Immunologic Activity in the Small Intestinal Mucosa of Pediatric Patients With Type 1 Diabetes

Mia Westerholm-Ormio,[1] Outi Vaarala,[2,3] Päivi Pihkala,[2] Jorma Ilonen,[4] and Erkki Savilahti[1]

Involvement of gut immune system has been implicated in the pathogenesis of type 1 diabetes. However, few studies have been performed on the gut mucosa from patients with type 1 diabetes. Thus, we characterized the stage of immune activation in jejunal biopsy samples from 31 children with type 1 diabetes by immunohistochemistry, in situ hybridization, and RT-PCR. We found enhanced expressions of HLA-DR, HLA-DP, and intercellular adhesion molecule-1 by immunohistochemistry even on structurally normal intestine of patients with type 1 diabetes and no signs of celiac disease. In addition, the densities of IL-1α– and IL-4–positive cells detected by immunohistochemistry and IL-4 mRNA–expressing cells evaluated by in situ hybridization were increased in the lamina propria in patients with type 1 diabetes and normal mucosa. Instead, the densities of IL-2, γ-interferon (IFN-γ), and tumor necrosis factor α–positive cells, the density of IFN-γ mRNA positive cells, and the amounts of IFN-γ mRNA detected by RT-PCR correlated with the degree of celiac disease in patients with type 1 diabetes. Our study supports the hypothesis that a link exists between the gut immune system and type 1 diabetes. *Diabetes* 52:2287–2295, 2003

Accumulating data suggest that the gut immune system plays a role in the development of type 1 diabetes (1), an autoimmune disease that results from the destruction of insulin-secreting pancreatic islet β-cells by autoreactive T-cells (2). In experimental studies, several indications of the involvement of gut immune system in the development of autoimmune diabetes have been established. First, diet modifies the development of autoimmune diabetes in BB rats and NOD mice (3–5). Second, the islet infiltrating T-cells express gut-associated homing receptor β7-integrin, and antibodies that block this receptor or its endothelial ligand mucosal addressin cell adhesion molecule-1 inhibit the development of autoimmune diabetes in NOD mice (6–8). Third, mesenterial lymphocytes from a young NOD mouse transfer autoimmune diabetes to the recipients, indicating that diabetogenic T-cells are present in the gut-associated immune system (9). Finally, feeding autoantigen may induce the development of autoreactive cytotoxic lymphocytes and acceleration of autoimmune diabetes (10,11).

In humans, a link between the gut immune system and type 1 diabetes has also been suggested: enhanced immune responses to several cow milk proteins have been reported in serologic studies on patients with newly diagnosed type 1 diabetes (12–14). We have also shown that GAD-specific T-cells in patients with type 1 diabetes express gut-associated homing receptor α4β7-integrin (15). Accordingly, T-cells derived from human diabetic pancreas have been demonstrated to show mucosal homing properties (16).

Furthermore, the association between type 1 diabetes and celiac disease (CD) has been recognized for some years (17,18), the prevalence of CD among children and adults with type 1 diabetes being as high as 2–8% (19–24). Both diseases are associated with the HLA class II alleles DQB1*0201 and DQA1*0501 (HLA-DQ2) (25), which may partly explain the association of diseases, but recently it has been proposed that long-term exposure to gluten could induce type 1 diabetes (26). In a previous study, we found markers of immune activation even in structurally normal small intestine of patients with type 1 diabetes (27). The expression of HLA class II antigens in the villous epithelium and the density of α4β7-expressing cells in the lamina propria were increased in patients when compared with control subjects. However, it is not clear how an inflammation in the gut is linked to the process that destroys islet β-cells, and to date very few studies have been performed in the small intestine of patients with type 1 diabetes.

Our aim was to characterize the immune activation in jejunal biopsy specimens from pediatric patients with type 1 diabetes. We investigated the protein expression of interleukin-1α (IL-1α), IL-2, IL-4, γ-interferon (IFN-γ), and tumor necrosis factor-α (TNF-α) in jejunal biopsies from 21 pediatric patients with type 1 diabetes. We also investigated the lymphocytes, measuring their activation as well as the expression of HLA class II antigens on the epithelium. Furthermore, in the same samples, we analyzed the mRNA expression of IL-4 and IFN-γ by in situ hybridization. The specific amounts of IL-2, IL-4, IFN-γ, TNF-α, chemokine receptor-4 (CCR-4), and CCR-5 mRNA were

From the [1]Hospital for Children and Adolescents, University of Helsinki, Helsinki, Finland; the [2]Department of Molecular Medicine, National Public Health Institute, Helsinki, Finland; the [3]Division of Pediatrics, Department of Molecular and Clinical Medicine, Faculty of Health Sciences, University of Linköping, Linköping, Sweden; and the [4]Department of Virology, University of Turku, Finland.

Address correspondence and reprint requests to Mia Westerholm-Ormio, Hospital for Children and Adolescents, Research Laboratory, Biomedicum Helsinki, B433b, P.O. Box 63, FIN-00014 University of Helsinki, Finland. E-mail address: mia.westerholm-ormio@hus.fi.

Received for publication 31 October 2002 and accepted in revised form 22 May 2003.

AEC, carbatzole; CCR, chemokine receptor; CD, celiac disease; EMA, endomysium antibodies; ICAM-1, intercellular adhesion molecule-1; IEL, intraepithelial lymphocyte; IFN-γ, γ-interferon; IL, interleukin; mAb, monoclonal antibody; TCR, T-cell receptor; TNF-α, tumor necrosis factor α; tTGA, tissue transglutaminase antibodies.

© 2003 by the American Diabetes Association.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

TABLE 1
Clinical findings of the patients with type 1 diabetes

Patients with type 1 diabetes and patient no.	Sex	Age (years)	Duration of diabetes	DQB1	DQA1	Morphology	Circulating antibodies			Methods		
							IgA-EMA*	IgA-tTGA†	Gliad-IgA/IgG‡	IHC	ISH	RT-PCR
Normal mucosa												
1	M	2.9	0.3	02/0302	05	Normal	Neg	Neg	−/−	−	−	+
2	M	4.6	1.3	x		Normal	NT	NT	−/+	−	−	+
3	F	5.2	1.8	02	03/05	Normal	Neg	Neg	−/−	−	−	+
4	M	5.4	2.1	02	03/05	Normal	Neg	Neg	+/−	+	+	+
5	F	9.0	6.0	0302	03	Normal	10	Neg	−/+	−	−	+
6	F	9.3	4.3	02/0302	0201	Normal	20	Neg	−/−	+	+	+
7	M	9.4	4.0	NT	NT	Normal	Neg	Neg	−/+	−	−	+
8	M	9.5	7.9	0301	05	Normal	Neg	Neg	+/+	+	+	+
9	F	10.2	9.0	02/0302		Normal	Neg	Neg	+/+	+	+	+
10	M	11.6	9.2	0302		Normal	Neg	Neg	+/−	−	−	+
11	M	12.1	10.4	0302/060	03	Normal	Neg	Neg	+/+	+	+	+
12	M	12.8	0.3	0302	03	Normal	Neg§	NT	−/+	−	−	+
13	F	15.6	11.6	0302		Normal	Neg	Neg	−/+	+	+	+
14	F	15.8	9.3	0602/3/4		Normal	Neg§	NT	+/−	−	−	+
15	F	18.1	7.2	02/0602	x	Normal	Neg	Neg	−/+	+	+	+
16	F	18.2	13	x	03	Normal	Neg	Neg	+/−	+	+	+
Potential CD												
17	F	6.0	0.3	02/0302	05	Normal	50	3	−/+	+	+	+
18	F	6.1	4.0	0302	03	Slight	400	NT	−/+	−	−	+
19	M	6.6	1.2	02	05	Normal	50	14	−/−	−	−	+
20	F	9.5	0.1	0302		Normal	100	16	+/+	+	+	+
21	M	10.0	2.1	02	05	Normal	50	23	−/−	−	−	+
22	M	11.5	1.2	02	05	Normal	400	45	−/−	+	+	+
23 (same as no. 31)	M	12.5	4.4	02/0302	03/05	Normal	1600	1129	−/−	+	+	+
24	M	13.2	1.4	02	03/05	Normal	400	39	−/−	+	+	+
CD												
25	F	4.1	2.4	0602		SVA	800	707	+/+	+	+	+
26	F	3.9	0.7	x		SVA	1600	NT	+/+	+	+	+
27	M	6.9	3.1	x		SVA	1600	NT	+/+	+	+	+
28	M	7.6	0.3	02	05	SVA	100	19	−/−	+	+	+
29	F	9.7	0.9	02/0302	03/05	PVA	100	33	−/−	+	+	-
30	F	10.5	6.2	02/0604	05	PVA	400	67	+/−	+	+	+
31 (same as no. 23)	M	14.6	6.5	02/0302	03/05	PVA	1600	652	+/−	+	+	+
32	F	15.8	6.5	02/4d	0201	SVA	Pos‖	NT	+/+	+	+	+

