Jejuna of patients with insulin-dependent diabetes mellitus (IDDM) show signs of immune activation

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SUMMARY

The roles of enteric viruses and food antigens as possible triggers in human insulin-dependent diabetes mellitus and the evidence that mucosal-associated homing receptors are important in both human and experimental diabetes prompted us to undertake an immunohistochemical study of intestinal specimens from patients with IDDM. We studied jejunal morphology and immunohistochemistry in 26 patients with IDDM, 13 of whom had the HLA-DQB1*0201 gene and therefore a higher risk of coeliac disease. The findings were compared with those in specimens from age-matched controls. Villous structure and the density of the intraepithelial lymphocytes were normal in every biopsy specimen. The extent of positivity with anti-DR and -DP antibodies in the villous epithelium was significantly greater in the specimens from patients than in those from controls ($P = 0.0002$ in both comparisons). The crypts were also more positive: for DR $P = 0.0001$, and for DP $P = 0.002$. The densities of T cells, CD4+, CD8+, and T cell receptor α/β^+ and γ/δ^+ cells in the epithelium and lamina propria were similar in patients and controls, but the patients had significantly more α_4/β_7 integrin+ cells in the lamina propria ($P = 0.006$). No difference was seen between HLA-DQB1*0201-positive and -negative patients. These findings reflect a stage of inflammation in the structurally normal intestines of patients with IDDM and suggest secretion of inflammatory Th1-type cytokines in the intestine.

Keywords insulin-dependent diabetes mellitus immunohistochemistry HLA class II antigens α_4/β_7 integrin

INTRODUCTION

The progressive extinction of insulin-secreting β cells in the pancreatic islets, mediated by cytotoxic T cells and leading to diabetes both in man and non-obese diabetic (NOD) mice has been shown to be produced by programmed cell death, apoptosis [1–3]. Transgenic NOD mice made to express Fas ligand showed heightened sensitivity to diabetogenic T cells due to increased apoptosis [1], while, on the other hand, NOD mice lacking Fas expression were protected from diabetes [4]. One mechanism for the expression of Fas in human β cells may be induction of nitric oxide production by IL-1β [3]. While the initiator(s) of the autoreactive T cells in diabetes remain enigmatic [5,6], in the mouse model glutamic acid decarboxylase (GAD)-reactive CD4+ Th1 cells alone are capable of inducing diabetes [7]. We recently showed

that the reactivity of the peripheral lymphocytes of patients with IDDM to GAD65 was markedly reduced when the α_4/β_7 integrin+ cells were depleted [8], suggesting that GAD-reactive cells may originate in the intestine. The mucosal homing pattern of infiltrating lymphocytes and the appearance of mucosal addressin in the endothelium of vessels in the pancreatic islets during the development of diabetes in NOD mice suggest that the antigens encountered in the intestine may be relevant to the autoimmune process [9–12]. A cross-reactive initial stimulation for both T and B cells may be caused by food antigens, such as cow's milk [13], or by infectious agents, recent data indicating the strongest candidate to be enterovirus [14,15]. B cells, too, are essential for the development of autoimmune diabetes in NOD mice [16,17]; and therefore, B cells originating from the gut may also be important as antigen-presenting cells in the pancreas.

In the present study we investigated lymphocytes in the jejuna of 26 patients with IDDM, measuring their subgroups, activation markers and homing receptors, and looked for vascular integrins and expression of class II antigens on the epithelium as markers of immune activation.

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PATIENTS AND METHODS

Patients

Patients were recruited from those attending the diabetes clinic of the Children's Hospital, University of Helsinki. They had all participated in an epidemiological study on childhood diabetes in Finland [18]. The study was designed to look for latent coeliac disease (CoD) in IDDM. We therefore invited equal numbers of patients who were either positive or negative for the HLA-DQB1*0201 gene allele; 13 patients in each group agreed to participate. None of the patients had gastrointestinal symptoms or growth retardation, and serologic tests for CoD (IgA- and IgG-antigliadin and IgA-reticulin antibodies) were negative [19].

