Enterovirus infections have been linked to type 1 diabetes in several studies. Enteroviruses also have tropism to pancreatic islets and can cause β-cell damage in experimental models. Viral persistence has been suspected to be an important pathogenetic factor. This study evaluates whether gut mucosa is a reservoir for enterovirus persistence in type 1 diabetic patients. Small-bowel mucosal biopsy samples from 39 type 1 diabetic patients, 41 control subjects, and 40 celiac disease patients were analyzed for the presence of enterovirus using in situ hybridization (ISH), RT-PCR, and immunohistochemistry. The presence of virus was compared with inflammatory markers such as infiltrating T cells, HLA-DR expression, and transglutaminase 2–targeted IgA deposits. Enterovirus RNA was found in diabetic patients more frequently than in control subjects and was associated with a clear inflammation response in the gut mucosa. Viral RNA was often detected in the absence of viral protein, suggesting defective replication of the virus. Patients remained virus positive in follow-up samples taken after 12 months’ observation. The results suggest that a large proportion of type 1 diabetic patients have prolonged/persistent enterovirus infection associated with an inflammation process in gut mucosa. This finding opens new opportunities for studying the viral etiology of type 1 diabetes.

Type 1 diabetes is one of the most common chronic diseases in developed countries, and its incidence has increased sharply since the second world war. It is caused by a selective destruction of insulin-producing β cells in the pancreas mediated by immunological mechanisms. Susceptibility to the disease is modulated by >40 different risk genes, with HLA genes contributing more than half of the genetic susceptibility. Environmental factors clearly influence the disease risk and contribute to the rapidly increasing incidence rates.

The connection between type 1 diabetes and enterovirus infections has been widely studied. Enteroviruses have been found in the blood and pancreas of type 1 diabetic patients in several studies, and they have also been associated with increased risk of type 1 diabetes in prospective studies (1–7). The recent discovery of the genetic polymorphisms of IFIH1 gene as diabetes risk-modulating factors has further strengthened this association, since these genes mediate the recognition of enteroviruses by the innate immune system (8,9). Diabetes-associated polymorphisms seem to be associated with a strong innate immune response, which may lead to enhanced inflammation response during virus infection (10). Other innate immune system genes (IRF7 network) also modulate the risk of T1D (11).

Frequent detection of the virus in diabetic patients has suggested a possible role of viral persistence. Enterovirus persistence has previously been described, e.g., in chronic cardiomyopathies (12) and in animal models (13–16), where enterovirus and other picornaviruses may persist for prolonged periods. These studies have suggested that enterovirus can persist as double-stranded RNA without clear protein synthesis (15,17). It is known from several viral diseases that persistent infection typically leads to a strong innate immune response, local inflammation, and immune-mediated tissue damage.

Celiac disease is characterized by small intestinal inflammation, mucosal damage, and increased mucosal permeability. The association between type 1 diabetes and celiac disease is well established. It is therefore intriguing to hypothesize whether enterovirus infection and enteropathy contribute to this association. In spite of the recent progress in this research field, it is not known how enteroviruses could cause selective damage in insulin-producing β cells. Their primary replication site is the gut mucosa, from which the virus can spread to the blood (primary viremia) and infect internal organs. However, the possible presence of enteroviruses in human intestine has not been studied in detail. We recently published the first such study suggesting that enterovirus may be present in the small intestine in type 1 diabetic patients (18). The current study evaluates the hypothesis that gut mucosa is a reservoir for enterovirus persistence in type 1 diabetic patients, maintaining a continuous inflammatory state that can spread to the pancreas and play a crucial role in enterovirus-induced diabetes.

**RESEARCH DESIGN AND METHODS**

**Study series.** The study series consisted of 120 individuals altogether who underwent gastroscopy because of various gastrointestinal complaints. Small-bowel mucosal biopsies were taken from distal duodenum for morphological analyses and for basic research. Samples were taken during the period 1995–2000 at the Department of Gastroenterology, Tampere University Hospital. Formalin-fixed samples were available from all 120 study subjects and frozen samples from 86 subjects.

The study subjects comprised 39 type 1 diabetic patients, 40 patients with celiac disease who did not have type 1 diabetes, and 41 control subjects, including subjects who had neither of these diseases but non-specific gastrointestinal
symptoms (25 dyspepsia, 10 irritable bowel syndrome, 3 aphthous stomatitis, 1 ulcerative colitis, 1 collagenous colitis, and 1 eventually healthy). Furthermore, one control subject had psoriasis and one IGA nephropathy. All control subjects were non-diabetic and showed no signs of celiac disease (Table 1). The diagnosis of type 1 diabetes was based on World Health Organization criteria (10), and all the patients were on insulin treatment. Sixteen also had celiac disease, 10 thyroid autoimmune disorder, 1 IGA nephropathy, 1 Addison disease, 1 alopecia, and 1 rheumatoid arthritis. The duration of diabetes varied from 0 to 38 years (median 20 years; exact information available from only 23 patients).

