed in longitudinal birth cohorts testing viral infections at frequent intervals before and during the development of islet autoimmunity. Five such studies, including between 11 and 41 cases of islet autoimmunity, have so far published results on enterovirus using various methods and testing strategies (more details are found in CONCLUSIONS). The evidence for involvement of enterovirus in type 1 diabetes pathogenesis comes predominantly from the Finnish population (4–6), whereas a study from Colorado (7) and one from Germany (8) did not find any significant association.

In view of these conflicting results, we aimed to test whether the presence of human enterovirus in monthly fecal samples predicted development of repeated positivity for two or more diabetes-associated islet autoantibodies in children with the HLA genotype conferring the highest risk for type 1 diabetes.

RESEARCH DESIGN AND METHODS

— The children prospectively observed in this study participate in the Norwegian cohort entitled "Environmental Triggers of Type 1 Diabetes: The MIDIA Study." The cohort was identified at birth from the general population based on genetic testing for the HLA genotype conferring the highest genetic risk of type 1 diabetes, *DRB1*0401-DQA1*03-DQB1*0302/DRB1*03-DQA1*05-DQB1*02*. Between 2001 and 2006, 911 children were included into the cohort. All subjects were followed up with stool samples, blood samples for autoantibody screening, and structured questionnaires. The study was approved by the Regional Committee for Medical Research Ethics and the Norwegian Data Inspectorate.

Blood samples taken at ages 3, 6, 9, and 12 months and every 12 months thereafter were processed, and the plasma was tested for autoantibodies against GAD 65, protein tyrosine phosphatase IA-2, and insulin, using radiobinding as-

says as described in detail earlier (9). Mailed questionnaires were administered at the same intervals. If a plasma sample was found to be positive for one autoantibody, the child was retested every 6 months; if a sample was positive for two or three antibodies, the child was retested every 3 months. The end point for this study, islet autoimmunity, was defined as positivity for two or more islet autoantibodies in two or more consecutive samples. Type 1 diabetes was diagnosed according to the World Health Organization criteria.

By December 2008, 27 of the 911 children in the cohort had reached the end point and were assigned as case subjects. The median age at onset of islet autoimmunity was 12.0 months (range 5.4–37.4 months). Of the 27 case children, diabetes was diagnosed in 10 by 1 September 2009, at a median age of 23.1 months (8.7–54.2 months). The timing of autoantibody seroconversion and age at diagnosis for each of the case subjects is shown in supplementary Table 1 (available in an online appendix at http://care.diabetesjournals.org/cgi/content/full/dc10-1413/DC1).

Two control subjects were randomly assigned per case subject, matched for the length of follow-up (at least as long as the time when the corresponding case subject developed multiple islet autoantibodies), date of birth within ±1 month (tolerating up to ±3 months if necessary), and county of residence (tolerating closest neighboring county if necessary). Children were ineligible as control subjects if they were repeatedly positive for one or more islet autoantibodies during follow-up. One control subject was transiently positive for a single autoantibody before the end point in the respective case subject; otherwise no control subjects developed positive autoantibodies (even after their case subject reached the end point). Data from one control child (matching group 27) are missing because the parents later withdrew the child from the study and refused any use of the collected data.

To test for enterovirus infections, we used stool samples obtained by the parents; they collected stool samples from their children every month from 3 to 35 months of age. These were sent by mail to our central laboratory, with a median transit time of 3 days. Parents also kept records of symptoms of infection in structured questionnaires. Of 704 planned blood samples, 637 were taken (91%); 2,173 of 2,482 scheduled stool samples

(88%) and 492 of 547 questionnaires were received (90%). The median duration of follow-up with stool samples was 28 months (range 7–35 months). The characteristics of the study participants are shown in Table 1.