Findings in the histochemical study were compared with those for age-matched controls studied in the same laboratory. The numbers of control biopsies available for the different stains ranged from eight to 24 owing to the loss of 16 control specimens (as the freezer had accidentally warmed up). None of the controls had gastrointestinal symptoms; their jejuna were studied because of retardation of growth. The morphology of the jejunum was found to be normal in every case.

Immunohistochemical methods

Jejunal biopsy specimens were taken with a Crosby–Kugler capsule from the ligament of Treitz or distal to it. Specimens were divided for routine histology and immunohistochemical study. The part for morphology was embedded in paraffin and stained with haematoxylin–eosin; the other part for immunohistochemistry was fixed in acetone for 10 min, then in chloroform for 30 min [20]. The specimens were evaluated without knowledge of the background of the specimen. The numbers of stained cells were counted under a light microscope with a calibrated graticule at ×1000 magnification. In the surface epithelium, the positive cells along the basement membrane of the epithelium were counted from the length of at least 30 fields (1·8 mm) and their densities expressed as cells/mm. In the lamina propria at least 30 fields between the epithelium and muscularis mucosae (0·1 mm^2) were measured and cell densities expressed as cells/mm^2. The proportion of epithelial cells staining with antisera to HLA-DR and HLA-DP was measured with point counting, with a grid with 100 points. At least 1500 points falling on epithelial cells were counted in each specimen, each point registered as positive or negative, and the percentage of positive points was calculated. With the same technique, the proportions of the lamina propria occupied by cells positive for intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) antisera were estimated. Proliferative (Ki-67$^+$) cells in the crypts were calculated as percentages of the crypt epithelial cells, at least 200 crypt cells being counted in each specimen. In the same specimens the densities of cells having α/β or γ/δ T cell receptors, and the CD3, CD4, CD8 or CD38 antigens were counted both in the epithelium and in the lamina propria.

Monoclonal antibodies

MoAbs to constant fragments of HLA-DR and -DP chains purchased from Becton Dickinson (Mountain View, CA) were used at dilutions of 1:1000 and 1:40, respectively. The anti-DQ MoAb (Serotec Ltd, Oxford, UK) reacting with a monomorphic determinant present on all HLA-DQ molecules [21] was used at a dilution of 1:400. The MoAb Ki-67 (DAKO-PC; Dakopatts, Glostrup, Denmark) recognizing a nuclear antigen present only in proliferating cells [22] was used at a dilution of 1:10. The MoAb TCRδ1 (T Cell Diagnostics, Inc., Woburn, MA), and the antibody βF1 (T Cell Diagnostics) were used at dilutions of 1:100. The MoAbs anti-Leu-4 (anti-CD3; Becton Dickinson), T4 (anti-CD4; Coulter Immunology, Hialeah, FL) and OKT8 (anti-CD8; Ortho Diagnostic Systems, Raritan, NJ) were used at a dilution of 1:400. Antiserum to CD38, anti-Leu-17, was obtained from Becton Dickinson and used at a dilution of 1:50. Antibodies to CD11a, recognizing the lymphocyte functional antigen-1 (LFA-1; Immunotech, Marseilles, France), were used at a dilution of 1:50, antibodies to its ligand, ICAM-1 (CD54) (T Cell Diagnostics) were used at a dilution of 1:2000; and antibodies to VCAM-1 (CD106) (T Cell Diagnostics) at a dilution of 1:1000. The MoAb to α_4/β_7 integrin (ACT-1) was received from Leukosite Inc. (Cambridge, MA) and used at a dilution of 1:200.

Determination of HLA antigens

The HLA-DQ alleles were determined in the context of a nationwide study on genetic and environmental factors in childhood diabetes in Finland, as described elsewhere [23].

Statistical analysis

Cell densities and the percentages of positive stainings in patients and controls were compared by non-parametric tests (Mann–Whitney U-test and χ^2 test) because of the non-linear distribution of the parameters. The values for patients either positive or negative for the HLA-DQB1*0201 allele were compared by the same tests.

