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An Easy Method to Quantify Plasma Cells in Caeliac Disease

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Abstract. *Background: Caeliac disease is a common immune-mediated condition in the proximal small intestine, generated by a permanent intolerance to cereal gluten proteins in genetically predisposed individuals. It has become apparent that abnormal microbiota proliferate in the duodenal lumen of patients with caeliac disease. Recently it was also noticed that an antibody against multiple myeloma oncogene 1/IRF4 (MUM1) stained plasma cells and their precursors. Materials and Methods: Eleven consecutive duodenal biopsies were investigated; four had villous atrophy (caeliac patients) and the remaining seven exhibited histologically normal mucosa (non-caeliac patients). Sections were stained with H&E and with anti-MUM1. A graticulated eyepiece (10 mm, divided into 10×10 squares) was used for counting of MUM1-expressing cells in the superficial compartment (SC) and in the deep compartment (DC) of the lamina propria mucosa (lpm). Results: In the duodenal mucosa of caeliac patients the mean number of MUM1-labelled cells in 12 areas of the lpm was 67.1 (range 37-88) in the SC and 61.5 (range 42-84) in the DC. In the duodenal mucosa of non-caeliac patients, the mean number of MUM1-labelled cells in 21 areas of the lpm was 7.6 (range 0-24) in the SC, and 29.2 (range 22-40) in the DC ($p<0.05$). Conclusion: These preliminary results showed that a significantly higher number of plasma cells/plasma cell precursors accumulate in the lpm in patients with caeliac disease, particularly in the SC. This abnormal accumulation of MUM1-expressing cells might be a defence mechanism against the alien bacterial flora recently reported in the duodenal microenvironment in caeliac patients. This appears to be the first report in which MUM1 immunostaining is applied to assess the frequency of plasma cell precursors in the duodenal mucosa in caeliac patients.*

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Caeliac disease is a common immune-mediated condition in the proximal small intestine, generated by a permanent intolerance to cereal gluten proteins in genetically predisposed individuals (1). In most Western countries, the prevalence of diagnosed caeliac disease in children is 0.5-1% (2). In Sweden, the epidemiology of childhood caeliac disease has undergone dramatic changes over recent decades (3). Up to the early 1980s, the incidence of caeliac disease in Swedish children was about 1/1000 (4). During the mid- and late 1980s, a sharp increase was observed at several pediatric clinics in this country. Today, caeliac disease is the second most common chronic disease in Swedish children, with an incidence of 3% (5).

Despite numerous sensitive and specific serological markers (6), duodenal biopsy remains the gold standard for diagnosing caeliac disease. Following diagnosis, a lifelong strict gluten-free diet is the treatment of choice to restore mucosal normality, thereby reverting symptoms and ameliorating possible complications.

Duodenal biopsies in these patients may display a wide range of abnormalities, from well-developed villi with increased number of intraepithelial lymphocytes (IELs) to villous atrophy with a more pronounced increase in IELs, crypt hyperplasia with increased numbers of mitoses, and mononuclear cell infiltration of the *lamina propria mucosa* (*lpm*) (7). The majority of the studies currently describing the histological features of caeliac disease focus on the number of IELs observed in the luminal epithelium. The general consensus is that this number should be in excess of 25 IELs/100 epithelial cells (8-11). Notwithstanding this, only a few studies in recent literature deal with other important histological parameters of caeliac disease, namely an increased number of round cells in the *lpm*. Earlier work showed that immunoglobulins IgA, IgG and IgM are increased in the duodenal mucosa in patients with caeliac disease (12-15). These immunoglobulins are secreted by plasma cells.

In a previous study (16), we investigated the occurrence of plasma cells/plasma cell precursors in liver biopsies from patients with autoimmune hepatitis by the aid of the interferon regulatory factor 4, a protein encoded by the *IRF4* gene, also known as *MUM1*. The *MUM1* gene contributes to

the regulation of immunoglobulin gene expression in B-cell differentiation within germinal centre light zones (12). Defects in IRF4 may cause multiple myeloma, a malignant tumour of plasma cells.

