In Vitro–Deranged Intestinal Immune Response to Gliadin in Type 1 Diabetes

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Dietary gluten has been associated with an increased risk of type 1 diabetes. We have evaluated inflammation and the mucosal immune response to gliadin in the jejunum of patients with type 1 diabetes. Small intestinal biopsies from 17 children with type 1 diabetes without serological markers of celiac disease and from 50 age-matched control subjects were examined by immunohistochemistry. In addition, biopsies from 12 type 1 diabetic patients and 8 control subjects were cultured with gliadin or ovalbumin peptic-tryptic digest and examined for epithelial infiltration and lamina propria T-cell activation. The density of intraepithelial $CD3^+$ and $\gamma\delta^+$ cells and of lamina propria $CD25^+$ mononuclear cells was higher in jejunal biopsies from type 1 diabetic patients versus control subjects. In the patients' biopsies cultured with peptic-tryptic gliadin, there was epithelial infiltration by $CD3^+$ cells, a significant increase in lamina propria $CD25^+$ and $CD80^+$ cells and enhanced expression of lamina propria CD54 and crypt HLA-DR. No such phenomena were observed in control subjects, even those with celiac disease–associated HLA haplotypes. In conclusion, signs of mucosal inflammation were present in jejunal biopsies from type 1 diabetic patients, and organ culture studies indicate a deranged mucosal immune response to gliadin. *Diabetes* 53: 1680–1683, 2004

Type 1 diabetes is considered a T-cell–mediated autoimmune disease in which T-cells activated against insulin, glutamate decarboxylase, and other islet antigens infiltrate the pancreas and destroy insulin-producing β-cells (1). An increased risk of type 1 diabetes has been associated with exposure to such environmental factors as infections and dietary proteins. This implicates the gut immune system in the pathogenesis of type 1 diabetes (2). In experimental and human diabetes, the tissue-specific homing properties of lymphocytes provide the immunological link between the gut and the inflamed pancreatic islets (3–5). An inflammatory state has been demonstrated in the structurally normal intestine of patients with type 1 diabetes (6,7), and the abnormal intestinal permeability found in these patients is further evidence that diabetes is of intestinal origin (8).

Dietary gluten has been advocated as a risk factor for type 1 diabetes. Exposure to soy proteins and wheat gluten seems to modify the incidence of diabetes in biobreeding (BB) rats (9) and nonobese diabetic (NOD) mice (10). Patients with type 1 diabetes are at a high risk of celiac disease (CD) (11), and the risk seems to be correlated with the duration of gluten exposure (12). Cellular immune responses to gluten were enhanced in peripheral blood mononuclear cells of type 1 diabetic patients (13). More recently, we demonstrated that ~20% of type 1 diabetic children react to rectal instillation of gliadin with a significant increment of lamina propria and epithelium $CD3^+$ and $\gamma\delta^+$ cells (14).

The aims of this study were as follows: to look for signs of inflammation in the small intestinal mucosa of type 1 diabetic patients who did not show serological signs of CD, to look for immunohistochemical evidence of activated mucosal cell-mediated immunity, and to investigate the in vitro intestinal immune response to gliadin using an established organ culture system (15).

RESEARCH DESIGN AND METHODS

We studied 17 type 1 diabetic patients who were on a gluten-containing diet (13 males, 4 females, median age 13 years, range 8–16); 50 age-matched individuals served as control subjects. The diagnoses in the control subjects were iron deficiency anemia, failure to thrive, gastroesophageal reflux, short stature, and recurrent abdominal pain. All patients and control subjects were negative for anti-endomysial and anti-human tissue transglutaminase antibodies.