Ethical considerations

Specimens from controls were taken for diagnostic purposes. The indication for the biopsy, i.e. the high incidence of asymptomatic CoD, was explained to patients with IDDM and their parents, who gave their consent for taking the biopsy specimen for this study. The study plan was accepted by the Ethics Committee of the Children's Hospital, University of Helsinki.

RESULTS

Histology of jejunal specimens

The morphology of all biopsy specimens was normal. The percentages of proliferative cells in the crypts of the specimens from patients and controls were similar, indicating normal turnover rates of epithelial cells (Table 1).

Intraepithelial lymphocytes

The density of intraepithelial lymphocytes was similar in patients and controls (Table 1). No difference was found between the patients with the DQB1*0201 allele and those without it.

The densities of CD3$^+$, α/β TCR$^+$, γ/δ TCR$^+$, CD8 and CD4$^+$ intraepithelial lymphocytes were similar in patients and controls. The density of α_4/β_7 integrin$^+$ cells tended to be greater in the patients ($P = 0·07$) (Table 1).

Lymphocytes in the lamina propria

The densities of CD3$^+$, CD4$^+$, CD8$^+$, CD11a$^+$, TCR α/β and γ/δ^+ cells in patients and controls did not differ; the density of CD38$^+$ cells being somewhat higher in the patients ($P = 0·065$) (Tables 1 and 2). The densities of α_4/β_7 integrin$^+$ cells, however, were significantly greater in the specimens from the patients than in those from the controls ($P = 0·006$) (Table 1).

Table 1. Density of intraepithelial lymphocytes, of leucocyte function antigen-1$^+$ (CD11a) and of homing receptor α_4/β_7 integrin$^+$ cells, and percentage of dividing crypt cells (Ki-67$^+$) in the jejuna of patients with IDDM and controls

Marker	Density of cells in the surface epithelium (cells/mm), for Ki-67 % of crypt cells, mean, 95% CI and number of patients		Density of cells in the lamina propria (cells/mm^2), mean, 95% CI and number of patients	
	Patients	Controls	Patients	Controls
CD11α	16	10	2408	2198
	10, 21 (26)	5·5, 15 (8)	2137, 2678 (26)	1606, 2789 (8)
Integrin α_4/β_7	6·0	2·3	3438	2009
	3·9, 8·1 (26)	0·4, 4·2 (8)	3077, 3799 (26)	1088, 2930 (8)
Intraepithelial lymphocytes	41	52		
	34, 49 (22)	43, 61 (11)		
Ki-67 (%)	17	13		
	14, 21 (25)	9, 17 (11)		

Staining with HLA class II antibodies
In biopsy specimens from patients with IDDM, the percentage of epithelium stained with anti-HLA-DR antiserum was significantly greater than in those from the controls ($P = 0·0002$) (Table 3). In 10 out of 24 patients the crypts stained positive with HLA-DR antiserum to a small extent (Fig. 1), while none of the eight control specimens showed positive staining ($P = 0·001$, χ^2 test). The area stained with anti-HLA-DP antiserum was similarly more extensive in the specimens from patients than in those from controls ($P = 0·0002$) (Table 3). In many specimens from patients the
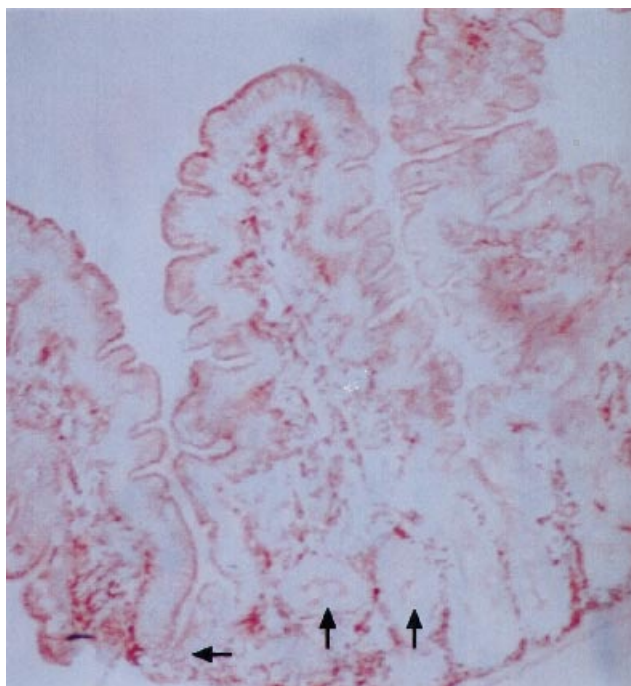