It has become apparent that abnormal microbiota proliferate in the duodenal lumen of patients with caeliac disease. Sanchez *et al.* (17) found that *Bifido bacterium* diversity was higher in patients with caeliac disease than in controls and that *Bifido bacterium adolescentis*/*Bifido bacterium animalis lactis* were more prevalent in patients with active caeliac disease than in patients with treated caeliac disease and control children. Schippa *et al.* (18) found that *Bacteroides vulgatus* and *Escherichia coli* more often found in caeliac patients than in controls. Recently Forsberg *et al.* (19) and Ou *et al.* (20) detected rod-shape bacteria attached to the intestinal epithelium in patients with caeliac disease. The role played by these abnormal bacteria in genetically-predisposed individual's remains to be elucidated.

Two main mechanisms might be activated to combat these bacteria in caeliac patients: one is the up-regulation the natural antimicrobial enzyme, lysozyme (21), and the other is the increase of numbers of immunoglobulin-producing plasma cells. Against this background, it was considered of interest to assess the frequency of plasma cells in duodenal biopsies from patients with caeliac disease. For this purpose, duodenal biopsies were challenged with MUM1 immunostaining.

Materials and Methods

Eleven consecutive duodenal biopsies were investigated: four had villous atrophy (caeliac patients) and the remaining seven were of histologically normal mucosa (non-caeliac patients).

Sections were stained with CD138 and MUM1 immunostains. Staining with CD138 was soon discontinued as it also labelled other structures, including part of the background.

MUM1 immunostaining. Sections (5 μm) were pre-treated with Enzyme 1 (Leica Microsystems, Wetzlar, Germany) for 5 min, incubated for 30 min with anti-human MUM1 antiserum (Dako A 0099, DAKO, Glostrup, Denmark) on a Leica Bond MAX (Leica Microsystems), were next diluted at 1:1600 in Antibody Diluent AR 9352 (Leica Microsystems), and developed in a Developing Kit (DS 9800, Bond Refine Polymer DAB Kit; Leica Microsystems) with haematoxylin-eosin (H&E). MUM1/IRF4 is expressed in the nuclei and cytoplasm of plasma cells and a small percentage of germinal centre (GC) B-cells is located in the light zone, as demonstrated by staining biopsies of the colon with chronic inflammation, carrying a lymphatic follicle; while MUM1 stained plasma cells, the lymphocytes in the follicles remained unstained. In the absence of plasma cells in H&E-stained sections, MUM1-expressing cells were regarded as plasma cell precursors.

Plasma cell counting. Well-oriented sections were investigated. A graticulated Olympus eyepiece was used (10 mm, divided into 10×10 squares) for partitioning a section of the view-field for counting. One of the sides of the frame was positioned on the most luminal aspect of the duodenal mucosa. Counting of MUM1-labeled plasma cells/plasma cell precursors was carried out within the luminal-*muscularis mucosae* axis: i) in the five most superficial squares of the grid, called the superficial compartment (SC) and ii) in the five remaining deeper squares of the grid, called the deep compartment (DC). The *lpm* in three different normal villi and deep crypts (villous atrophy) exhibiting *a priori* the highest number of MUM1-positive plasma cells were investigated at high-power (×40 objective). All MUM1-positive cells present within the two aforementioned zones, framed by the ocular grid, were counted.

Statistical analysis. The non-parametric Mann–Whitney test was used to compare difference between groups. Statistical significance was defined as $p<0.05$.

The Regional Ethical Committee approved the study.

Results

Following MUM1 immunostaining of sections from duodenal biopsies of patients with caeliac disease, high numbers of dark-stained plasma cells/plasma cell precursors were found in the superficial and in the deep aspects of the *lpm* (Figure 1). On the other hand, fewer MUM1-immunostained cells were found in duodenal biopsies from patients without caeliac disease (Figure 2).