Processing and molecular testing of stool samples

The processing and testing of stool samples in this study were described earlier (10). In brief, the samples were received by postal service, diluted, and centrifuged. The supernatants were frozen at $-80°C$ until copurification of RNA and DNA. The extraction protocol used the 96-well QIAamp plates vacuum-processed under the QIAamp Viral RNA Mini protocol (Qiagen, Hilden, Germany). West Nile virus Armored RNA (Asuragen, Austin, TX) was added in a constant quantity to the lysis buffer, which was used in the first step of the protocol. This exogenous internal control was used to monitor the success of RNA extraction and detection. Testing for human enterovirus RNA was performed in duplicate in 20-μl-volume one-step real-time RT-PCR with a primer-probe combination specific for the conserved 5′-untranslated region of human enteroviruses. This combination does not react with the rhinovirus species. Serial dilutions of enterovirus Armored RNA (Asuragen) were used to construct a sev-

en-point standard curve from 24 to 10^5 copies/μl. The threshold of positivity used in this study was set to 100 copies/μl RNA, a quantity that could be consistently and reliably detected.

Statistical analysis

To optimize the use of information in repeated samples collected from each individual, we compared the percentage of enterovirus RNA–positive samples collected from case subjects with those collected from control subjects, and tested this result using a mixed-effect logistic regression model with random intercept for each individual to account for potential intraindividual correlation (clustering) in risk of enterovirus positivity (xtmelogit in Stata 11). The primary analysis involved only samples collected up to seroconversion for the case subjects and the corresponding age in the matched control subjects. In case subjects who first tested positive for a single autoantibody, this first occurrence of autoantibody positivity was regarded as the onset of autoimmunity. The estimated odds ratio (OR) (with 95% CI) from this model is interpreted as the odds that a fecal sample is positive for enteroviral RNA given that it came from a child who later developed islet autoimmunity, relative to the odds that a sample is enterovirus-positive given that it came from a control child. Planned (secondary) subgroup analyses involved time win-

Table 1—*Characteristics of the case subjects and control subjects in this study*

	Case subjects	Control subjects
n	27	53
Age at onset of islet autoimmunity (months)*	12.1 (5–37)	12.3 (5–37)
Female sex	17 (63)	23 (43)
No. of other children in the family (siblings, half-siblings, step siblings)		
None	5 (18.5)	16 (30.2)
≥1	22 (81.5)	37 (69.8)
First-degree relative with diabetes		
None	17 (63)	50 (94.3)
Yes, of that	10 (37)	3 (5.7)
Sibling only	3	0
Father only	3	2
Mother only	2	1
Multiple family members	2	0
Progression from islet autoimmunity to type 1 diabetes		
Yes	10	None
Stool samples		
Total	627	1,417
Before development of islet autoimmunity*	339	692

Data are median (range), *n* (%), and *n*. *For matched control subjects: before the age at which the corresponding case subject seroconverted for islet autoantibodies.

dows of 6 and 12 months before seroconversion in case subjects (and corresponding ages in matched control subjects), samples collected before 1 year of age, and samples collected after seroconversion. We also adjusted for other variables by including them in the regression model, as reported in RESULTS. In separate analyses only the first enterovirus RNA-positive samples among series of two or more consecutively positive samples was counted, assuming that they were part of the same infectious episode.

We also analyzed the data according to a formal nested case-control study design using conditional logistic regression (accounting for the matched design with a fixed intercept for each matching group), modeling the cumulative number of enterovirus RNA–positive fecal samples before seroconversion (grouped as 0, 1, 2, or ≥3) as the exposure variable. With the given study design, the measure of association from this analysis is interpreted as the relative risk of islet autoimmunity per increase in cumulative number of enterovirus RNA–positive samples, with a corresponding 95% CI.