Fig. 1. Biopsy specimen from a patient with IDDM treated with antibodies to HLA-DR, original mag. ×10. Positive staining is seen in the apical parts of epithelial cells throughout the villi and also in many crypts, indicated by arrows.

epithelium of the villi was positive with anti-HLA-DP antibodies from the tip to the base (Fig. 2), but in the controls only granular spots of some cells in the apex of the villi were positive (Fig. 3). Of 22 patients, 19 had some staining of the crypt epithelium (Fig. 4), while two out of seven control specimens also showed weak staining ($P = 0·002$).

Staining of the epithelial cells with anti-DQ antiserum was seen in two of the 11 controls studied, while 19 of 26 patients had similar positive staining ($P = 0·001$); in all, the staining was weak and seen only in the apical parts of the villi.

When the patients were divided according to the presence or absence of HLA-DQB1*0201 allele, no difference was seen in the staining pattern for HLA-DR or -DP (data not shown).

Staining with anti-ICAM-1 (CD54) and VCAM-1 (CD106) antibodies
In both patients and controls, anti-CD54 antibodies were bound to a large proportion, $>20\%$, of the surface of the lamina propria, the majority of positive staining was on cells, but some was also seen extracellularly (Table 3). The percentage of structures in the lamina propria positive for anti-CD106 antibodies was much smaller, in patients about 6%, and in controls 3% ($P = 0·1$).