*Class IgA endomysium antibodies, upper normal limit 1:5. †Class IgA tissue transglutaminase antibodies, upper normal limit 8 arbitrary units (a.u.) (28). ‡Class IgA and IgG gliadin antibodies, classified according to Kolho et al. (29). §Negative class IgA reticuline antibodies. ‖Reticuline IgA 1:50. IHC, immunohistochemistry; ISH, in situ hybridization; NT, not tested; PVA, partial villous atrophy; RT-PCR, real-time quantitative RT-PCR; SVA, subtotal villous atrophy; x, alleles investigated not found.

evaluated by RT-PCR from the same mucosal samples and from 12 additional children with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Patients. Because of positive finding in the annual CD screening test, including the less specific positive gliadin antibodies, or because of gastrointestinal symptoms, 32 small intestinal biopsy specimens were obtained from 31 pediatric patients with type 1 diabetes. These patients were divided into three groups: patients with type 1 diabetes and normal villous structure and without markers of CD; patients with type 1 diabetes and normal villous structure and positive endomysium (EMA) or tissue transglutaminase (tTGA) IgA antibodies, designed as potential CD; and patients with type 1 diabetes and untreated CD. We considered an EMA titer of 1:50 or higher as positive, because a titer of 1:5–1:50 is the range for weak positivity (29). Partial or subtotal villous atrophy with crypt hyperplasia was defined as CD (villous crypt ratio <2).

Of the 32 type 1 diabetes jejunal specimens, 16 showed normal mucosal morphology with negative circulating CD autoantibodies (eight girls; mean age 10.6 years; range 2.9–18.2). Fourteen patients were negative for both EMA and tTGA antibodies, 2 of which were negative and 12 were positive for gliadin IgA or IgG antibodies. None of these 14 patients had had positive EMA or tTGA antibody titers before the biopsy or during follow-up after the biopsy. Two patients showed EMA titers of >1:5<1:50 at time of biopsy (Table 1, patients 5 and 6). Both of these patients had negative tTGA antibodies, a normal jejunal villous architecture, and normal values of intraepithelial lymphocyte counts, and the EMA and tTGA titers have remained negative for 2 years after the biopsy. Mean age at onset of type 1 diabetes was 4.1, and mean duration of type 1 diabetes at time of biopsy was 6.1 years in this group. Eight patients had a diagnosis of CD (five girls; mean age 9.1 years; range 3.9–15.8). Mean age at onset of type 1 diabetes was 5.8, and mean duration of type 1 diabetes at time of biopsy was 3.3 years in patients with CD. Eight patients had signs of potential CD (three girls; mean age 9.4; range 6.0–13.2); the mean age at onset of type 1 diabetes was 7.6, and mean duration of type 1 diabetes at time of biopsy was 1.8 years in this group. At the time of the biopsy, all of the patients were on a normal gluten-containing diet. From one CD patient (Table 1, patient 31), two biopsies were available. In the first one, villous structure was normal but with positive EMA and tTGA IgA antibodies; this specimen was included in the potential CD study group (Table 1, patient

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

TABLE 2
Clinical findings of the healthy control subjects

Healthy control subjects	Sex	Age (years)	Circulating antibodies			Methods		
			IgA-EMA*	IgA-tTGA†	Gliad-IgA/IgG‡	IHC	ISH	RT-PCR
1	F	1.6	Neg	Neg	Neg/Pos	+	+	−
2	M	2.6	Neg§	NT	Neg/Pos	+	−	−
3	F	3.8	NT	NT	Neg/Pos	+	+	−
4	F	4.4	Neg	Neg	Pos/Pos	+	+	+
5	M	5.0	Neg	Neg	Neg/Neg	+	+	+
6	M	6.4	Neg	Neg	Pos/Pos	+	+	−
7	F	7.0	Neg	Neg	Neg/Neg	+	+	−
8	F	8.2	NT	NT	Neg/Pos	+	+	+
9	F	9.3	Neg	Neg	Pos/Pos	+	+	+
10	M	10.1	Neg	NT	Neg/Pos	+	+	+
11	F	12.7	Neg	Neg	Neg/Pos	+	+	+
12	F	13.7	Neg§	NT	Neg/Pos	+	+	+

*Class IgA endomysium antibodies, upper normal limit 1:5. †Class IgA tissue transglutaminase antibodies, upper normal limit 8 arbitrary units (a.u.) (28). ‡Class IgA and IgG gliadin antibodies, classified according to Kolho et al. (29). §Negative class IgA reticuline antibodies. IHC, immunohistochemistry; ISH, in situ hybridization; NT, not tested.

23). The second biopsy specimen, 2.1 years later, showed partial villous atrophy. Clinical data are summarized in Table 1.

Jejunal biopsies on 12 age-matched control pediatric patients (mean age 7.1 years; range 1.6–13.7) were performed because of growth retardation, gastrointestinal symptoms, positive antigliadin antibodies, or any combination of these. The healthy control subjects had no history of CD or dermatitis herpetiformis in the family, they were not on medication, and they did not have any chronic diseases. The morphology of the jejunum was normal, and the EMA and tTGA antibodies were negative in all control children. Clinical data are summarized in Table 2.

Samples. A Watson biopsy device was used to take a specimen from the proximal jejunum or a gastroscope from the distal duodenum. The specimens were divided for routine histology and immunohistochemical studies as previously described (30). Frozen tissue samples were cut into 8-μm sections, coded, and evaluated without knowledge of the specimen. The part of the mucosal specimen still left was used for RT-PCR. Some of the patients were enrolled later in the study and were included only in the RT-PCR study (Table 1).

HLA genotyping. HLA genotyping was performed in 11 patients from peripheral venous blood sample and in 19 patients from jejunal paraffin blocks. HLA-DQB1, HLA-DQA1, and HLA-DRB1 analysis was performed by a technique developed for screening type 1 diabetes susceptibility on the basis of the presence of alleles associated with a risk for or with protection against type 1 diabetes. This two-step screening technique is based on the hybridization of relevant PCR products with lanthanide-labeled probes detected by time-resolved fluorometry (31).

Immunohistochemistry. The avidin-biotin immunoperoxidase system was used on cryostat sections as previously described (32). For immunostaining of cytokines before incubation with monoclonal antibodies (mAbs), permeabilization was performed by incubation of the slides in 0.1% PBS-Tween 20 for 10 min at room temperature and diluted in 1% normal horse sera in 0.1% PBS-Tween20 for 1 h at 37°C.