Table I shows that in the duodenal mucosa of patients with caeliac disease, the mean number of MUM1-labelled cells in 12 areas of the *lpm* was 67.1 (range 37-88) in the SC and 61.5 (range 42-84) in the DC. The Table also shows that in the duodenal mucosa of non-caeliac patients, the mean number of MUM1-labelled cells in 21 areas of the *lpm* was 7.6 (range 0-24) in the SC, and 29.2 (range 22-40) in the DC. The difference between the number of MUM1-expressing plasma cells/plasma cell precursors in the superficial and deep aspects of the *lpm* in patients with and in those without caeliac disease was significant ($p<0.05$).

Discussion

These preliminary results showed that the number of plasma cells/plasma cell precursors in the SC and the DC in the *lpm* of the duodenal mucosa from patients with caeliac disease was significantly higher than in the *lpm* of the duodenal mucosa of non-caeliac patients. As in a previous study with liver biopsies (16), it was found that plasma cells/plasma cell precursors were strongly highlighted by MUM1 immunostaining, whereas lymphocytes remained unstained. Intestinal plasma cells are derived from conventional B-cells in the Peyer's patches (22). In animals, they confer specific immunity to luminal pathogens through natural polyspecific antibodies encoded by germ-line IgVH genes (23). Binding to multiple pathogens, these cells provide broad-spectrum protection (24). The existence of a memory response in this system is consistent with the involvement of germinal

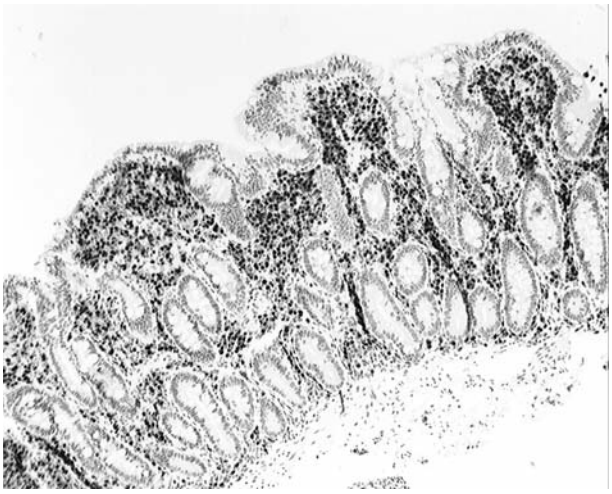

Figure 1. *Duodenal mucosa in a patient with caeliac disease. Note the high frequency of plasma cells/plasma cell precursors in the superficial and deep aspects of the lamina propria mucosa (MUM1 immunostain, ×10).*

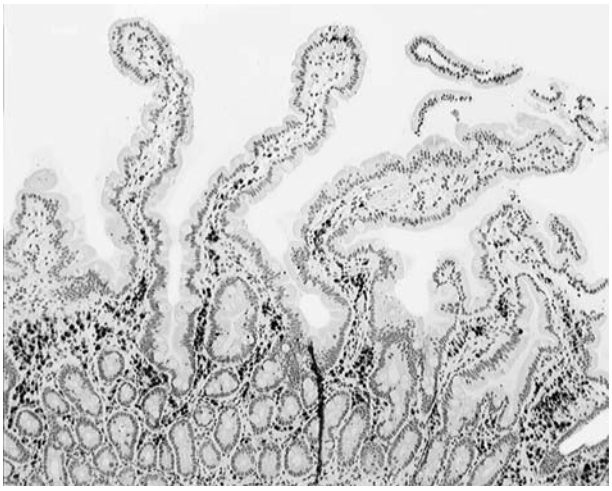

Figure 2. *Duodenal mucosa in a patient without caeliac disease. Note the absence of plasma cells/plasma cell precursors in the superficial aspect of the lamina propria mucosa (MUM1 immunostain, ×10).*