DISCUSSION

In patients with IDDM, compared with age-matched controls, we found a marked extension of the expression of both HLA-DR and HLA-DP antigens in the surface epithelium of the jejunum. HLA-DQ was also more frequently expressed in the patients' jejuna than in control specimens. In the normal human jejunum, HLA-DR and -DP antigens are present only in the upper part of the villi [24], as seen in the controls of the present study. In inflammatory conditions, such as active CoD, DR and DP expression in the epithelium is greatly extended, involving not only the villi but also the crypts [25–27]. The major inducers of this extensive expression of HLA class II molecules are interferon-gamma (IFN-γ) and tumour necrosis factor-alpha (TNF-α), as shown in tissue culture experiments [28,29]. Thus, in the jejunum of a patient with diabetes, the same Th1-dependent cytokine secretion pattern prevails as is essential for the development of diabetes in the pancreas
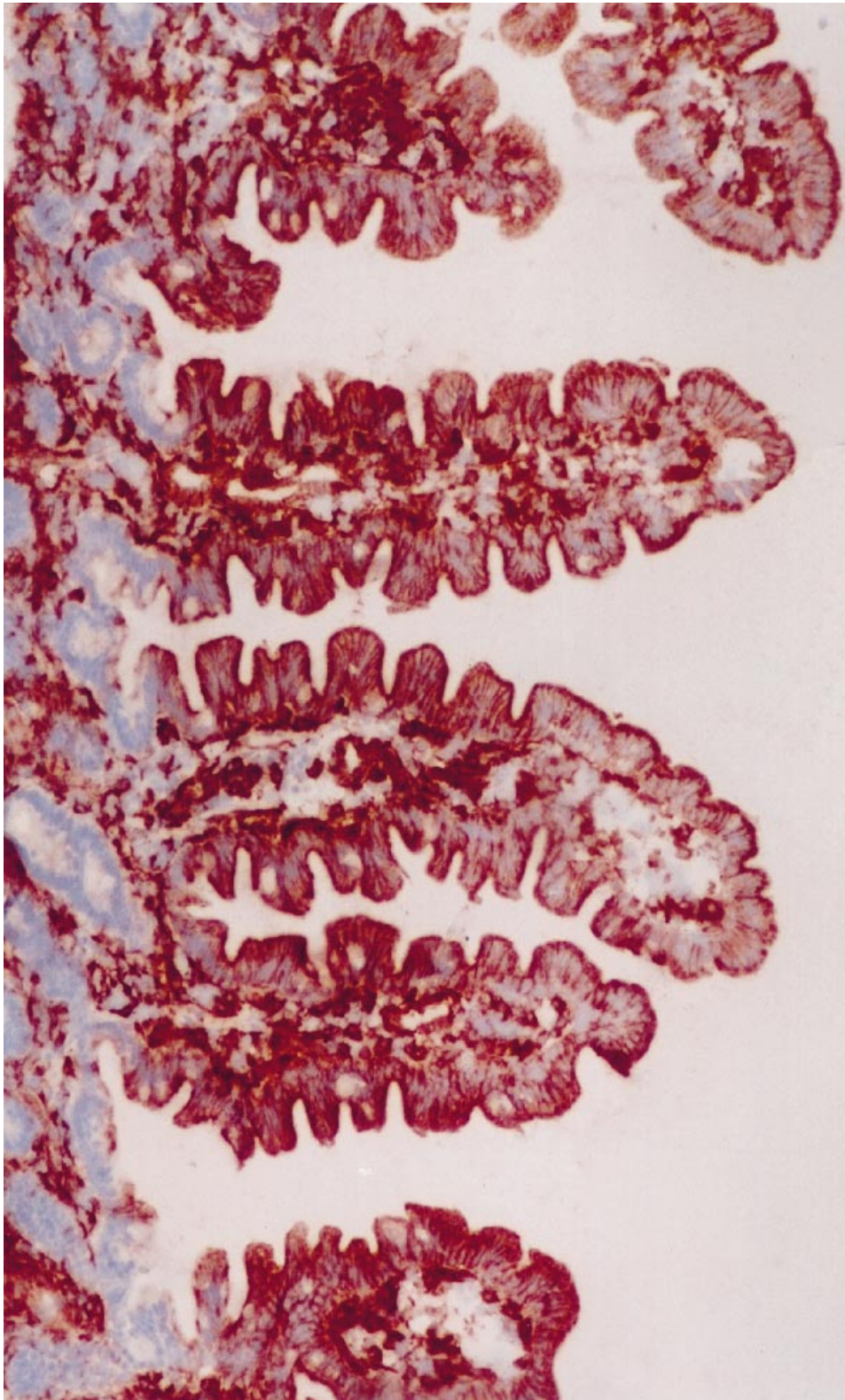
Fig. 2. Biopsy specimen from a patient with IDDM treated with antibodies to HLA-DP, original mag. ×10. Intensive, positive staining is seen throughout the epithelial cells of villi; on average, 81% of the area of epithelial cells was positive.

Table 2. Density of CD3$^+$, CD4$^+$, CD8$^+$, T cell receptor (TCR) α/β^+, TCR γ/δ^+ and CD38$^+$ cells in the jejuna of patients with IDDM and of controls