RESULTS

Frequency of human enterovirus before development of autoimmunity

The frequency of human enterovirus RNA in stool samples before the development of islet autoimmunity did not differ between case subjects (12.7%) and control subjects (13.6%). Results were similar even after adjustment for age, sex, month of sampling, year of sample, number of siblings, breastfeeding, and first-degree relatives with type 1 diabetes (Table 2). Likewise, no association was seen when only infections before 12 months of age (OR 1.02 [95% CI 0.51–2.04]) or various time windows before seroconversion in case subjects were analyzed: with a 6-month window, the frequency was 20 of 142 (14.1%) in case subjects vs. 42 of 308 (13.6%) in control subjects (1.05 [0.54–2.04]) and with a 12-month window the frequency was 31 of 214 (14.5%) in case subjects vs. 62 of 454 (13.7%) in control subjects (1.09 [0.62–1.92]). The use of infectious episodes rather than number of positive stool samples (i.e., consecutive positive samples were deemed as a single episode) did not appreciably alter the above figures. The results were similar when a conditional logistic regression model estimating the OR per increase in infections before de-

velopment of islet autoimmunity was used (OR 1.12 [0.66–1.91]).

Quantity of human enterovirus RNA

The effect of viral load was assessed by dividing the positivity into two categories: low to moderate (quantity of 100–9,999 enterovirus copies/μl RNA) and high (≥10,000 enterovirus copies/μl RNA). No association with islet autoimmunity was found in this type of analysis (Table 3). In the 43 enterovirus-positive samples from the preautoimmunity period among case subjects, the median estimated human enterovirus quantity was 18,000 copies/μl RNA compared with a median of 12,000 copies/μl RNA among 94 enterovirus-positive samples from matched control subjects from the corresponding

periods (Mann-Whitney nonparametric test $P = 0.37$). Similar results were seen in the samples collected after the onset of autoimmunity. Among the 30 new enterovirus episodes during the preautoimmune period of case subjects, 13 (43.3%) were followed by at least one additional consecutive enterovirus-positive sample, compared with 29 of 65 (44.6%) among the control subjects (χ^2 test $P = 0.73$).

Occurrence of human enterovirus during the whole observation period

In total, we tested 2,044 stool samples from the case subjects (627) and control subjects (1,417) in the study. Human enterovirus was detected in 80 of 627 (12.8%) samples from case subjects and 210 of 1,417 (14.8%) samples from con-

Table 2—Frequency of human enterovirus fecal samples collected before islet autoimmunity

	Case subjects	Control subjects	OR (95% CI)*	
			Unadjusted	Adjusted†
n	27	53		
Enterovirus RNA				
Negative samples	296	598	1.00 (reference)	1.00 (reference)
Positive samples	43 (12.7)	94 (13.6)	1.01 (0.59–1.72)	1.09 (0.61–1.96)
Total	339	692		
New enterovirus infection episode				
No	296	598	1.00 (reference)	1.00 (reference)
Yes	30 (9.2)	65 (9.8)	0.94 (0.59–1.52)	0.92 (0.54–1.57)
Total‡	326	663		

Data are *n*, *n* (%), and ORs (95% CI). *Estimated from logistic mixed-effects logistic regression models with random intercept for each subject to control for intraindividual correlation (no significant random intercept in model for enterovirus episodes, but highly significant in model for enterovirus positivity). The unadjusted OR in ordinary logistic regression ignoring intraindividual correlation in infections was 0.92; ‡Adjusted for sex, calendar month of sample collection, year of sample collection (2001–2003, 2004–2006, or 2007–2008), age (continuous), number of siblings (0 vs. ≥1), breast-feeding, and first-degree family history of type 1 diabetes (yes/no); †Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

Table 3—Semiquantitative testing of the stool samples: frequency of enterovirus infections with high and low viral load in the children who subsequently developed repeated positivity of multiple autoantibodies vs. matched control subjects who did not develop autoimmunity

	Case subjects	Control subjects
n	27	53
Enterovirus RNA-negative	296	598
Enterovirus RNA-positive, low-moderate quantity*	18 (5.3)	46 (6.6)
Enterovirus RNA-positive, high quantity*	25 (7.4)	48 (6.9)
Total	339	692
Enterovirus RNA-negative	296	598
New infection episode, low-moderate quantity*	11 (3.4)	31 (4.7)
New infection episode, high quantity*	19 (5.9)	34 (5.1)
Total†	326	663