The mAbs were used at the following dilutions: CD3, 1:200 (T-cell marker; Becton-Dickinson, San Jose, CA); T-cell receptor-γδ (TCRγδ), 1:200 (anti-γδ-TCR; Endogen, Woburn, MA); TCRbF1, 1:40 (anti-αβ-TCR, clone 8A3; Endogen); HLA-DR, 1:500, and HLA-DP, 1:40 (both from Becton-Dickinson); intercellular adhesion molecule (ICAM-1), 1:1,500 (anti-intercellular adhesion molecule-1, clone VF27; Endogen); Ki-67, 1:100 (reacting with nuclear antigens in proliferating cells; DAKO, Glostrup, Denmark); IL-1α, 1:50 (clone 20B8; Biosource International, Camarillo, CA); IL-2, 1:50 (clone 7A3; Biosource International); IL-4, 1:50 (IL-4II, clone 12.1; Mabtech, Nacka, Sweden); IFN-γ, 1:50 (clone 1-D1K; Mabtech); and TNF-α, 1:50 (clone 68B6A3; Biosource International). Nonimmune mouse IgG1 (DAKO) was used as negative primary antibody control and incubation with 1% normal horse sera on additional sections as negative control for reagents in the immunoperoxidase system.

Microscopic evaluation. The numbers of positively stained cells were counted under a light microscope through a calibrated graticule at ×1,000 magnification, as described previously (27,32). In the same specimen, the positive cells in at least 30 fields either along the epithelium or comprising the lamina propria were counted, and cell densities were expressed as cells/mm or cells/mm^2, respectively.

HLA-DR and -DP were used to stain epithelial cells for HLA class II expression. Epithelial staining was graded from 2–6 according to their cellular distribution (1–3) and area of staining (1–3) (33). The proportion of the lamina propria that stained positively for ICAM-1 was estimated and graded from 1–3, 1 representing faint and 3 strong staining. Ki-67–positive cells were calculated as percentages of crypt cells, with at least 200 crypt cells counted in each specimen.

Radioactive RNA in situ hybridization. The sections were subjected to in situ hybridization for human IL-4 and IFN-γ riboprobes obtained from cDNAs described earlier (30). Tissue sections were incubated with 1.2×10^6 cpm of [^{33}P]-labeled (1,000–3,000 Ci/mmol; Amersham, Life Technologies, Arlington Heights, IL) antisense or sense riboprobe in a total volume of 80 μl after the in situ protocol, described previously (34).

Microscopic evaluation of RNA in situ hybridization. Positive IL-4 or IFN-γ mRNA–expressing cells were counted through a calibrated graticule at ×400 magnification and expressed as the number of positive cells/area of epithelium or lamina propria. A cell was considered positive when expressing seven or more positive cytoplasmic grains, which always corresponds to more than twice the background level. The lamina propria was assessed from the area immediately below the surface epithelium, excluding areas with lymphatic aggregates. For the specific probes, a minimum of two sections were prepared for each patient.

Total RNA isolation and cDNA synthesis. Total RNA was isolated from the mucosal samples with GenElute Mammalian total RNA kit (Sigma, St. Louis, MO) following the manufacturer's instructions. cDNA was synthesized using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) in reaction volume of 75 μl. The reaction mixture consisted of 1× TaqMan RT Buffer, 5.5 mmol/l $MgCl_2$, 500 μmol/l of each dNTP, 2.5 μmol/l Random Hexamer primer, 0.4 IU/μl RNase Inhibitor, and 10 ng/μl template RNA. The solution was treated with 2 IU of DNAse at 37°C for 30 min followed by 5 min of inactivation at 75°C. After addition of 25 IU of MultiScribe Reverse Transcriptase, the RT reaction was carried out at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min, and samples were stored at −20°C until used.

Real-time PCR quantification (TaqMan). Real-time quantitative PCR was performed using specific TaqMan PDAR Primer/Probes and the ABI-Prism 7700 Sequence Detection System (Applied Biosystems). TaqMan PDARs IL-2 (PN 4309882P), IL-4 (PN 4309883P), IFN-γ (PN 4309890P), TNF-α (PN 4309891P), CCR-4 (PN 4312819P), and CCR-5 (PN 4316064P) were used. For normalizing differences in sample sizes and amount of RNA degradation, PDAR for ribosomal RNA 18S (PN 4310893E) was applied as an endogenous control. A validation experiment described elsewhere (35) was performed to confirm that the amplification efficiencies of the target genes and the endogenous control were approximately equal.

The reaction mixture consisted of 1× TaqMan Universal PCR Master Mix, 1× TaqMan Primer/Probe, and 50 ng (for target gene) or 5 ng (for endogenous control) of template cDNA. PCR was performed under the following conditions: 50°C for 2 min, 95°C for 10 min; 50 cycles of 95°C for 15 s and 60°C for 1 min. Analysis of a homemade calibrator cDNA sample and controls without template was also performed on each PCR plate. Each measurement was set up in triplicate.

Quantitative analysis was done using the comparative C_T (ΔC_T) method, in

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

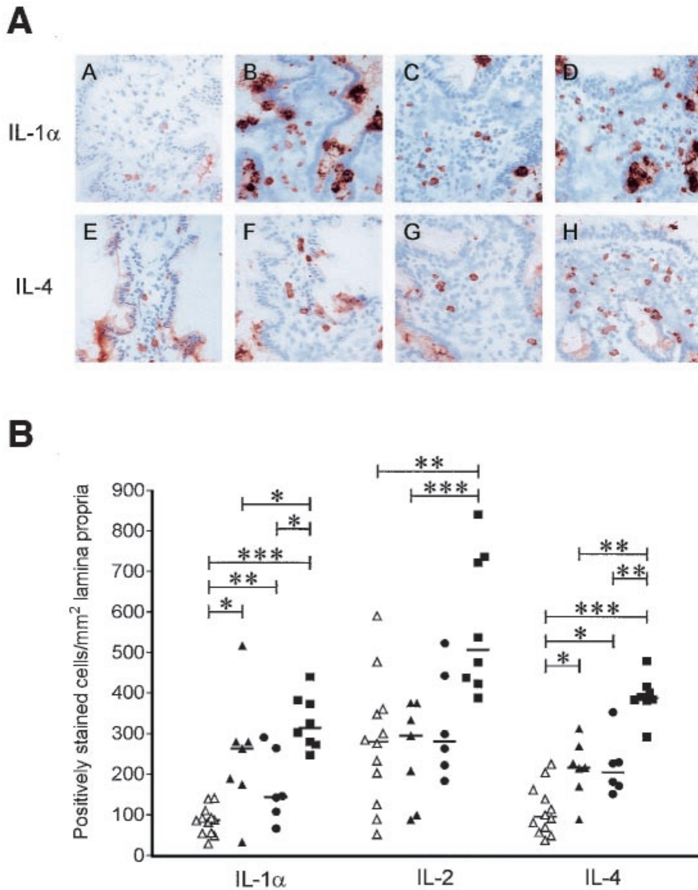

FIG. 1. ***A*****: Immunoperoxidase stainings for IL-1α (*****A–D*****) and IL-4 (*****E–H*****) in jejunal biopsy specimens from healthy control subjects (*****A*** **and** ***E*****) and from patients with type 1 diabetes and normal jejunal mucosa (*****B*** **and** ***F*****), potential CD (*****C*** **and** ***G*****), or CD (*****D*** **and** ***H*****). Positive cells are seen with typical paranuclear staining in higher densities in the type 1 diabetes specimens; also visible is some residual staining, which is easily identifiable, and except for that associated with goblet cells, clearly noncellular in its association. Carbatzole (AEC)-hematoxylin stain, magnification ×200.** ***B*****: Densities of IL-1α–, IL-2–, and IL-4–positive cells in the lamina propria detected by immunohistochemistry in jejunal specimens of healthy control subjects (△), type 1 diabetic patients with normal jejunal mucosa (▲), with potential CD (●), or with CD (■). Horizontal lines show medians for each study group. *P < 0.05, **P < 0.01, ***P < 0.001.**

which C_T value is defined as the cycle number in which the detected fluorescence exceeds the threshold value (35). ΔC_T means the difference between C_T of a target gene and the endogenous control, whereas $\Delta\Delta C_T$ is the difference between ΔC_T of the analyzed sample and the calibrator. Calculation of $2^{-\Delta\Delta CT}$ then gives a relative amount of target compared with the calibrator, both normalized to an endogenous reference (18S).