Marker	Density of cells in the surface epithelium (cells/mm), mean, 95% CI and number of patients		Density of cells in the lamina propria (cells/mm^2), mean, 95% CI and number of patients	
	Patients	Controls	Patients	Controls
CD3	29	29	2184	1872
	23, 34 (25)	19, 38 (24)	1950, 2502 (25)	1509, 2234 (24)
CD4	2·1	3·3	1493	1640
	1·3, 3·0 (26)	1·9, 4·7 (22)	1286, 1619 (26)	1319, 1961 (22)
CD8	26	24	829	996
	21, 32 (26)	18, 31 (21)	676, 1001 (26)	780, 1410 (22)
TCR α/β	25	27	1933	1862
	20, 29 (26)	17, 36 (22)	1677, 2090 (26)	1508, 2216 (24)
TCR γ/δ	3·3	3·4	90	98
	2·0, 4·6 (26)	2·3, 4·6 (24)	59, 120 (26)	70, 125 (24)
CD38	16	20	3285	2812
	12, 27 (19)	12, 27 (5)	3076, 3494 (19)	2434, 3192 (5)

[30]. In such an environment, *in vitro* experiments have demonstrated that the antigen presentation by epithelial cells is enhanced and the cells are more leaky [29]. Both changes may relate to the pathogenesis of IDDM.

Another striking finding in the jejuna of our patients with IDDM was the increased density of mononuclear cells bearing α_4/β_7 integrin, significantly in the lamina propria and to a lesser extent in the epithelium. The α_4/β_7 integrin is a homing receptor of lymphocytes, directing them to mucosal tissues, especially to the intestine, by binding to mucosal vascular addressin cellular adhesion molecule-1 [31]. Subpopulations of CD4$^+$ memory cells [32] and B cell blasts [33] express particularly high levels of α_4/β_7 integrin. In addition, we have shown that B cells activated by oral vaccines [34] or by enteric infections also express this receptor [35]. Therefore, the present finding in the jejuna of patients with IDDM, that expression of α_4/β_7 integrin by mononuclear cells is increased, suggests that in these patients more T and B memory cells have accumulated in the intestinal mucosae. The necessity of B cells for the initiation of experimental diabetes has recently been

Table 3. Staining of the jejunal epithelium with HLA-DR and -DP antibodies, and of the structures in the lamina propria with anti-intercellular adhesion molecule (ICAM) and -vascular cell adhesion molecule (VCAM) antibodies in jejuna of patients with IDDM and controls

Marker	Percentage, mean, 95% CI and number of patients	
	Patients	Controls
HLA-DR	40	11
	32, 48 (26)	7, 18 (8)
HLA-DP	37	12
	28, 37 (26)	0·1, 24 (8)
ICAM-1	23	21
	21, 35 (26)	16, 25 (8)
VCAM-1	5·8	2·6
	3·7, 7·9 (26)	0·2, 5·1 (8)

demonstrated [16,17]. We previously showed that α_4/β_7^+ cells were responsible for the greater part of T cell stimulation by GAD65 [8], showing the importance of mucosally activated T cells in the autoimmune response in IDDM. In the NOD mouse model, the endothelium of the pancreatic venules expresses the mucosal addressin cellular adhesion molecule-1, and mononuclear cells infiltrating the pancreas bear the homing receptor interacting with it, integrin $\alpha_4\beta_7$ [9]. Another marker suggestively different in patients and controls was CD38; its antibodies recognized the majority of T and B cells. This molecule is also involved in the trafficking of lymphocytes through high endothelial venules [36].

