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Detection of a Low-Grade Enteroviral Infection in the Islets of Langerhans of Living Patients Newly Diagnosed With Type 1 Diabetes

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The Diabetes Virus Detection study (DiViD) is the first to examine fresh pancreatic tissue at the diagnosis of type 1 diabetes for the presence of viruses. Minimal pancreatic tail resection was performed 3–9 weeks after onset of type 1 diabetes in six adult patients (age 24–35 years). The presence of enteroviral capsid protein 1 (VP1) and the expression of class I HLA were investigated by immunohistochemistry. Enterovirus RNA was analyzed from isolated pancreatic islets and from fresh-frozen whole pancreatic tissue using PCR and sequencing. Nondiabetic organ donors served as controls. VP1 was detected in the islets of all type 1 diabetic patients (two of nine controls). Hyperexpression of class I HLA molecules was found in the islets of all patients (one of nine controls). Enterovirus-specific RNA sequences were detected in four of six patients (zero of six controls). The results were confirmed in various laboratories. Only 1.7% of the islets contained VP1$^+$ cells, and the amount of enterovirus RNA was low. The results provide evidence for the presence of enterovirus in pancreatic islets of type 1 diabetic patients, which is consistent with the possibility that a low-grade enteroviral infection in the pancreatic islets contributes to disease progression in humans.

Despite intensive research efforts over the past century, the precise causes of type 1 diabetes are still unknown, although it is well established that the illness results from a complex interplay among genetic predisposition, the immune system, and various environmental factors (1). One such influence is viral infection, first postulated in 1927 by Gundersen (2) who observed an increase in the incidence of type 1 diabetes following mumps epidemics. Since, numerous studies addressed the possible role of viruses as causative agents in type 1 diabetes, and infection with enteroviruses emerged as associated with disease development (3–6). However, the question of causality remains open, and data confirming the presence of enterovirus in the pancreata of type 1 diabetic patients would significantly strengthen the conclusions.

One of the principal factors limiting progress in the field has been the lack of availability of well-preserved tissue samples for study (7). The majority of published studies have made use of pancreatic tissues collected at

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autopsy from type 1 diabetic patients with varying durations of diabetes (8–10). Much of the material was affected by postmortem changes and was preserved in the form of formalin-fixed, paraffin-embedded blocks, which means that many of the more modern techniques used to detect viruses cannot be applied with confidence. Well-preserved human organ samples would be preferable because these should be more amenable to the detection of viral RNA sequences, viral antigen production, and the cellular responses to viral infection (11).

The main objective of the Diabetes Virus Detection study (DiViD) was to collect pancreatic tissue from living subjects very soon after the diagnosis of type 1 diabetes to investigate the presence of viruses. The sampling procedures and clinical data from the recruited patients have been previously reported (12). Here, we provide evidence that the islets of Langerhans of these patients display features consistent with a low-grade enteroviral infection. The study was approved by the government's regional ethics committee in Norway.

RESEARCH DESIGN AND METHODS

Six type 1 diabetic patients (three women, three men) age 24–35 years (median 28 years) were recruited to the study after giving written informed consent. Pancreatic biopsy samples (~3 cm of the tail) were taken 3–9 weeks after diagnosis of type 1 diabetes (median 5 weeks). Details regarding the patients are shown in Table 1.

The biopsy samples were processed under sterile conditions and immediately divided into multiple smaller pieces, snap frozen in liquid nitrogen in the operating theater (Table 1), and subsequently stored at −80°C. Formalin-fixed, paraffin-embedded tissue blocks were prepared simultaneously. Other parts were allocated for the purification of living pancreatic islets in ViaSpan medium. Nine otherwise healthy, nondiabetic, cadaver Caucasian organ donors (two women, seven men, age range 18–38 years [mean 25.2 years]) collected by the Network for Pancreatic Organ Donors with Diabetes (nPOD) were used as controls for immunohistochemistry analyses, all being negative for anti-GAD, IA2, insulin, and ZnT8 autoantibodies. In addition, six nondiabetic organ donors from Uppsala, Sweden (age range 55–70 years [median 67 years]), also negative for anti-GAD and IA2 were used as methodological controls in virus detection from isolated islets using PCR. Demographic details regarding age, sex, BMI, autoantibodies, and HLA of the controls are shown in Supplementary Table 1.