Statistical analysis. Cell densities quantified by immunohistochemistry or in situ hybridization and the specific amounts of mRNA within the four subject groups were compared by nonparametric tests (Mann-Whitney *U* test) because of the nonlinear distribution of the parameters. $P < 0.05$ was considered significant. Because of the small size of the study groups, values outside the 25–75th percentiles were considered abnormal for the group.

Ethical considerations. Specimens from pediatric patients were taken for diagnostic purposes. Use of biopsy specimens in this study was approved by the ethics committee of the Hospital for Children and Adolescents, University of Helsinki. In addition, after an oral explanation of the study plan, a written parental consent with a signature was obtained from the parents of all children.

RESULTS

HLA genotyping. The patients' type 1 diabetes–associated HLA DQB1*0201 and 0302 risk alleles are shown in Table 1. The heterodimer HLA-DQ2 associated with CD was found in 4 of 8 patients with type 1 diabetes and CD, in 6 of 8 patients with type 1 diabetes and potential CD, and in 3 of 16 patients with type 1 diabetes and normal mucosa. When the patients with type 1 diabetes were divided according to the presence of the HLA-DQ2 heterodimer, the patients carrying HLA-DQ2 showed increased densities of CD3 (median 1,688: 25–75th percentiles 1,250–2,125 vs. 961: 813–1,266; $P = 0.041$) and γδ-TCR–positive cells (469: 406–491 vs. 113: 81–176; $P < 0.0001$) in the lamina propria and γδ-TCR–positive intraepithelial lymphocytes (IELs; 46: 20–62 vs. 4.5: 1.3–33; $P = 0.023$). No other HLA-genotype association was found (data not shown).

Immunohistochemistry

Intraepithelial compartment. Densities of CD3-, γδ-TCR–, and αβ-TCR–positive cells were higher in the epithelium of patients with type 1 diabetes and CD or potential CD than in patients with type 1 diabetes and normal mucosa and in control subjects. Patients with type 1 diabetes and CD showed only higher density of CD3-positive IELs when compared with patients with type 1 diabetes and potential CD. Furthermore, intraepithelial IFN-γ–positive cells were significantly increased in patients with type 1 diabetes and CD or potential CD when compared with patients with type 1 diabetes and normal mucosa and with control subjects. As a group, patients with type 1 diabetes and normal intestinal mucosa did not differ from control subjects regarding the density of IELs (Table 3).

Lamina propria. The density of CD3-, γδ-TCR–, and αβ-TCR–positive cells in the lamina propria was higher in patients with type 1 diabetes and CD than in patients with type 1 diabetes and normal mucosa and in control subjects. These cells were also increased in patients with type 1 diabetes and potential CD compared with control subjects but not compared with patients with type 1 diabetes and normal intestine (Table 3).

The densities of IL-1α– and IL-4–positive cells in the lamina propria were greater in all three type 1 diabetes study groups than in control subjects. In addition, higher densities of IL-1α– and IL-4–positive cells could be seen in

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

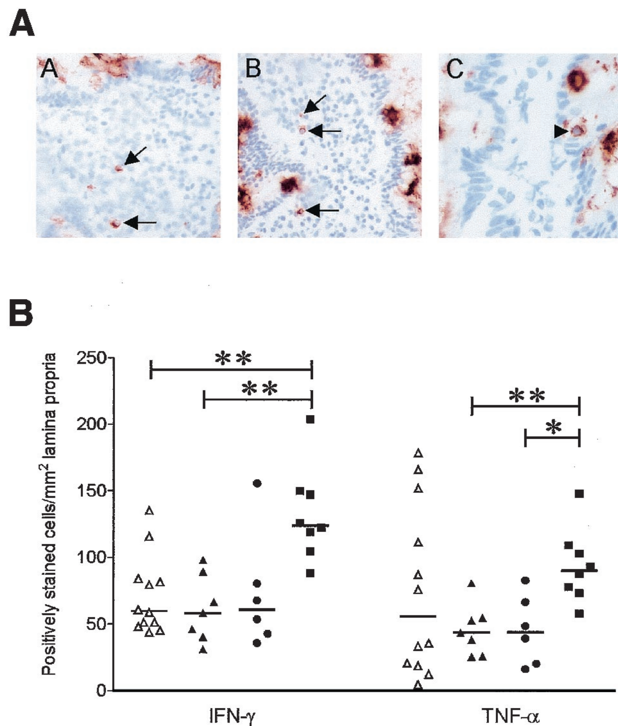

FIG. 2. Immunoperoxidase staining for IFN-γ in jejunal biopsy specimens from patients with type 1 diabetes and CD (*A*) or with potential CD (*B* and *C*). Positive cells are seen with typical paranuclear staining in the lamina propria (*A* and *B*) and are indicated by arrows. One IFN-γ–positive intraepithelial cell is seen in higher magnification in the surface epithelium of a biopsy specimen from a patients with type 1 diabetes and potential CD (indicated by arrowhead in *C*). AEC-hematoxylin stain, magnification ×200 in *A* and *B*, ×400 in *C*. *B*: Densities of IFN-γ– and TNF-α–positive cells in the lamina propria detected by immunohistochemistry in jejunal specimens of healthy control subjects (△) and patients with type 1 diabetes and normal jejunal mucosa (▲), potential CD (●), or CD (■). Horizontal lines show medians for each study group. *$P < 0.05$, **$P < 0.01$.