In the present study we set out to find markers of latent CoD [37]. The great majority of patients with IDDM have normal jejunal morphology, but the risk of CoD is greatly increased [19,38,39]. This increase is seen particularly in those patients with IDDM whose HLA class II antigens are similar to those of patients with CoD [39]. Recently it was shown that, of the patients with IDDM found to have CoD, a significant proportion had developed it only after the onset of clinical diabetes [19,40]. All our patients with IDDM were on a normal gluten-containing diet. In Finland, young patients with IDDM have been shown to have a higher dietary intake of cereal proteins than control children [41]. It is to be expected that patients liable to develop CoD will show subtle changes, best distinguished by immunohistochemical methods, similar to those described in many family members of patients with CoD [42,43]. A constant finding in the jejunal epithelium of such patients is a markedly higher density of T cell receptor γ/δ^+ cells [20,44,45]. This is definitively a marker of latent CoD [46], and is associated with the disease-specific HLA-DQ genes in the family members of patients with CoD [42]. Among the patients with IDDM, and even among those having the DQB1*0201 allele, a high density of γ/δ^+ cells was not more frequent than in the controls. This is probably due to the relative rarity of latent CoD in IDDM among those having already had diabetes for 5 years. In our study [19], 1·2% of patients had unrecognized silent CoD when IDDM became clinically manifest and an equally high percentage developed CoD within 2 years, resulting in an incidence of 2·4% in patients having had diabetes for 2–3 years. As the incidence in