Islet Isolation

Pancreatic islets were isolated in Uppsala using methods previously developed for clinical islet isolation (13). Isolated islets were cultured for a few days, and aliquots of the culture medium were collected 1, 3, and 6 days postisolation for enterovirus PCR analyses. For practical reasons, the PCR studies were not blinded, but both the

Table 1—Clinical data of patient cases with newly diagnosed type 1 diabetes

Case	Age (years)	Sex	BMI (kg/m^2)	Diagnosis to biopsy (weeks)	HbA_{1c} at biopsy [% (mmol/ mol)]	Insulin (units/kg/day)	Anti-GAD (<0.08 ai)	Anti-insulin (<0.08 ai)	Anti-ZnT8 (<0.12 ai)	Anti-IA2 (<0.10 ai*)	HLA risk allele†	Time to snap freezing of pancreatic tissue (s)
1	25	F	21.0	4	6.7 (50)	0.5	1.76	0.7	0.28	0.16	Yes	110
2	24	M	20.9	3	10.3 (89)	0.35	0.79	<0.01	0.44	>3	Yes	150
3	34	F	23.7	9	7.1 (54)	0.17	1.77	<0.05	1.45	>3	Yes	150
4	31	M	25.6	5	7.4 (57)	0.4	0.77	0.1	<0.01	2.54	Yes	160
5	24	F	28.6	5	7.4 (57)	0.36	0.46	0.1	0.06	>3	Yes	240
6	35	M	26.7	5	7.1 (54)	0.52	1.85	<0.05	<0.01	<0.04	Yes	190

ai, antibody index. *Arbitrary units according to Diabetes Antibody Standardization Program (24). †Presence of HLA DR3-DQ2, HLA DR4-DQ8, or both.

patient cases and controls were studied in parallel in the same PCR runs in two laboratories (Uppsala and Tampere, Finland).

Detection of Enterovirus

Immunostaining with clone 5D8/1 (Dako, Glostrup, Denmark) to detect enterovirus capsid protein 1 (VP1) was performed in two laboratories (Tampere and Exeter, U.K.). Consecutive 4-μm sections from two different paraffin blocks from each patient case and from one block from each control were processed and labeled using a standard immunoperoxidase technique for formalin-fixed paraffin-embedded sections. For details regarding the methods, see Supplementary Data.

RESULTS

All six type 1 diabetic patients were positive for enterovirus in the endocrine pancreas by at least one of the three methods used to detect either viral protein or RNA (Table 2). Four patients were enterovirus positive in the pancreas by two methods and one by three methods.

Detection of Enterovirus Genome

Enterovirus RNA was detected by RT-PCR in the medium harvested from the enriched islet preparation in three of the six type 1 diabetic patients in both the Uppsala and Tampere laboratories. Virus was detected in the medium harvested from islet cultures on days 1 and/or 3 but not on day 6 (Table 2). In addition, islet culture medium from one patient was detected as positive on day 3 in Tampere, and this patient was also positive in the remaining enriched exocrine cells, also containing some islets, in Uppsala. None of the islet cultures from six nondiabetic controls were enterovirus positive. A snap-frozen pancreas sample containing 30 mg whole tissue was enterovirus positive in one patient in both laboratories. The same patient was also virus positive in the enriched islets and exocrine cell fractions as described previously (Table 2). The amount of enterovirus RNA was low in all positive samples. The viral genome was partially sequenced in all four virus-positive cases, and the sequence showed a perfect match with enterovirus sequences (Table 3). Because of the low virus titer, we were able to sequence only the conserved region of the genome; therefore, the exact genotype of the virus could not be identified. All patient cases and controls were PCR negative for rhinovirus, norovirus, rotavirus, and parechovirus in both enriched islets and exocrine cells as well as in snap-frozen whole-tissue samples. High-throughput sequencing of total RNA extracted from whole frozen tissue from each patient did not detect any viral sequences.

Detection of Enterovirus Protein

Pancreatic islets from all six type 1 diabetic patients were immunopositive for VP1 (Table 2), whereas this protein was detected in two of nine controls (100% vs. 22%, $P < 0.01$). Only 1.7% of the patient islets contained intense $VP1^+$ cells (42 of a total 2,492 islets; immunostaining of consecutive sections for insulin, glucagon, and VP1). Altogether, 60 intense $VP1^+$ cells were identified in the islets.

Expression of Class I HLA Molecules

Hyperexpression of class I HLA molecules was observed to be homogeneous in the islets of all six type 1 diabetic patients but in only one of the nine controls ($P < 0.01$) (Table 2). Among the six patients, all insulin-containing islets showed class I HLA hyperexpression irrespective of the presence of enterovirus VP1 protein. Figure 1 shows an insulin-containing islet with hyperexpression of class I HLA and the presence of enterovirus VP1 protein.