centres. Once a B-cell binds an antigen, it may proliferate in germinal centres. B-Cells are subsequently selected according to the affinity of the antibody encoded by the mutated *Ig* genes for each antigen. Surviving B-cells may undergo further rounds of mutation and selection until clonally-related cells emerge from the germinal centres as plasma cell precursors or memory cells (22). Following oral immunization of humans with cholera vaccine, plasma cells secreting anti-cholera toxin IgA are found in the duodenal mucosa (25). Thus, human plasma cells secrete specific neutralizing antibacterial immunoglobulins. It should be kept in mind that the majority of the plasma cells in the human body are found in the intestinal *lpm* (26). Wood *et al*. (15) claimed that increased mucosal plasma cell counts in the duodenal mucosa in patients with caeliac disease mirrored increased local production of immunoglobulins. This increase, however, was not reflected in the immunoglobulin concentrations in the serum, emphasizing the importance of studying the immune function of the gut in caeliac disease, in tissue sections rather than immunological abnormalities in serum. Scott *et al*. (12) suggested that in mucosa of patients with caeliac disease, the increase of IgA and IgM plasma cells indicated that extracellular deposits of IgA and IgM were locally produced.

One significant negative finding in this study was that in the atrophic mucosa of caeliac patients, none of the IELs was positive for MUM1 immunostaining. This was in contrast with the high number of MUM1-expressing cells flocked in the SC, strongly suggesting that plasma cells/plasma cell precursors in patients with caeliac disease had been summoned to the most superficial aspect of the *lpm*, close to the luminal microenvironment (where both the noxious gluten products and the abnormal bacterial flora are present). Why immunoglobulin specific-producing plasma cells/plasma cell precursors are not present in the duodenal epithelium of caeliac patients remains unclear, as specific immunoglobulins

Table I. *The numbers of plasma cells/plasma cell precursors in three different areas of the lamina propria mucosa/case, in duodenal biopsies from four patients with caeliac disease and from seven individuals without caeliac disease. The mean is given in brackets. The superficial compartment refers to the five most superficial squares of an ocular grid (10 mm, divided into 10×10 squares) and the deep compartment, to the five remaining deeper squares of the grid.*

	Caeliac patients		Non-caeliac patients	
Cases	Superficial compartment	Deep compartment	Superficial compartment	Deep compartment
1	80, 76, 74 (76.7)	61, 58, 63 (60.7)	0, 2, 0 (0.7)	22, 24, 26 (24.0)
2	37, 43, 44 (41.3)	84, 77, 79 (80.0)	6, 8, 8 (7.3)	25, 22, 27 (24.7)
3	85, 88, 76 (83.0)	48, 56, 62 (53.3)	17, 21, 24 (20.7)	40, 31, 32 (34.3)
4	63, 67, 72 (67.3)	42, 59, 49 (50.0)	4, 0, 7 (3.7)	23, 27, 21 (23.7)
5			12, 10, 15 (12.3)	37, 32, 29 (32.7)
6			2, 4, 2 (2.7)	29, 39, 33 (33.7)
7			4, 8, 6 (6.0)	35, 27, 32 (31.3)
All	805/12 (67.1)	738/12 (61.5)	160/21 (7.6)	613/21 (29.2)

could be even more effective if closer to the proliferating noxious bacteria in the duodenal microenvironment. The scenario that the luminal duodenal epithelium cannot house (besides IELs) more intraepithelial intruders, such as plasma cells/plasma cell precursors, appears unlikely.

In conclusion, these preliminary results showed that a significantly higher number of plasma cells/plasma cell precursors accumulate, particularly in the upper aspect of the *lpm*, in patients with caeliac disease. This abnormal accumulation of MUM1-expressing cells might be a defence mechanism against the alien bacterial flora recently reported in the duodenal microenvironment in such patients. The results substantiate the notion of a continuous cross-talk between the mucosa and the bacteria present in the immediate microenvironment.

This appears to be the first report in which MUM1 immunostaining is applied in order to assess the frequency of plasma cell precursors in the duodenal mucosa in patients with caeliac disease.

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