Jejunal biopsy and immunohistochemical analysis. Jejunal biopsies were obtained with a Watson biopsy device or a gastroscope. Each biopsy specimen was sliced into two parts. One part was fixed in 10% formalin, embedded in paraffin wax, cut into 5-μm thick sections, and stained with hematoxylin-eosin. The other part was immediately embedded in an optimal cutting temperature compound (BioOptica) and stored at −80°C. For immunohistochemistry, biopsy specimens were sectioned into 4-μm slices, which were fixed in acetone for 10 min. After a 20-min preincubation with normal rabbit serum (1:200, Dako), sections were incubated for 1 h with anti-CD3 (1:200; Dako), anti-CD25 (1:20; Dako), anti-TCRγδ (1:80; Thema), anti–HLA-DR (1:100; Dako), anti-CD54 (1:200; Dako), or anti-CD80 B7-1 (BB1) (Pharmingen) monoclonal antibodies and then exposed to rabbit anti-mouse immunoglobulin for 30 min. Monoclonal antibodies were diluted in Tris, pH 7.4; all incubations were carried out at room temperature in a humidity chamber. As a negative control, mouse IgG1 (1:100 Dako) was used instead of a primary antibody. After washing with Tris, pH 7.4, the sections were layered with monoclonal mouse PAP (peroxidase antiperoxidase) (Dako) or monoclonal mouse APAAP (alkaline phosphatase antialkaline phosphatase) (Dako) for 30 min. We used 2-amino-9-ethyl-carbazole (Sigma) or New Fuchsin for the peroxidase and alkaline phosphatase reactions, respectively. Sections were counterstained with Mayer's hematoxylin and mounted with Aquamount (BDH Laboratory Supplies, Poole, U.K.). Costaining experiments were set up to detect activated T-cells. Cryosections were fixed in acetone and preincu-

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CD, celiac disease.

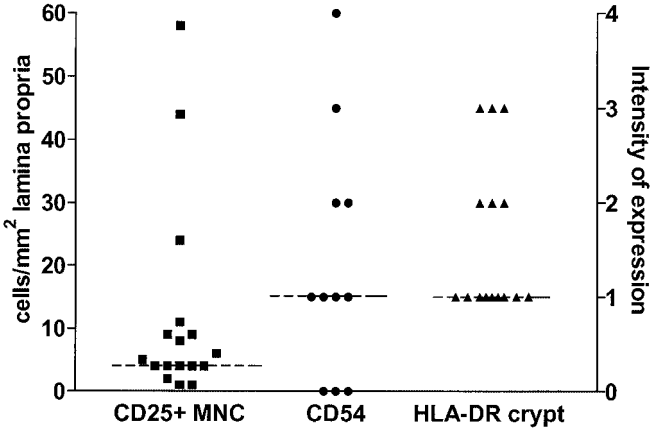

FIG. 1. Immunohistochemical analysis of jejunal biopsy from type 1 diabetic patients: number of lamina propria CD25$^+$ mononuclear cells (MNC), expression of lamina propria CD54 (intracellular adhesion molecule 1), and crypt enterocyte HLA-DR. The dotted line indicates the 90th percentile of the control group.

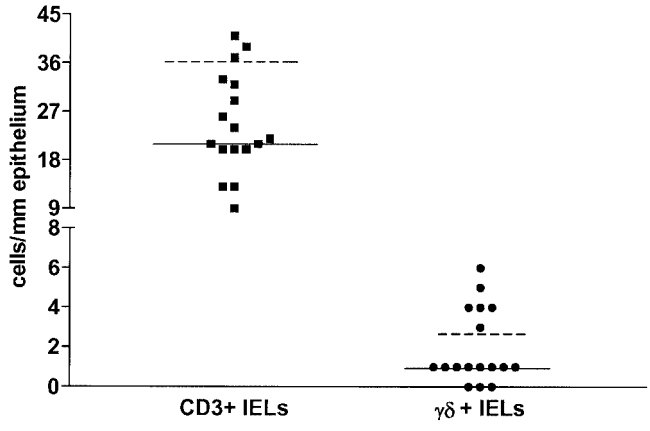

FIG. 2. Immunohistochemical analysis of jejunal biopsy from type 1 diabetic patients: CD3$^+$ and γδ$^+$ intraepithelial lymphocytes (IELs). The continuous line indicates the median of type 1 diabetic patients; the dotted line indicates the 90th percentile of the control group.

bated with a mixture of goat and swine normal serum (1:100; Dako). The sections were first incubated with mouse monoclonal antibody anti-CD25 (1:25; Dako) and rabbit polyclonal antibody anti-CD3 (1:100; Dako) and then with a mixture of swine anti-rabbit IgG conjugated to fluorescence in situ hybridization (1:30; Dako) and goat anti-mouse IgG conjugated to Texas Red (1:100; Molecular Probes). Finally, the sections were mounted in glycerol/PBS (1:10). The immunofluorescence of the three colors was observed with an Axioscope2 microscope (C. Zeiss) linked to an analysis image system (Siemens).