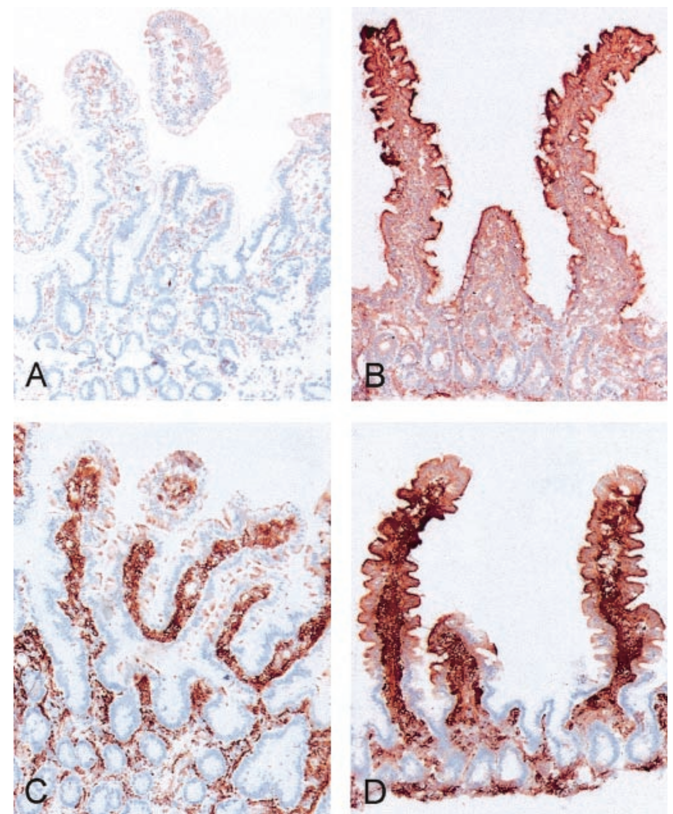

FIG. 3. Immunoperoxidase stainings for HLA-DR (*A* and *B*) and HLA-DP (*C* and *D*) in jejunal biopsy specimens from a healthy control subject (*A* and *C*) and from a patient with type 1 diabetes and normal jejunal mucosa (*B* and *D*). Intensive, positive HLA-DR staining is seen throughout the epithelial cells of the villi and also in many crypt cells in the specimen from a patients with type 1 diabetes (*B*), whereas the specimen from a control patient shows only faint HLA-DR staining in the apical parts of the epithelial cells at the tip of the villi (*A*). Biopsy specimen from a control patient who was treated with HLA-DP antibody shows scattered, positive granules in the apical parts of the epithelial cells at the tip of the villi (*C*), whereas strong positive staining is seen throughout the villous epithelial cells in the specimen from a patient with type 1 diabetes (*D*). No positive staining is seen with either HLA-DR or HLA-DP in crypt cells in specimen from control patient (*A* and *C*). AEC-hematoxylin stain, magnification ×50.

patients with type 1 diabetes and CD than in the two other type 1 diabetes study groups (Fig. 1). The densities of IL-2 and IFN-γ cells were greater in patients with type 1 diabetes and CD than in patients with type 1 diabetes and normal mucosa and in control subjects (Figs. 1*B* and 2). TNF-α–positive cells were found in higher density in patients with type 1 diabetes and CD than in patients with type 1 diabetes and potential CD or normal mucosa (Fig. 2*B*).

Staining with HLA class II antibodies and anti–ICAM-1. Strong positive staining of epithelial cells with HLA-DR, HLA-DP, and ICAM-1 was present in all patients with type 1 diabetes (medians for the three type 1 diabetes study groups, HLA-DR >5.5/6, HLA-DP >5/6, and ICAM-1 3/3). In control subjects, the staining with these antibodies showed faint to moderate positivity (medians, HLA-DR 2/6, HLA-DP 3/6, and ICAM-1 2/3), a significant difference when compared with the three type 1 diabetes study groups (HLA-DR $P < 0.003$, HLA-DP $P < 0.02$, and ICAM-1 $P < 0.03$ for all three comparisons). In addition, patients with type 1 diabetes and CD expressed stronger staining of HLA-DP than did the other two type 1 diabetes study groups ($P = 0.006$ and $P = 0.001$; Fig. 3).

Staining with Ki-67. The percentage of proliferative cells in the crypts was higher in specimens of patients with type 1 diabetes and CD or potential CD than in those from patients with type 1 diabetes and normal mucosa or from control subjects. The percentages of proliferative cells in patients with type 1 diabetes and normal mucosa did not differ from control subjects (Table 3).

Radioactive RNA in situ hybridization

Intraepithelial compartment. IL-4 mRNA–positive hybridization signal was located mainly in the lamina propria, with little or no signal in the surface epithelium. IFN-γ mRNA–expressing cells were found in the intraepithelial compartment throughout the surface and the crypt epithelium. These cells were scarcely found in the normal specimens, the median being only 1 cell/10 areas of surface epithelium. In the type 1 diabetes specimens with CD or potential CD, IFN-γ mRNA–positive IELs were higher than in normal type 1 diabetes specimens or controls. In addition, the CD specimens showed higher density of IFN-γ mRNA–positive IELs than the potential CD specimens did (Table 4, Fig. 4).

Lamina propria. Mononuclear cells expressing IL-4 mRNA were present in all the jejunal samples. The density of IL-4 mRNA–expressing cells was greater in patients with type 1 diabetes and CD or potential CD than in

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

TABLE 3

Densities of IFN-γ–, CD3-, TCR γ/δ–, and TCR α/β–positive cells in the surface epithelium (cells/mm) and of CD3-, TCRγ/δ–, and TCRα/β–positive cells in the lamina propria (cells/mm^2) in the jejunal mucosa of patients with type 1 diabetes and normal mucosa, potential CD, or CD, and of control subjects

Patients with type 1 diabetes and:	Cells in the surface epithelium				Cells in the lamina propria			% of dividing crypt cells
	IFN-γ	CD3	TCR γ/δ	TCR α/β	CD3	TCR γ/δ	TCR α/β	Ki-67
Normal mucosa (n = 8)	1.4 (1.0–1.8)	9.1 (6.0–15.6)	1.6 (1.2–2.8)	4.9 (3.0–9.5)	879 (704–1143)	92 (70–195)	645 (471–796)	15 (11–19)
Potential CD (n = 5)	5.4 (3.7–5.4)*	96 (69–115)*	20 (6.0–31)*	46 (45–67)†	1250 (893–1688)‡	406 (144–491)‡	938 (750–1420)‡	30 (29–40)†
CD (n = 8)	5.5 (5.0–6.8)*	175 (154–182)*§	42 (37–59)*	110 (91–130)*	1567 (1237–2135)†	322 (173–469)†	1239 (1075–1312)†	36 (29–39)*
Controls (n = 12)	1.2 (1.0–1.6)	9.4 (5.3–12.7)	1.4 (1.0–2.3)	4.2 (3.3–7.7)	600 (522–757)	56 (44–86)	542 (488–625)	16 (7.4–26)

Data are percentages of proliferating crypt cells (Ki-67), medians (25–75th percentile). *$P < 0.01$ vs. patients with type 1 diabetes and normal mucosa and control subjects. †$P < 0.03$ vs. patients with type 1 diabetes and normal mucosa and control subjects. ‡$P < 0.05$ vs. control subjects. §$P < 0.02$ vs. patients with type 1 diabetes and potential CD.

patients with type 1 diabetes and normal mucosa and control subjects. In addition, IL-4 mRNA–positive cells were increased in patients with type 1 diabetes and normal mucosa compared with control subjects.

In situ hybridization with the antisense probe for IFN-γ showed that positively expressed cells were found mainly in the superficial lamina propria and occasionally in the deeper parts. Densities of IFN-γ mRNA–expressing cells in the lamina propria were greater in all type 1 diabetes groups than in control subjects. However, among patients with type 1 diabetes, IFN-γ mRNA positive cells were 5.0 and 2.7 times more abundant in patients with type 1 diabetes and CD or potential CD than in those with normal intestine. Furthermore, patients with type 1 diabetes and CD showed increased densities of IFN-γ mRNA–positive cells compared with the other two type 1 diabetes study groups. The difference between patients with type 1 diabetes and potential CD and patients with type 1 diabetes and normal mucosa did not reach statistical significance (Table 4, Fig. 4). All sections hybridized with the sense probes showed only background signals.

RT-PCR. Signals of IL-2, IFN-γ, TNF-α (data not shown), CCR-4, and CCR-5 mRNA were detectable by RT-PCR in all jejunal specimens. IL-4 mRNA was not detectable in the controls, whereas it was detected in 3 of 16 of the type 1 diabetes specimens with normal mucosa, in 3 of 8 potential CD, and in 5 of 8 of the patients with type 1 diabetes and CD. The amount of IFN-γ mRNA was greater in patients with type 1 diabetes and CD or potential CD compared with patients with type 1 diabetes and normal mucosa and with control subjects, correlating with the degree of CD. In addition, CCR-4 was expressed in higher amounts in patients with type 1 diabetes and CD than in patients with type 1 diabetes and normal jejunal mucosa (Fig. 5).