DISCUSSION

This study of fresh pancreatic tissue collected close to the time of diagnosis of type 1 diabetes suggests that a low-grade enteroviral infection is sustained within the islets of Langerhans. Thus, enterovirus RNA was amplified successfully by RT-PCR from four of the six patients in

Table 2—Detection of enterovirus protein, enterovirus RNA, and expression of class I HLA molecules in the pancreata of newly diagnosed type 1 diabetic patients

	Enterovirus VP1 protein expression		Enterovirus genome				Class I HLA expression
Methodology	IHC		Enterovirus-specific RT-PCR			RNA sequencing	IHC
Tissue	Pancreatic islets		Supernatant from cultured purified pancreatic islets		Snap-frozen pancreas 30 mg	Snap-frozen pancreas 30 mg	Pancreatic islets
Laboratory	Tampere	Exeter	Uppsala	Tampere	Tampere and Uppsala	Oslo	Exeter
Case							
1	Positive	Positive	Negative	Negative	Negative	Negative	Hyperexpression
2	Positive	Positive	Positive*	Positive†	Negative	Negative	Hyperexpression
3	Positive	Positive	Negative	Negative	Negative	Negative	Hyperexpression
4	Positive	Negative	Positive*	Positive†	Negative	Negative	Hyperexpression
5	Positive	Positive	Positive‡	Positive‡	Negative	Negative	Hyperexpression
6	Positive	Positive	Negative§	Positive‡	Positive	Negative	Hyperexpression

IHC, immunohistochemistry. *Positive day 1 and 3. †Positive day 1. ‡Positive day 3. §Positive in RNA extracted from culturing of the remaining cells, containing both exocrine and an unknown number of islets.

Table 3—Sequence alignment of PCR-products, Uppsala

Case2	GTACCTTTGTGCGCCTGTTTTATTACCCCCTCCCCTAACTGTAACTTAGAAGTAATACACACCGATCAACAGTAAGTGTGGCACGCCAGCCATGCTCCGATCAAGCACTTCTGTTCCCCC
Case4	----------
Case5	----------
Case6	----------
Case2	GGACCGAGTATCAATAGACTGCCCACGCGGTTGAAGGAGAAAGTCCGTTATCCGGCCAACTACTTCGAGAAGCCCAGTAACACCATGGAAGTTGCAGAGTGTTTCGCTCAGCACAACC
Case4	----------
Case5	----------
Case6	----------
Case2	CCAGTGTAGATCAGGTCGATGAGTCACTGCATACCCCACGGGTGACCGTGGCAGTGGCTGCGTTGGCGGCCTGCCTATGGGGAAGCCCATAGGACGCTCTAATACAGACATGGTGTGAAG
Case4	----------
Case5	----------
Case6	----------
Case2	AGTCTATTGAGCTAGTTGGTAGTCCTCCGGCCCCTGAATGCGGCTAATCCTAACTG**T**GGAGCA**T**A**CG**CCC**T**CAA**G**CCAG**G**GGGC**AG**T**G**TGTCGTAA**C**GGG**C**AACTCTGCAGCGGAACCGA
Case4	----------CCCTGAATGCGGCTAATCCTAACTG**T**GGAGCA**C**A**TG**CCC**A**CAA**A**CCAG**T**GGGT**GG**T**G**TGTCGTAA**C**GGG**T**AANTNNNNNGNNGAACCGA
Case5	----------
Case6	----------CCCTGANTGCGGCTAATCCTAACTG**C**GGAGCA**N**A**TA**CCC**A**CAA**A**CCAG**T**GGGC**AG**T**C**TGTCGTAA**T**GGG**T**AACTCTGCAGCGGAACCGA
Case2	CTAC----------
Case4	CTACTTTGGGTGTCCGTGTTTCT**A**TTTA**T**TC**C**T**A**TA**C**NG**G**C----------
Case5	----------AANAGTAAAAGAAACACGGACACCCAAAGTAGTCGGTTCCGCTGCNNAGTTACCCATTACG
Case6	CTACTTTGGGTGTCCGTGTTTCT-TTTA**C**TC**TT**T**A**T**TG**T**CACCATAAGC AGC C AATAAAAGAGTAAAAGAAACACGGACACCCAAAGTAGTCGGTTCCGCTGCAGAGTTACCCATTACG
Case2	----------
Case4	----------
Case5	ACAGACTGCCCACTGGCTTGTGGGTNNCTGCTCCGCAGTTAGGATTA
Case6	ACAGACTGCCCAC----------

Variable nucleotide sites between cases appear in boldface.