Organ culture studies. Additional biopsy fragments were obtained from 12 type 1 diabetic patients and 8 control subjects for organ culture studies. The fragments were placed on a stainless steel mesh positioned over the central well of an organ culture dish with the villous surface of the specimens uppermost. They were cultured for 24 h with medium alone, with the peptic-tryptic digest of gliadin from wheat bread (1 mg/ml), obtained as reported elsewhere (16), or with the peptic-tryptic digest of ovalbumin (1 mg/ml). The specimens were harvested, snap frozen in isopenthane, cooled in liquid nitrogen, and prepared for cryosectioning as described for immunohistochemistry.

Morphometric analysis. The density of cells expressing CD3 and TCRγδ$^+$ in the intraepithelial compartment was determined by counting the number of stained cells per millimeter of epithelium and compared with standard parameters. The number of lamina propria mononuclear cells expressing CD25 or CD80 was evaluated in an area of 1 mm^2 lamina propria, using a microscope with a calibrated eyepiece aligned parallel to the muscolaris mucosae and compared with standard parameters. Lamina propria staining of CD54 and staining of epithelial cells by anti–HLA-DR was evaluated in terms of staining intensity and graded on an arbitrary scale of weak staining: (−) = 0, (+/−) = 1, (+) = 2, (++) = 3, and (+++) = 4. The counts were independently analyzed in a blinded manner by two observers.

HLA typing. All type 1 diabetic patients and control subjects were genotyped for HLA class DRB1 and DQB1 molecules. Dynal Allset$^+$ SSP DR and SSP DQ low-resolution kits and Dynal Allset$^+$ SSP DQB103 and Dynal Allset$^+$ SSP DQA1 kits were used for typing, and the results were recorded after 2% agarose gel electrophoresis.

Ethical considerations. The indication for biopsy, i.e., the high incidence of asymptomatic CD in cases of type 1 diabetes, was explained to patients and their parents, who then gave their consent to biopsy sampling for the study. The University Ethics Committee approved use of the biopsy specimens in this study.

Statistical analysis. Being normally distributed, the data were compared by the Student's t test. P values <0.05 were considered significant.

RESULTS

Immunohistochemical analysis of jejunal biopsies. All the biopsies from type 1 diabetic patients had a normal jejunal architecture. The small intestinal mucosa from type 1 diabetic patients showed signs of activated cell-mediated mucosal immunity. The density of lamina propria CD25$^+$ mononuclear cells (means ± SD: 11.5 ± 15.9 mm^2) was significantly higher ($P < 0.05$) in patients versus control subjects (2.5 ± 2.8); as shown in Fig. 1, in 14 of 17 (82.3%) patients, values exceeded the 90th percentile of the control group (4 cells/mm^2 lamina propria). CD54 and crypt HLA-DR expression was enhanced in 4 of 11 (36%) and 8 of 15 (53%) patients, respectively.

The epithelial compartment was also significantly ($P < 0.05$) infiltrated by CD3$^+$ (24.7 ± 9.3 per millimeter epithelium) and γδ$^+$ cells (1.9 ± 1.9 per millimeter epithelium) with respect to control subjects (22.2 ± 11.2 and 1.1 ± 1.2, respectively). As shown in Fig. 2, densities of CD3$^+$ and γδ above the 90th percentile of the control group (37 and 3 cells per millimeter epithelium) were observed in 3 of 17 (17.6%) and 6 of 17 (35.2%) type 1 diabetic patients, respectively.

Organ culture studies. The inflammatory response was more pronounced in gliadin-challenged biopsies. In fact, CD25$^+$ mononuclear cells were significantly ($P < 0.001$) increased in biopsies exposed to gliadin versus those exposed to medium alone (Fig. 3). CD25$^+$ cells are a heterogeneous population, which in most cases are morphologically similar to macrophages. We costained biopsy specimens from patients with anti-CD3 and anti-CD25 monoclonal antibodies to determine the number of T-cells

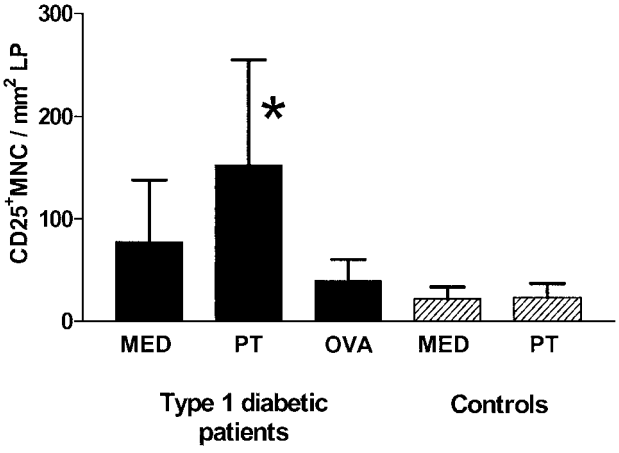

FIG. 3. Organ culture studies in type 1 diabetic patients (■) and control subjects (▨): lamina propria (LP) CD25$^+$ mononuclear cells (MNC). Bars indicate the mean and SD in biopsies cultured with only medium (MED), gliadin (PT), or ovalbumin digest (OVA). *P < 0.001.