DISCUSSION

We found that the small intestine in pediatric patients with type 1 diabetes shows enhanced immune activation. The increased expression of MHC class II antigens and ICAM-1 detected by immunohistochemistry even in patients with structurally normal intestine confirms our earlier findings (27). The expression of HLA-DR and -DP was expanded throughout the villous surface and crypts in addition to the normal expression seen only on the upper villi. In addition, we found a higher density of IL-1α– and IL-4–positive cells in the lamina propria in patients with type 1 diabetes than in control subjects, irrespective of the morphology of the intestine. The findings were not restricted to patients carrying the CD associated HLA-DQB1*0201/HLA-DQA1*0501 risk alleles, suggesting that activation of the gut immune system may be associated with type 1 diabetes and not only with the genetic risk allele shared with CD.

It is interesting that in evaluation by in situ hybridization, IL-4 mRNA expression was higher in type 1 diabetes groups than in control subjects, and IL-4 mRNA was detected by RT-PCR in some biopsy samples from patients with type 1 diabetes but in none of the control subjects. IL-4 is mainly known as the Th2 driving cytokine. IL-4 is spontaneously secreted by gut-derived immune cells, and it enhances epithelial permeability, which is suggested to be increased in type 1 diabetes (36,37). On the other hand,

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

TABLE 4
Radioactive in situ hybridization in the mucosal samples

Patients with type 1 diabetes and:	Positive cells in the epithelium (cells/area)	Positive cells in the lamina propria (cells/area)	
	IFN-γ	IFN-γ	IL-4
Normal mucosa (n = 8)	0.17 (0.11–0.29)	0.37 (0.27–0.58)*	0.44 (0.40–0.48)*
Potential CD (n = 5)	0.69 (0.59–0.93)†	1.01 (1.0–1.16)*	0.48 (0.48–0.67)†
CD (n = 8)	2.37 (1.61–2.81)‡§	1.88 (1.43–2.08)‡§	0.87 (0.67–0.95)†
Controls (n = 12)	0.10 (0.09–0.13)	0.12 (0.11–0.13)	0.11 (0.09–0.16)

Data for study groups are medians (interquartile ranges). *$P < 0.01$ vs. control subjects. †$P < 0.05$ vs. patients with type 1 diabetes and normal mucosa and control subjects. ‡$P < 0.001$ vs. patients with type 1 diabetes and normal mucosa and control subjects. §$P < 0.05$ vs. patients with type 1 diabetes and potential CD.

IL-4 has also been shown to be critical for the maturation of dendritic cells and for the upregulation of antigen uptake and presentation by macrophages (38). Recently, IL-4 was shown to trigger type 1 diabetes by activating the autoimmune BDC2.5 T-cells in the pancreas of ins-IL-4/BDC2.5 transgenic mice. It was postulated that IL-4 triggers self-antigen presentation within the pancreatic islets by enhancing the antigen uptake in macrophages (39). In CD, Maiuri et al. (40) demonstrated during an in vitro gliadin challenge of cultured intestinal specimens a rapid release of IL-4 from degranulated mast cells, followed later by production of Th1-type cytokines. As we did not perform double-staining methods, we can only hypothesize on the origin of IL-4–positive cells. On the basis of the size of their cytoplasm, however, some of the IL-4 mRNA–positive cells detected by in situ hybridization showed resemblance of macrophages.

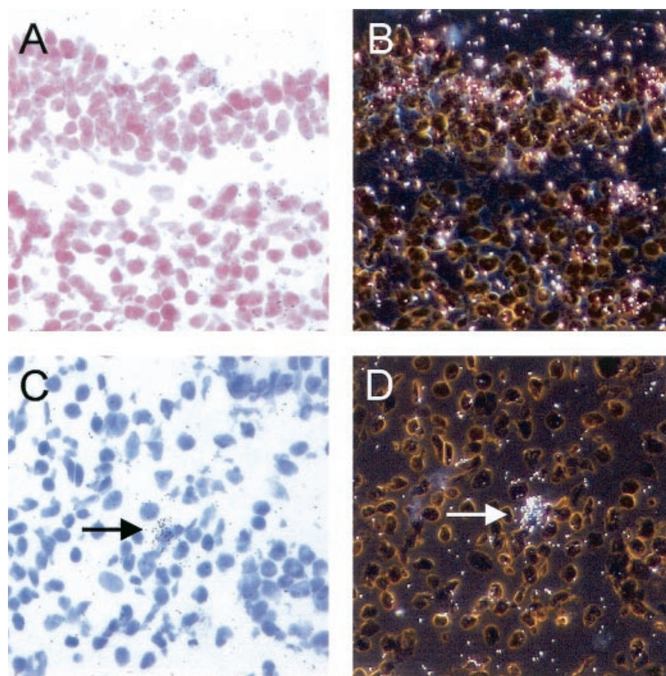

FIG. 4. Radioactive in situ hybridization of IFN-γ and IL-4 mRNA in jejunal biopsies; bright (*A* and *C*) and dark (*B* and *D*) field views. Hybridization with IFN-γ in a specimen from a patient with type 1 diabetes and CD (*A* and *B*) shows mRNA-expressing cells in the epithelium and in the lamina propria. Hybridization with IL-4 (*C* and *D*) shows a strongly positive cell in the lamina propria of a biopsy specimen from a patient with type 1 diabetes and normal jejunal mucosa. Arrows indicate the strongly IL-4–positive cell, morphologically resembling macrophages (*C* and *D*). Magnification ×400.

IL-1α is a proinflammatory interleukin secreted by monocyte lineage of immunologic cells and additionally by nonimmunologic cells such as epithelial cells. It has been revealed to participate in the gastrointestinal inflammatory process (41,42). IL-1 contributes to the regulation of dendritic cells and hence in antigen presentation. The increased densities of IL-1α–positive cells in all patients with type 1 diabetes may reflect the inflammation process activated at the level of innate immune system in the intestine of patients with type 1 diabetes.

Increased densities of IFN-γ mRNA–positive cells in lamina propria were also detected by in situ hybridization in patients with type 1 diabetes and normal mucosa, but this increased IFN-γ activation was not confirmed by the other methods used. In the gut epithelium, activation of HLA class II antigens and ICAM-1 was found, indicating enhanced capacity of antigen presentation. Furthermore, our results suggest that lymphocyte activation in the small intestine of patients with type 1 diabetes and no CD is found in the lamina propria and includes activation of Th2 immune cells. Studies performed in NOD mice and in patients with type 1 diabetes suggest that the islet-infiltrating, autoreactive cells express gut-associated homing receptor (6–9,15). Thus, the lymphocytes may recirculate between the gut and pancreas. The circulating mono-

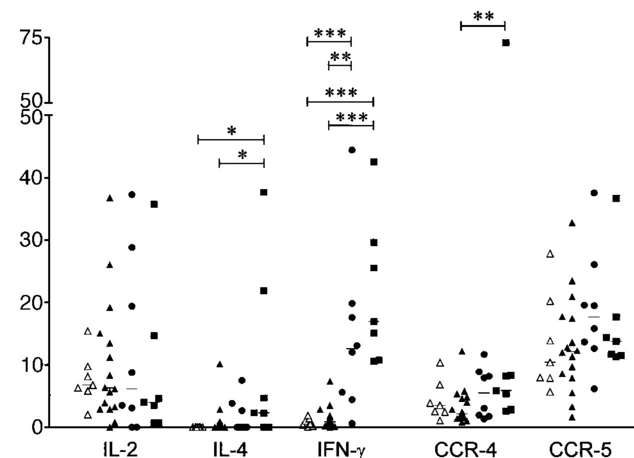

FIG. 5. Cytokine and chemokine receptor–specific mRNA detected by RT-PCR in jejunal specimens of healthy control subjects (△; n = 7) and in patients with type 1 diabetes and normal jejunal mucosa (▲; n = 16), potential CD (● n = 8), or CD (■; n = 7). Individual results are shown as relative amount ($2^{-\Delta\Delta CT}$) of target compared with the calibrator, both normalized to an endogenous reference (18S). Horizontal lines show medians for each study group. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

nuclear cells of the gut immune system home to lamina propria, whereas intraepithelial lymphocytes expressing α4bϵ receptor do not normally escape to circulation. Transfer experiments in the NOD mice model suggest that the diabetogenic immune cells are activated in the mesenterial immune system already before the infiltration of pancreatic islets, emphasizing the primary role of the gut immune system in type 1 diabetes (9). Dietary prevention of autoimmune diabetes in animal models suggests that changes in the gut immune system may induce the autoimmune destruction of β-cells in pancreas (4,5). Our findings of immune aberrancies in the gut suggest poor development of oral tolerance in children who are prone to type 1 diabetes (12–14).