Data are *n* or *n* (%). *Negative, <100 copies/ml enterovirus RNA; low-moderate quantity, 100–9,999 copies/ml enterovirus RNA; high quantity, ³10,000 copies/ml enterovirus RNA; †Excluding consecutively positive samples that may have been part of the same infectious episode as in the previous positive sample.

trol subjects; the overall occurrence did not differ between case subjects and control subjects (OR 0.84 [0.58–1.22]). Looking only at samples taken after the start of islet autoimmunity gave similar results (0.74 [0.45–1.22]). Only 11 subjects did not shed enterovirus in their stool during their entire observation period (4 case subjects and 7 control subjects). The remaining children had various numbers of positive monthly samples, from only 1 ($n = 7$) up to 8–9 ($n = 7$). Infections and their distribution over the observational period in case subjects and control subjects of the 27 matching groups are shown in supplementary Fig. 1 (available in an online appendix at http://care.diabetesjournals.org/cgi/content/full/dc10-1413/DC1).

Seasonal variation of infections

There was a pronounced seasonality of infections with a peak in autumn (October with 27% positive samples) and a smaller peak in July (with 24% positive samples) and a dip in March (with 3% positive samples). Supplementary Fig. 1 shows several episodes of increased density of infections that can be observed across the case-control matching group. The occurrence of infections was also age-dependent: a rise was noted from the 5th to 9th month of age and during the first half of the 2nd year of life.

Molecular typing of enterovirus strains using partial VP1 sequencing

VP1 genotypes were determined for selected positive samples (97 samples) to distinguish prolonged infections with one strain against multiple consecutive infections. The distribution of the 17 different serotypes found is shown in supplementary Table 2 (available in an online appendix). Because the sequenced samples were not representative of the whole case-control dataset, direct comparison of the serotype repertoire between case subjects and control subjects was not possible. A phylogenetic tree constructed from the dataset is shown in supplementary Fig. 2 (available in an online appendix).

CONCLUSIONS — We tested enterovirus RNA in >2,000 monthly fecal samples from children who developed repeated positivity for multiple islet autoantibodies and their matched control subjects, all with a single *HLA-DQ, -DR* genotype, conferring the highest risk of type 1 diabetes. We found no evidence to support a higher frequency of enterovirus in case subjects than in control subjects either before or after seroconversion for islet autoantibodies. It must be kept in mind that the study population consisted only of very young children; thus, the conclusions might not apply to older individuals.

This study is the first to use a quantitative assay for testing the viral load, enabling us to distinguish between low- and high-quantity infections and follow the dynamics of the viral load. Our cohort includes only the highest risk *HLA-DQ, -DR* genotype and is thus more genetically restricted than previously reported studies. The generalizability of our results might be questioned if the HLA genotype influenced the risk of enterovirus infection and/or immune response. However, preliminary results from our pilot study, which also included a group without the high-risk HLA genotype, indicated only a moderate difference in frequency of fecal enterovirus shedding (11). To our knowledge, none of the previous cohort studies of enterovirus and islet autoimmunity has found any significant difference in association depending on HLA genotype.

We have also used a strict definition of islet autoimmunity, requiring repeated positivity for two or three islet autoantibodies, which is known to be strongly predictive of type 1 diabetes in genetically susceptible children. The number of case subjects and sample size could indeed be increased with a less strict definition of autoimmunity. However, the power of the study might actually decrease by including subjects with milder autoimmunity who are less likely to eventually develop type 1 diabetes.

Regular monthly sampling from all participants and high completeness are important strengths, because shedding duration is thought to be ~3–4 weeks (12); the necessity of frequent stool sampling is further supported by our earlier study showing that excretion usually lasted <3 months (13). Detection of viral RNA in serum would probably underestimate the true infection frequency, because enterovirus RNA is present in serum for a much shorter period (12) than is the usual time span between blood samples. On the other hand, it is probable that viremia reflects more closely the spreading of the virus to the target organ, so frequent sampling of both stool and blood samples would be ideal.