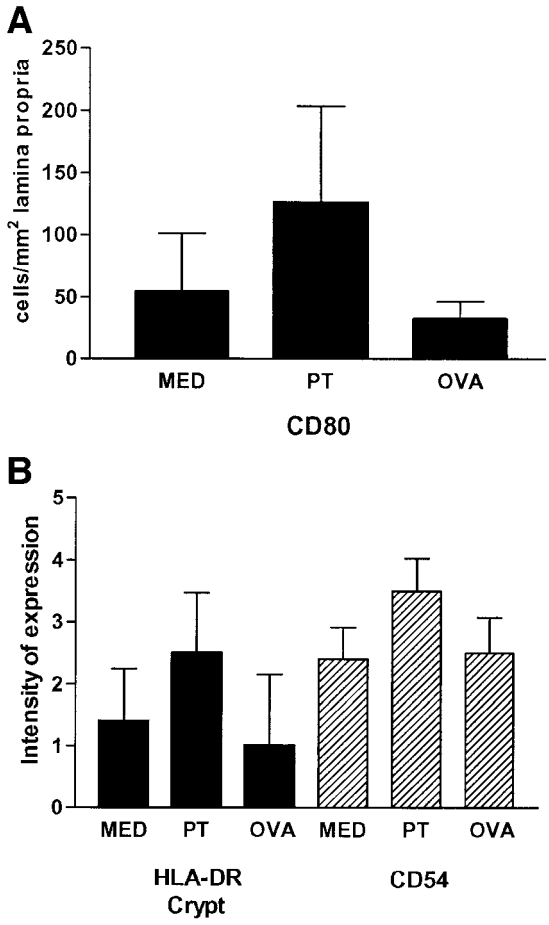

FIG. 4. Organ culture studies in type 1 diabetic patients. *A*: Lamina propria CD80$^+$ cells. *B*: Lamina propria expression of CD54 (intracellular adhesion molecule 1) and crypt enterocyte HLA-DR expression. Bars indicate the mean and SD in biopsies cultured with only medium (MED), gliadin (PT), or ovalbumin digest (OVA).

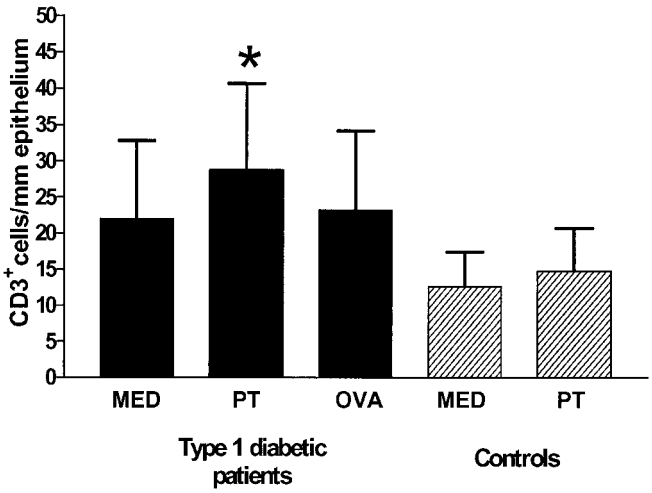

FIG. 5. Organ culture studies in type 1 diabetic patients (■) and control subjects (▨): intraepithelial CD3$^+$ cells. Bars indicate the mean and SD in biopsies cultured with only medium (MED), gliadin (PT), or ovalbumin digest (OVA). *$P < 0.001$.

activated by gliadin. CD3$^+$CD25$^+$ cells were counted as a percentage of total lamina propria CD25$^+$ cells. After the in vitro gluten challenge, the percentages of CD25$^+$ T-cells were not significantly changed (15.9 ± 4.3 vs. 19.7 ± 6.8), but the number of lamina propria T-cells expressing CD25 was increased (22.2 ± 15.2) versus fragments not exposed to gluten (9.7 ± 7.3). Similarly, the expression of lamina propria CD80 (Fig. 4*A*) and CD54 and crypt HLA-DR was enhanced (Fig. 4*B*) in gliadin-challenged biopsies. No such changes were detected in type 1 diabetic biopsies cultured with ovalbumin peptides or in control biopsies cultured with gliadin.