Celiac disease was diagnosed in study subjects by a positive endomysial antibody result and by small-bowel mucosal villous atrophy and crypt hyperplasia. Celiac disease had recently been diagnosed in all the patients, and all were on a normal gluten-containing diet at the time of the biopsy. One celiac disease patient had IGA nephropathy, and one had hyperthyroidism.

The study protocol was approved by the ethical committee of the Tampere University Hospital. All subjects gave informed consent.

**Table 1: Summary of study subjects**

<table>
<thead>
<tr>
<th>TID patients*</th>
<th>Celiac disease patients</th>
<th>Control subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (N)</td>
<td>39</td>
<td>40</td>
</tr>
<tr>
<td>Males/females (M/N)</td>
<td>11/28</td>
<td>13/27</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43 (18–63)</td>
<td>41 (18–75)</td>
</tr>
<tr>
<td>HLA DQ2 and/or DQ8 (%)</td>
<td>57</td>
<td>100</td>
</tr>
<tr>
<td>Age at diabetes diagnosis (years)**</td>
<td>18 (4–51)</td>
<td></td>
</tr>
<tr>
<td>Diabetes duration (years)**</td>
<td>20 (0–38)</td>
<td></td>
</tr>
</tbody>
</table>

Data are median (range) unless otherwise indicated. TID, type 1 diabetic. *Sixteen of the type 1 diabetic patients also had celiac disease. **Exact information from only 23 type 1 diabetic patients available. Others were diagnosed early in childhood.

RESULTS

Small intestinal biopsy samples were first screened for the presence of enterovirus genome using ISH method. Seventy-four percent of the type 1 diabetic patients compared with 29% of the control subjects were found to be virus positive (P < 0.001) (Table 2 and Fig. 1). A higher frequency of clearly positive hybridization signals was seen in diabetic patients especially (51 vs. 5%; P < 0.001) (Table 2). Enterovirus RNA was mainly located in the epithelial cells of villi and crypts; however, occasional staining in lamina propria was also observed. A follow-up sample was available from three diabetic patients who were enterovirus positive in the initial sample (taken 1 year after the initial sample). All of these remained enterovirus positive. The detection of enteroviruses was not associated with age, sex, HLA DQ2 and/or DQ8 alleles, or duration of diabetes or celiac disease (data not shown).

Next, the presence of viral RNA was confirmed using RT-PCR. A freshly frozen biopsy sample was available from 26 of the diabetic patients, 34 celiac disease patients, and 31 control subjects, and RT-PCR found the virus in 19, 15, and 10% of them, respectively. The virus was detected more frequently in ISH-positive than in ISH-negative subjects (12 of 50 vs. 1 of 41; P < 0.01).

In the next phase, we analyzed whether the presence of viral RNA was associated with the production of viral VP1 protein (see Supplementary Table 5). The majority of subjects who were positive for virus RNA in ISH were negative for VP1 protein in immunohistochemistry. Overall, VP1 protein was found in 22, 20, and 22% of altogether 30 diabetic patients, 37 celiac disease patients, and 41 control subjects, respectively (Fig. 1). VP1 protein was mainly observed in the epithelial cells of the crypts. The proportion of VP1-negative subjects among all RNA-positive subjects tended to be higher among diabetic and celiac disease patients than in control subjects (78 and 86 vs. 66%).
Finally, the presence of viral RNA (ISH) was correlated with inflammation markers in the intestinal mucosa. A total of 23 type 1 diabetic patients, 40 celiac disease patients, and 41 control subjects was analyzed for the presence of CD3+, αβ+, and γδ+ intraepithelial lymphocytes and HLA-DR+ cells. Viral RNA was associated with the infiltration of αβ+, γδ+, and HLA-DR+ cells (Table 3). The presence of inflammation markers was associated with enterovirus RNA positivity in the whole study cohort, and the trend was also seen in diabetic patients in γδ IELs (46 vs. 17%, respectively) and HLA-DR expression (46 vs. 0%), but because of the low number of diabetic patients positive for inflammation markers, the results did not reach statistical significance (data not shown). The mean γδ+-to-CD3+ cell ratio was also higher in virus-positive than in virus-negative subjects (0.24 vs. 0.17; P = 0.043). Besides T-cell–mediated inflammation, antibody-mediated inflammation (IgA deposits) was more frequent in virus-positive than in virus-negative subjects (54 vs. 31%; P = 0.023) (Table 3). As expected, the inflammatory markers were most frequent in celiac disease patients in general, but diabetic patients who did not have celiac disease also had a tendency of elevated γδ+ IELs, HLA-DR expression, and IgA deposits compared with control subjects (Table 4 and Supplementary Figs. 2 and 3). They also had a higher mean γδ+-to-CD3+ cell ratio (0.18 vs. 0.09; P = 0.028). However, the inflammation was clearly much weaker in the diabetes group than in the celiac disease group.