The increased densities of IFN-γ–, TNF-α–, and IL-2–positive cells and amount of IFN-γ mRNA detected by in situ hybridization and RT-PCR correlated with either full-blown or potent CD than that of type 1 diabetes, which is in agreement with previous findings (43–46). This was also the case for the densities of IELs. T-cells in the celiac mucosa have been reported to represent for the most part Th1 cells producing IFN-γ (47), and in this respect, patients with type 1 diabetes and CD did not differ from patients with CD. However, in this study, we have also demonstrated an increase in IFN-γ–positive cells in potential CD as previously described (30). Enhanced Th1 cytokine responses in the gut may cause gut inflammation and damage, and high expression of Th1 cytokines is associated with increased permeability in patients with CD, Crohn's disease, and other chronic gut inflammatory conditions (48).

The CD-associated HLA-DQ2 heterodimer is found in at least 90% of white patients with CD. Surprisingly, only four of eight of our patients with type 1 diabetes and CD expressed this heterodimer; two of them also expressed DR4. Shanahan et al. (49) found no DR4-positive subjects among 17 cases of type 1 diabetes and CD, whereas in our previous study, one of seven patients with type 1 diabetes and CD was positive for DR4 (19). This finding may be explained by the enrichment of DR4 among the Finnish type 1 diabetes population. Of the patients with type 1 diabetes and potential CD, six of eight expressed HLA-DQ2 (one patient of which is shared with the CD group). The follow-up time since the intestinal biopsies for the patients with potential CD is now in average 3.1 years (range 1.3–5.5), during which time only the patient who was already included in the type 1 diabetes patient group with CD has developed overt CD.

As a sign of enhanced immune activation, we found increased expressions of HLA-DR, HLA-DP, and ICAM-1 even on structurally normal intestine of patients with type 1 diabetes when compared with control subjects. Interestingly, patients with type 1 diabetes showed increased densities of IL-1α– and IL-4–positive cells in the lamina propria, whereas the densities of IL-2, IFN-γ, and TNF-α were associated with the degree of CD. The findings were not restricted to CD-associated HLA-DQ2 risk alleles in type 1 diabetes. Our study supports the hypothesis that a link exists between the gut immune system and type 1 diabetes.

ACKNOWLEDGMENTS

This study was financially supported by the Academy of Finland, the Finnish Medical Association, the Foundation of the Friends of the University Children's Hospitals in Finland, the Sigrid Jusélius Foundation, and the Juvenile Diabetes Research Foundation.

We are indebted to Ilkka Julkunen, MD, PhD, National Public Health Institute, Helsinki, for the kind gift of IL-4 and IFN-γ cDNAs and to Prof. Markku Heikinheimo and Ilkka Ketola, MD, Hospital for Children and Adolescents, University of Helsinki, for their expertise with radioactive RNA in situ hybridization. The skillful technical assistance of Sirkku Kristiansen and Harry Lybeck is acknowledged.

REFERENCES

1. Vaarala O: Gut and the induction of immune tolerance in type 1 diabetes. *Diabetes Metab Res Rev* 15:353–361, 1999
2. Atkinson MA, Maclaren NK: The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 331:1428–1436, 1994
3. Elliott RB, Reddy SN, Bibby NJ, Kida K: Dietary prevention of diabetes in the non-obese diabetic mouse. *Diabetologia* 31:62–64, 1988
4. Scott FW, Cloutier HE, Kleemann R, Wöerz-Pagenstert U, Rowsell P, Modler HW, Kolb H: Potential mechanisms by which certain foods promote or inhibit the development of spontaneous diabetes in BB rats: dose, timing, early effect on islet area, and switch in infiltrate from Th1 to Th2 cells. *Diabetes* 46:589–598, 1997
5. Scott FW, Rowsell P, Wang GS, Burghardt K, Kolb H, Flohé S: Oral exposure to diabetes-promoting food or immunomodulators in neonates alters gut cytokines and diabetes. *Diabetes* 51:73–78, 2002
6. Yang XD, Michie SA, Tisch R, Karin N, Steinman L, McDevitt HO: A predominant role of integrin alpha 4 in the spontaneous development of autoimmune diabetes in nonobese diabetic mice. *Proc Natl Acad Sci U S A* 91:12604–12608, 1994
7. Hänninen A, Salmi M, Simell O, Jalkanen S: Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior. *Diabetes* 45:1173–1180, 1996
8. Yang XD, Sytwu HK, McDevitt HO, Michie SA: Involvement of beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in the development of diabetes in obese diabetic mice. *Diabetes* 46:1542–1547, 1997
9. Hänninen A, Jaakkola I, Jalkanen S: Mucosal addressin is required for the development of diabetes in nonobese diabetic mice. *J Immunol* 160:6018–6025, 1998
10. Blanas E, Carbone FR, Allison J, Miller JF, Heath WR: Induction of autoimmune diabetes by oral administration of autoantigen. *Science* 274:1707–1709, 1996
11. Bellmann K, Kolb H, Rastegar S, Jee P, Scott FW: Potential risk of oral insulin with adjuvant for the prevention of type I diabetes: a protocol effective in NOD mice may exacerbate disease in BB rats. *Diabetologia* 41:844–847, 1998
12. Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Åkerblom HK, Dosch HM: A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. *N Engl J Med* 327:302–307, 1992 (published erratum appears in *N Engl J Med* 327:1252, 1992)
13. Vaarala O, Klemetti P, Savilahti E, Reijonen H, Ilonen J, Åkerblom HK: Cellular immune response to cow's milk beta-lactoglobulin in patients with newly diagnosed IDDM. *Diabetes* 45:178–182, 1996
14. Cavallo MG, Fava D, Monetini L, Barone F, Pozzilli P: Cell-mediated immune response to beta casein in recent-onset insulin-dependent diabetes: implications for disease pathogenesis. *Lancet* 348:926–928, 1996
15. Paronen J, Klemetti P, Kantele JM, Savilahti E, Perheentupa J, Åkerblom HK, Vaarala O: Glutamate decarboxylase-reactive peripheral blood lymphocytes from patients with IDDM express gut-specific homing receptor α4β7-integrin. *Diabetes* 46:583–588, 1997
16. Hänninen A, Jalkanen S, Salmi M, Toikkanen S, Nikolakaros G, Simell O: Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. *J Clin Invest* 90:1901–1910, 1992
17. Savilahti E, Simell O, Koskimies S, Rilva A, Åkerblom HK: Celiac disease in insulin-dependent diabetes mellitus. *J Pediatr* 108:690–693, 1986
18. Cronin CC, Shanahan F: Insulin-dependent diabetes mellitus and coeliac disease. *Lancet* 349:1096–1097, 1997