Similar results were obtained with intraepithelial CD3$^+$ cells. These cells were significantly ($P < 0.001$) increased compared with fragments cultured with medium alone. Again, no such changes were seen in type 1 diabetic biopsies cultured with ovalbumin peptides or in control biopsies cultured with gliadin (Fig. 5).

HLA typing. All 13 type 1 diabetic patients for whom HLA typing was available were HLA-DQ2$^+$ (HLA-DQA*05/DQB*02) and/or HLA-DQ8$^+$ (DQA*0301/DQB*0302). Only four control subjects had these alleles. However, HLA-DQ2$^+$ or HLA-DQ8$^+$ control subjects did not differ from the other control subjects in the in vitro immune response to gliadin.

DISCUSSION

Our data demonstrate mucosal inflammation in small intestinal biopsies from patients with type 1 diabetes. They are consistent with enhanced intracellular adhesion molecule 1 and epithelial HLA class II expression previously detected by immunohistochemistry (6). These findings suggest increased mucosal levels of proinflammatory cytokines as a result of local altered permeability (8) or immune dysregulation. Also, the epithelial compartment shows signs of increased infiltration by CD3$^+$ and γδ$^+$ cells. Similar findings have been observed in the intestinal mucosa of patients with Hashimoto's thyroiditis (17) and could be a general feature of autoimmune disorders (18).

More intriguing is the enhanced mucosal immune response to gliadin observed in biopsies from type 1 diabetic patients. Both an increased risk of diabetes (10) and intestinal inflammation have been reported in rodent models of type 1 diabetes in response to dietary gluten (19). In type 1 diabetic patients, peripheral lymphocyte proliferation increases in response to gliadin (13), and, at the intestinal level, rectal challenge with gliadin results in local mucosal recruitment of lymphocytes (14). Here we demonstrate a mucosal response that is clearly gliadin specific. A pattern resembling that seen in small biopsies from treated CD patients when in vitro challenged with gliadin peptides (20) was observed in all our type 1 diabetic patients. They all showed signs of T-cell activation in the lamina propria, i.e., interleukin-2 receptor (CD25) expression on lamina propria mononuclear cells (including T-cells), enhanced lamina propria expression of costimulatory (CD80) and adhesion molecules (CD54), and infiltration of the epithelium by CD3$^+$ cells.

The presence of tissue infiltration in the absence of clinical disease is not surprising. In fact, CD patients can have severe mucosal damage but no symptoms, i.e., the so-called "silent CD." More complex is the relationship with autoantibodies. We did not find endomysium or tissue transglutaminase autoantibodies in the organ culture supernatant; however, the method we used may not have been sufficiently sensitive, and studies are underway to try to detect these antibodies in the mucosa (21).

All our type 1 diabetic patients had CD-associated HLA haplotypes (HLA-DQ2 and -DQ8), but the phenomena observed were unrelated to these alleles because biopsies from HLA-DQ2$^+$ or HLA-DQ8$^+$ control subjects did not react to gliadin peptides. Overall, our data suggest that type 1 diabetes is associated with a failure in oral tolerance mechanisms, particularly versus gliadin.

The organ culture data indicate a prevalence of potential CD higher than suspected. The association between CD and type 1 diabetes is well established. Some diabetic patients who are negative for CD-associated autoantibodies will go on to become seropositive and will eventually develop frank enteropathy (22). The conditions influencing this process are poorly understood and, in this perspective, type 1 diabetes may represent a model with which to clarify the pathogenesis of CD.

From a practical viewpoint, there are no indications for withdrawing gluten from the diet of type 1 diabetic patients. A gluten-free diet protects mice that are genetically susceptible to diabetes (10). Differently, there are conflicting findings about the clearance of diabetes-related autoantibodies subsequent to a gluten-free diet in humans (23,24). In view of potential prevention strategies for type 1 diabetes, it remains to be established to what extent intestinal inflammation is gluten dependent and if it precedes the occurrence of diabetes.

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