Although the serotypes detected were not representative for all samples, we observed no preponderance of a strain, se-rotype, or group in either case subjects or control subjects. Several serotypes previously reported as possibly diabetogenic (e.g., Coxsackie B) were observed both in case subjects and in control subjects. Although some types may seem to be more prevalent, this is mostly due to repeatedly positive stool samples from a small geographical area during a short period, reflecting local epidemics.

Two previous studies assessed fecal shedding of enterovirus RNA. The Finnish Type 1 Diabetes Prediction and Prevention (DIPP) study used equally frequent sampling of stool as we did, reporting data from 12 case subjects with islet autoimmunity and 53 control subjects (14). The other study was the Diabetes Autoimmunity Study in the Young (DAISY) in Colorado, for which rectal swabs were collected at longer intervals (at ages 9, 12, 15, and 24 months and then annually) from 26 case subjects and 39 control subjects (7). In both studies, there was no significant difference in the frequency of fecal enterovirus RNA shedding between case subjects with islet autoimmunity and control subjects, which is consistent with our findings. However, in contrast with our findings, the DIPP study reported that samples from case subjects were more frequently positive in consecutive samples than were samples from control subjects.

A publication from DAISY (7) and a separate publication from the DIPP study including 41 case subjects and 196 control subjects with 3- to 6-month sample intervals (4) also analyzed enterovirus RNA in serum. In both these studies there was no significant difference in the frequency of serum enterovirus RNA, but when serum RNA and a series of enterovirus antibodies were combined as indicators of infection, there was a significant difference in the DIPP study, particularly in the 6-month interval before seroconversion in case subjects. Although we did not assess enterovirus RNA or antibodies in serum, no indication of a clustering of infections before seroconversion was found.

Two other Finnish studies reported a significant difference between case subjects with islet autoimmunity and control subjects in frequency of indicators of enterovirus infection in serum, namely the Childhood Diabetes in Finland (DiMe) study assessing 11 prediabetic siblings of patients with type 1 diabetes and 34 autoantibody-negative control subjects (6), and the Trial to Reduce IDDM in Geneti-

cally at Risk (TRIGR) study assessing 19 case subjects and 84 control subjects from birth to 2 years of age (5). Note, however, that enterovirus RNA in serum accounted for 23% of the identified infections (increases in enterovirus antibodies accounted for the remaining) and that the difference in enterovirus RNA was borderline (not) significant (14 vs. 8.4%, $P = 0.07$). Finally, no significant association was found in the German BABYDIAB study, which tested antibodies against Coxsackie viruses in blood samples collected at the age of 9 months and at 2, 5, and 8 years in 28 case subjects with persistent islet antibodies and 51 matched control subjects (8).

None of the previous studies contradicts our finding that fecal shedding of enterovirus RNA in general does not strongly predict islet autoimmunity. Although moderate effects (OR 1.5–2.0) cannot be ruled out from our data, the 95% CIs around the OR estimated from our actual data suggest that strong associations (OR >2) are unlikely. However, we cannot exclude a possible role of a subgroup of enterovirus infections (particular strains) perhaps influencing viremia and ability to spread from the gut (the primary site of replication) to the target organ. This ability was seemingly unlinked to the viral load or duration of gut infections, as judged from our results. Other relevant factors may potentially influence the level and duration of viremia and the ability to invade the islets and their β-cells.

In summary, there was no evidence to support a major role of frequency, timing, or quantity of fecal enterovirus shedding in prediction of advanced islet autoimmunity and no evidence that islet autoimmunity predicted increased susceptibility to fecal enterovirus shedding. Further research should be focused on the character of viremia and the ability of enterovirus to invade the target pancreatic tissue in much larger sample sets.

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