**DISCUSSION**

The current study suggests that the intestinal mucosa of type 1 diabetic patients is frequently infected by enteroviruses. The findings fit well with viral persistence, a phenomenon commonly observed in picornavirus infections and that can lead to prolonged inflammation and tissue damage (26). This observation also confirms our earlier study showing a similar phenomenon in a smaller subset of diabetic patients and control subjects (18).

Several factors suggest that this observation may have clinical significance. First, the presence of enterovirus RNA was associated with increased inflammation activity in the gut mucosa, including both cell-mediated and antibody-mediated inflammation (accumulation of IELs and IgA deposits). Second, the virus was more frequently detected in type 1 diabetic patients than in the control subjects, suggesting that it may play a role in the disease process. Third, our findings are consistent with prolonged infection and viral persistence in gut mucosa; the virus was found in the follow-up sample taken after 1 year’s observation in all
three diabetic patients who were initially virus positive and from whom such follow-up samples were available. Viral persistence was also supported by the fact that the viral genome (RNA) occurred frequently in the absence of viral proteins, and this was seen more often in the patient group than in the control group. Such an imbalance between viral RNA and protein expression has earlier been linked to enterovirus persistence, showing that enterovirus capsid protein synthesis is decreased in persistent infection and that virus replication occurs mainly on the RNA level without producing infectious virus (15,17). An analogous finding in human endomyocardial tissue shows a presence of enteroviral RNA without production of viral VP1 protein in idiopathic dilated cardiomypathy and chronic coronary disease (27). Altogether, the current study fits well with previous observations in orally infected mice showing that enterovirus can persist in intestinal mucosa (14,28). Accordingly, the virus detected in these patients may have persisted for a longer period. However, in light of the current study, it is not possible to conclude whether it infected these individuals before or after the diagnosis of type 1 diabetes. Studies have shown that a large proportion of type 1 diabetic patients are already positive for enterovirus in blood at the diagnosis of diabetes, and even earlier during the pathogenesis, close to the appearance of islet auto-antibodies (6,7,29,30).

It is possible that individuals susceptible to type 1 diabetes are prone to enterovirus infections and their persistence. In theory, such susceptibility could be mediated by genetic factors conferring susceptibility to type 1 diabetes. It is known that some of these susceptibility genes, such as HLA and IFIH1 genes, influence immune responses against enteroviruses (9,31). However, there are no studies available on the possible role of these genes in the development of enterovirus persistence. In the current study, the detection of enterovirus RNA was not associated with HLA risk genes for type 1 diabetes, arguing against a major effect of HLA genes. In addition to genetic factors, hyperglycemia is known to predispose to infections. Therefore, we cannot exclude the possibility that diabetes itself could predispose to prolonged enterovirus infections. However, the detection of enterovirus was not associated with the duration of type 1 diabetes, which indirectly speaks against this possibility.

The intensity of staining and the number of positive cells in ISH varied between the study groups, with patients with type 1 diabetes showing significantly stronger positivity than celiac disease patients and control subjects. This suggests that the virus replicates at a higher level in diabetic subjects, producing more RNA molecules and infecting more cells. The very weak positives were relatively frequent also among disease control subjects, suggesting that a low-grade infection may have contributed to the nonspecific gastrointestinal symptoms in some of them.

Virus RNA was found using different techniques including ISH and RT-PCR assays. Even though they gave parallel results, it was evident that enterovirus positivity was more frequent in ISH than in RT-PCR. This could be explained by the fact that two separate biopsy samples were needed for these two methods, as well as by the methodological differences in the pretreatment of the samples and possible presence of PCR inhibitors, which impair the sensitivity of PCR (18).

In conclusion, we propose that persistent enterovirus infection in gut mucosa may play a role in the pathogenesis of type 1 diabetes. Gut mucosa may be an important virus reservoir from which the virus can spread to the pancreas, which is anatomically very close and has common lymphatic and vasculature networks. Also, the viral persistence in gut mucosa may facilitate autoimmunity in enterovirus-induced myocarditis through inhibiting T regulatory cell activity (32).

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M.O. designed the study, participated in the development of the methods, analyzed data, contributed to data...
interpretation, and helped prepare the manuscript. S.T. designed the study, contributed to data interpretation, and helped prepare the manuscript. S.O. and T.H. participated in the development of the methods and helped prepare the manuscript. P.C. analyzed data and helped prepare the manuscript. I.R. helped prepare the manuscript. M.M. collected samples, contributed to data interpretation, and helped prepare the manuscript. K.K. collected samples, analyzed data, contributed to data interpretation, and helped prepare the manuscript. H.H. designed the study, contributed to data interpretation, and helped prepare the manuscript. M.O. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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