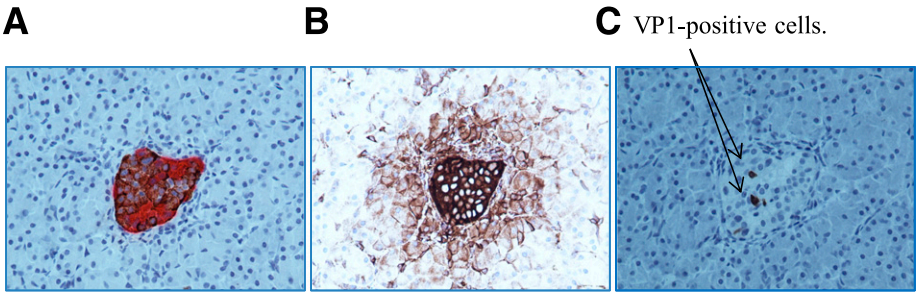

Figure 1—Pancreatic islet from one type 1 diabetic patient. Stained for insulin (brown) and glucagon (red) (*A*), class I HLA molecules (*B*), and enterovirus protein VP1 (*C*). Cells positive for enterovirus protein are marked by arrows.

two independent laboratories. The presence of enterovirus sequences was also confirmed by sequencing the PCR products. Moreover, the enterovirus capsid protein VP1 was detected immunohistochemically in islet cells of all six patients. The expression of VP1 is known to be most intense during the acute phase of an enterovirus infection (14), whereas it is reduced during persistent infection (15). This is because enteroviral persistence is characterized by naturally occurring deletions at the 5′ terminus of the genome (16,17), which reduce the replication rate of the virus. It was recently shown that these terminally deleted viruses also can persist after inoculation in murine pancreas in the absence of cytopathic virus weeks after the acute infectious period (16). Hence, the present results are consistent with the possibility that a low-grade enterovirus infection was established and maintained in the islet cells of the patients with type 1 diabetes. The biopsy samples were taken from the pancreatic tail, not discarding that the infection could be affecting other parts first before slowly disseminating to the rest of the tissue. The lack of virus-induced cytopathic effects in islets and exocrine cell clusters during 3–5 days of culture indicates that the virus is not powerfully cytolytic, implying that the virus might be rendered replication deficient during the development of a persistent infection.

It is well understood that the amplification achieved by PCR allows for the detection of even very small quantities of target RNA; thus, a significant finding is that four of the type 1 diabetic patients were positive for viral RNA by RT-PCR in two different laboratories. The detection of positive signals required as many as 40 cycles of amplification or the use of a nested RT-PCR method, indicating that only very small amounts of viral RNA were present. Sequencing confirmed that amplified sequences originated from enteroviruses.

The high sensitivity of PCR makes it susceptible for false-positive results due to viruses that may contaminate the samples during the analysis. Several actions were taken to avoid such contaminations and enable their detection if they occur. First, the two virus laboratories got the same results even though they carried out all RNA extraction and RT-PCR steps independently and used different primers and PCR protocols. Second, the amplified enterovirus sequences differed from each other, suggesting that they originated from different enterovirus strains and thus excluding a common contaminating virus. Third, exocrine cells isolated from the same pancreata in the same time and place as well as virus-negative internal control samples included in each test run were all PCR negative. In addition, all pancreas samples were PCR negative for all other tested viruses. Finally, pancreata from nondiabetic controls were PCR negative as determined using exactly the same PCR procedures and in the same laboratory as the samples from diabetic patients.

The antibody used to detect VP1 is known to recognize this protein from multiple enteroviruses in formalin-fixed samples (18). However, it has also been shown that under some conditions, the antibody may label certain human proteins (19). We were careful to use the antibody under conditions optimized to avoid such interactions without compromise of virus-specific binding (20). Thus, we are confident that the immunolabeling achieved in human pancreas sections is likely to represent the presence of viral protein.

In addition to virus-specific markers, the expression of class I HLA molecules was upregulated in the islets of all type 1 diabetic patients. This fits with previous observations showing that pancreatic islets of type 1 diabetic patients hyperexpress class I HLA molecules and interferon α (21,22). This might indicate ongoing virus-induced interferon secretion in the islets. We have previously shown that enterovirus infection in human pancreatic islets leads to such class I HLA hyperexpression in vitro partly mediated by secretion of type 1 interferons (23).

This study did not include pancreatic biopsy samples from healthy living individuals. Although not ideal, the nondiabetic organ donors from nPOD are clinically well defined and age matched to the patient cases. The mean age of the subjects from whom islets were isolated for the culture studies was higher than the patient cases, but we would emphasize that they served mainly as methodological controls in PCR analyses, being negative for all tested viruses in PCR.

In conclusion, DiViD is the first study of living, newly diagnosed type 1 diabetic patients to demonstrate the

presence of enterovirus in pancreatic islets using multiple techniques across several independent laboratories, including the detection of enterovirus-specific sequences in the islets. The results do not prove causality between enterovirus infection and type 1 diabetes, but they support the view that a low-grade enteroviral infection is present in the islets of Langerhans at diagnosis of type 1 diabetes. These findings should encourage studies in which antiviral medication and/or vaccines against enteroviruses could be tested to reduce disease progression and prevent type 1 diabetes.

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