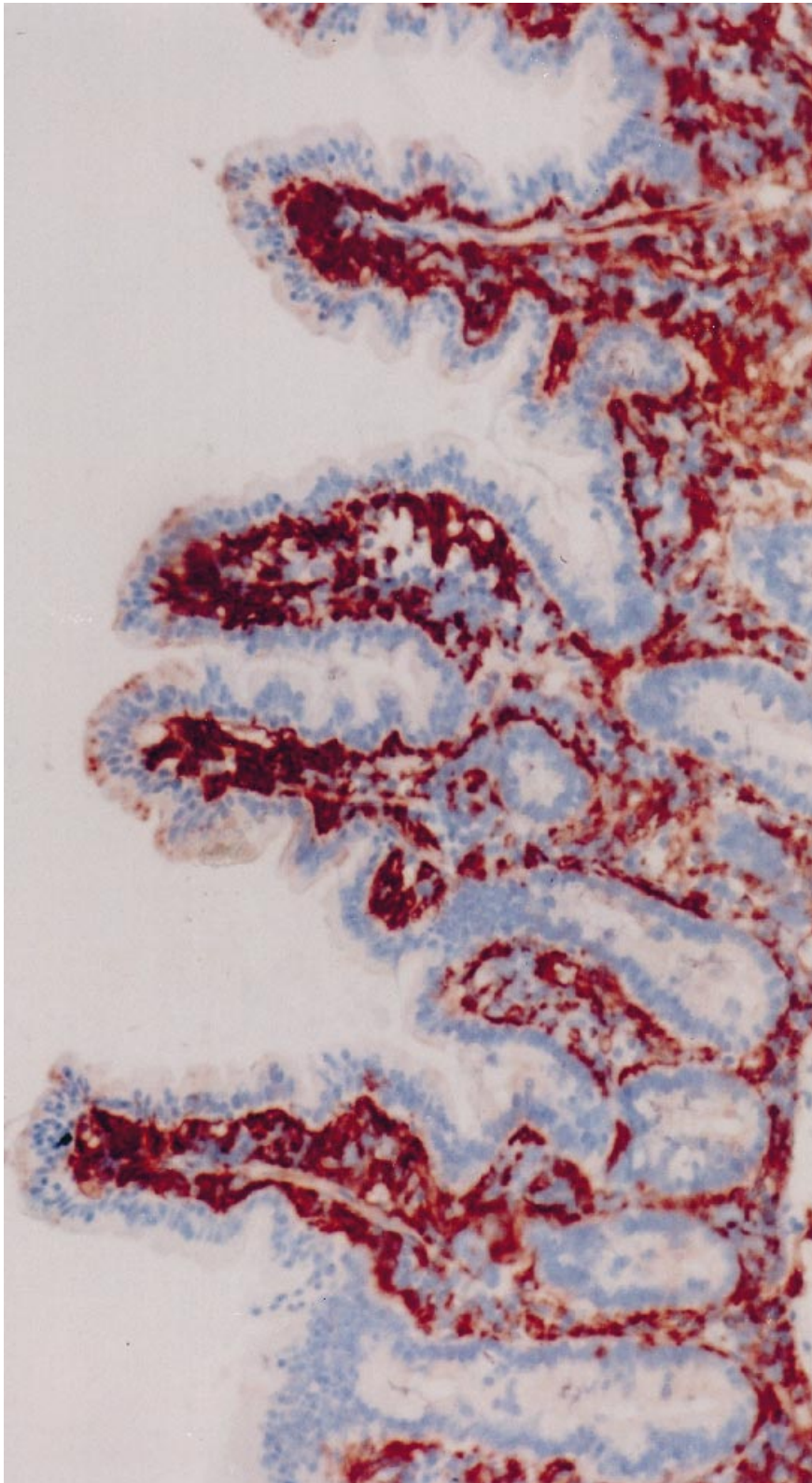
Fig. 3. Biopsy specimen from a control patient treated with antibodies to HLA-DP, original mag. ×10. Only a few positive granules are seen in the apical parts of the epithelial cells at the tip of villi; on average, 1% of the area of epithelial cells was positive.
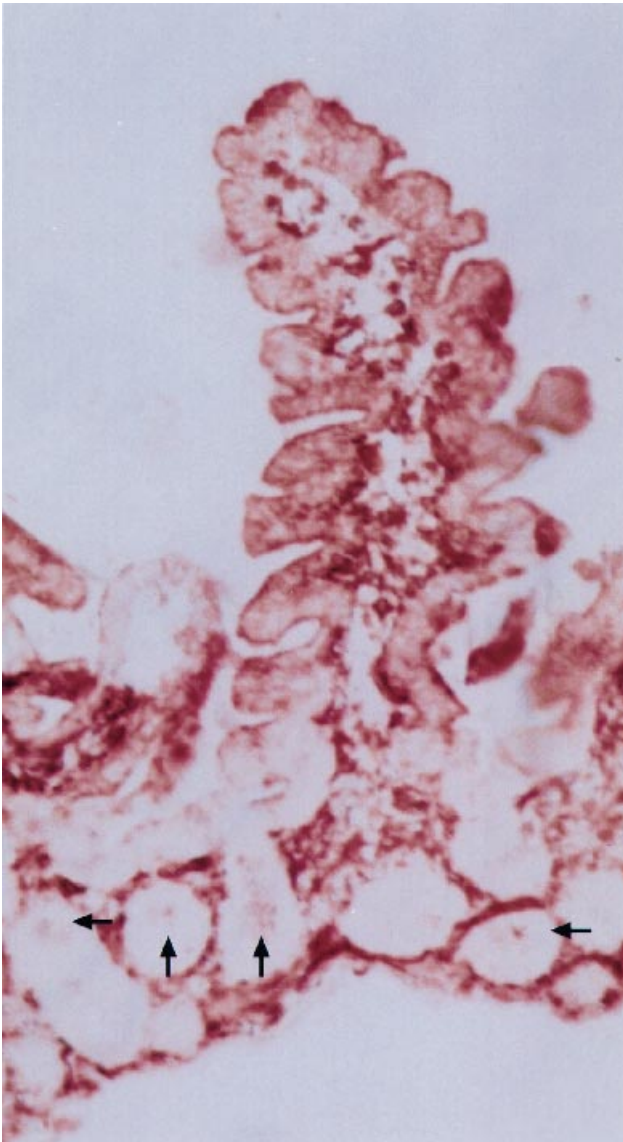
Fig. 4. Biopsy specimen from a patient with IDDM treated with antibodies to HLA-DP, original mag. ×10. In addition to positive staining in epithelial cells of villi, cells in several crypts are stained (indicated with arrows).

Finnish adults with IDDM is only slightly higher, 4·1% [47], the latent CoD in the study population may be expected to be quite low.

Thus even when routine histology shows nothing abnormal, immunohistochemistry suggests a jejunal abnormality in many patients with IDDM. The antigen presentation by the epithelial cells of the jejunum may be enhanced in these patients compared with that of non-diabetic subjects, and the lamina propria contains more mononuclear cells with mucosal homing receptors. These changes suggest that a similar cytokine secretion pattern prevails in the jejunum as in the prediabetic pancreas. It may relate to disturbed induction of tolerance to oral antigens in the gut.

ACKNOWLEDGMENTS

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