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023

19. Saukkonen T, Savilahti E, Reijonen H, Ilonen J, Tuomilehto-Wolf E, Åkerblom HK: Coeliac disease: frequent occurrence after clinical onset of insulin-dependent diabetes mellitus. Childhood Diabetes in Finland Study Group. *Diabet Med* 13:464–470, 1996
20. Carlsson AK, Axelsson IE, Borulf SK, Bredberg AC, Lindberg BA, Sjöberg KG, Ivarsson SA: Prevalence of IgA-antiendomysium and IgA-antigliadin autoantibodies at diagnosis of insulin-dependent diabetes mellitus in Swedish children and adolescents. *Pediatrics* 103:1248–1252, 1999
21. Hansen D, Bennedbæk FN, Hansen LK, Høier-Madsen M, Hegedüs LS, Jacobsen BB, Husby S: High prevalence of coeliac disease in Danish children with type I diabetes mellitus. *Acta Paediatr* 90:1238–1243, 2001
22. Barera G, Bonfanti R, Viscardi M, Bazzigaluppi E, Calori G, Meschi F, Bianchi C, Chiumello G: Occurrence of celiac disease after onset of type 1 diabetes: a 6-year prospective longitudinal study. *Pediatrics* 109:833–838, 2002
23. Aktay AN, Lee PC, Kumar V, Parton E, Wyatt DT, Werlin SL: The prevalence and clinical characteristics of celiac disease in juvenile diabetes in Wisconsin. *J Pediatr Gastroenterol Nutr* 33:462–465, 2001
24. Rensch MJ, Merenich JA, Lieberman M, Long BD, Davis DR, McNally PR: Gluten-sensitive enteropathy in patients with insulin-dependent diabetes mellitus. *Ann Intern Med* 124:564–567, 1996
25. Sollid LM, Thorsby E: HLA susceptibility genes in celiac disease: genetic mapping and role in pathogenesis. *Gastroenterology* 105:910–922, 1993 (published erratum appears in *Gastroenterology* 106:1133, 1994)
26. Ventura A, Magazzù G, Greco L: Duration of exposure to gluten and risk for autoimmune disorders in patients with celiac disease. SIGEP Study Group for Autoimmune Disorders in Celiac Disease. *Gastroenterology* 117:297–303, 1999
27. Savilahti E, Örmälä T, Saukkonen T, Sandini-Pohjavuori U, Kantele JM, Arato A, Ilonen J, Åkerblom HK: Jejuna of patients with insulin-dependent diabetes mellitus (IDDM) show signs of immune activation. *Clin Exp Immunol* 116:70–77, 1999
28. Stern M: Comparative evaluation of serologic tests for celiac disease: a European initiative toward standardization. Working Group on Serologic Screening for Celiac Disease. *J Pediatr Gastroenterol Nutr* 31:513–519, 2000
29. Kolho KL, Savilahti E: IgA endomysium antibodies on human umbilical cord: an excellent diagnostic tool for celiac disease in childhood. *J Pediatr Gastroenterol Nutr* 24:563–567, 1997
30. Westerholm-Ormio M, Garioch J, Ketola I, Savilahti E: Inflammatory cytokines in small intestinal mucosa of patients with potential coeliac disease. *Clin Exp Immunol* 128:94–101, 2002
31. Nejentsev S, Sjöroos M, Soukka T, Knip M, Simell O, Lövgren T, Ilonen J: Population-based genetic screening for the estimation of type 1 diabetes mellitus risk in Finland: selective genotyping of markers in the HLA-DQB1, HLA-DQA1 and HLA-DRB1 loci. *Diabet Med* 16:985–992, 1999
32. Savilahti E, Örmälä T, Arato A, Hacsek G, Holm K, Klemola T, Nemeth A, Mäki M, Reunala T: Density of gamma/delta+ T cells in the jejunal epithelium of patients with coeliac disease and dermatitis herpetiformis is increased with age. *Clin Exp Immunol* 109:464–467, 1997
33. Klemola T, Savilahti E, Arato A, Örmälä T, Partanen J, Eland C, Koskimies S: Immunohistochemical findings in jejunal specimens from patients with IgA deficiency. *Gut* 37:519–523, 1995
34. Heikinheimo M, Ermolaeva M, Bielinska M, Rahman NA, Narita N, Huhtaniemi IT, Tapanainen JS, Wilson DB: Expression and hormonal regulation of transcription factors GATA-4 and GATA-6 in the mouse ovary. *Endocrinology* 138:3505–3514, 1997
35. Heid CA, Stevens J, Livak KJ, Williams PM: Real time quantitative PCR. *Genome Res* 6:986–994, 1996
36. Carol M, Lambrechts A, Van Gossum A, Libin M, Goldman M, Mascart-Lemone F: Spontaneous secretion of interferon gamma and interleukin 4 by human intraepithelial and lamina propria gut lymphocytes. *Gut* 42:643–649, 1998
37. Di Leo V, Yang PC, Berin MC, Perdue MH: Factors regulating the effect of IL-4 on intestinal epithelial barrier function. *Int Arch Allergy Immunol* 129:219–227, 2002
38. Kodelja V, Müller C, Politz O, Hakij N, Orfanos CE, Goerdt S: Alternative macrophage activation-associated CC-chemokine-1, a novel structural homologue of macrophage inflammatory protein-1 alpha with a Th2-associated expression pattern. *J Immunol* 160:1411–1418, 1998
39. Falcone M, Yeung B, Tucker L, Rodriguez E, Krahl T, Sarvetnick N: IL-4 triggers autoimmune diabetes by increasing self-antigen presentation within the pancreatic Islets. *Clin Immunol* 98:190–199, 2001
40. Maiuri L, Picarelli A, Boirivant M, Coletta S, Mazzilli MC, De Vincenzi M, Londei M, Auricchio S: Definition of the initial immunologic modifications upon in vitro gliadin challenge in the small intestine of celiac patients. *Gastroenterology* 110:1368–1378, 1996
41. Sartor RB: Cytokines in intestinal inflammation: pathophysiological and clinical considerations. *Gastroenterology* 106:533–539, 1994
42. Youngman KR, Simon PL, West GA, Cominelli F, Rachmilewitz D, Klein JS, Fiocchi C: Localization of intestinal interleukin 1 activity and protein and gene expression to lamina propria cells. *Gastroenterology* 104:749–758, 1993
43. Kontakou M, Przemioslo RT, Sturgess RP, Limb GA, Ellis HJ, Day P, Ciclitira PJ: Cytokine mRNA expression in the mucosa of treated coeliac patients after wheat peptide challenge. *Gut* 37:52–57, 1995
44. Kontakou M, Sturgess RP, Przemioslo RT, Limb GA, Nelufer JM, Ciclitira PJ: Detection of interferon gamma mRNA in the mucosa of patients with coeliac disease by in situ hybridisation. *Gut* 35:1037–1041, 1994
45. Przemioslo RT, Lundin KE, Sollid LM, Nelufer J, Ciclitira PJ: Histological changes in small bowel mucosa induced by gliadin sensitive T lymphocytes can be blocked by anti-interferon gamma antibody. *Gut* 36:874–879, 1995
46. Troncone R, Gianfrani C, Mazzarella G, Greco L, Guardiola J, Auricchio S, De Berardinis P: Majority of gliadin-specific T-cell clones from celiac small intestinal mucosa produce interferon-gamma and interleukin-4. *Dig Dis Sci* 43:156–161, 1998
47. Nilsen EM, Lundin KE, Krajci P, Scott H, Sollid LM, Brandtzaeg P: Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon gamma. *Gut* 37:766–776, 1995
48. MacDonald TT, Bajaj-Elliott M, Pender SL: T cells orchestrate intestinal mucosal shape and integrity. *Immunol Today* 20:505–510, 1999
49. Shanahan F, McKenna R, McCarthy CF, Drury MI: Coeliac disease and diabetes mellitus: a study of 24 patients with HLA typing. *Q J Med* 51:329–335, 1982

Downloaded from http://diabetesjournals.org/diabetes/article-pdf/52/9/2287/650116/db0903002287.pdf by guest on 03 December 2023