Morphometric analysis of intestinal mucosa. V Quantitative histological and immunocytochemical studies of rectal mucosae in gluten sensitivity

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Abstract

To study changes in rectal mucosa that might be attributable to the effects of gluten, rectal biopsy specimens from untreated and treated gluten sensitised subjects were analysed morphometrically and by immunohistochemical techniques and were compared with a series of disease control mucosae. Although morphometry showed increased populations of plasma cells, lymphocytes, and mast cells in the mucosae of untreated patients, which were reduced (except for mast cells) by dietary gluten restriction, immunohistochemical techniques were far more sensitive in defining these changes. There were highly significant increases in $CD3^+$ and $\gamma\delta^+$ lymphocytes within both the lamina propria and the epithelium while neutrophils ($CD15^+$ cells) were not at all prominent. Activated ($CD25^+$) lymphocytes expressing interleukin (IL)-2 receptors were increased in lamina propria, usually subjacent to basal lamina, although a few $IL\text{-}2R^+$ intraepithelial lymphocytes were found: other $IL\text{-}2R^+$ cells were deemed to be macrophages ($CD68^+$). These results clearly indicate that in untreated, gluten sensitised subjects the rectal mucosa shows a lymphoplasmacytoid reaction that is responsive to gluten restriction. The absence of neutrophilia suggests that this lesion is not a conventional inflammatory type proctitis, but rather one presumed to be induced by gluten antigen(s) present in the faecal stream – that is, a cell mediated form of response.

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In previous studies from this laboratory,[1-4] the use of computerised image analysis in the numerical quantitation and comparison of jejunal mucosae from patients with gluten sensitivity[5 6] have been described. Mucosal pathology in gluten sensitivity seems to be dependent on T cell mediated reactivity in genetically predisposed subjects.[4 6] In those with severe, flat-destructive (type 3) mucosal lesions[6] and evident malabsorption, however, it would not be surprising if changes in rectal mucosa caused, perhaps, by the passage of residual antigenic fragments of gliadin through the colon, were seen. Indeed, in two of the earliest reports on this subject,[7 8] many gluten sensitised subjects were considered subjectively to have an abnormal rectal mucosa and were thus presumed to be showing a local immune response to dietary gluten proteins.

This subject has also been addressed in two other studies. In one, a retrospective review of 438 patients with coeliac disease and diarrhoea,[9] 14 of a total of 42 rectal biopsies performed (30%) were deemed to be abnormal. Eleven showed severe inflammatory changes (proctitis) and the remainder features reminiscent of ulcerative colitis. In another study of 135 gluten sensitive patients,[10] seven rectal biopsy specimens from a total of 21 biopsies performed were, on subsequent review, considered to be abnormal – two were compatible with 'colitis', four with microscopic lymphocytic colitis, and the seventh a mixed picture. There is a well known, although rare, association between gluten sensitivity and ulcerative colitis, as well as a five to 15 fold increased risk for ulcerative colitis in first degree relatives of patients with coeliac disease,[11 12] which raises the possibility of an aetiological environmental link between both conditions.

In view of these reports indicating some kind of distal colonic abnormality, it was decided to evaluate the rectal mucosa in gluten sensitive patients on a prospective basis: (i) to determine whether there is a coeliac disease associated inflammatory proctitis, (ii) to define its characteristics, and (iii) to analyse the effects of gluten withdrawal.

Methods

PATIENTS

Patients were drawn from the outpatient service of the University Department of Medicine, Hope Hospital, Salford and comprised 38 disease control subjects and 65 patients with proven gluten sensitivity, of whom 32 were untreated at the time of study. Eighteen of the disease control subjects had irritable bowel syndrome, five had bacterial overgrowth of small intestine from pseudo-obstruction (three) or advanced scleroderma (two), and three had Crohn's disease of the terminal ileum. The remainder comprised three patients with iron deficiency, one with carcinomatosis, two with oral aphthous ulceration, two normal subjects considered to have been incorrectly diagnosed with gluten sensitivity as children, one patient with mild microscopic colitis, one patient with giardiasis, and two seemingly normal relatives of patients with coeliac disease.

PROCESSING OF MUCOSAL BIOPSIES SPECIMENS

Specimens were obtained with grab forceps via a rigid, illuminated sigmoidoscope. Specimens

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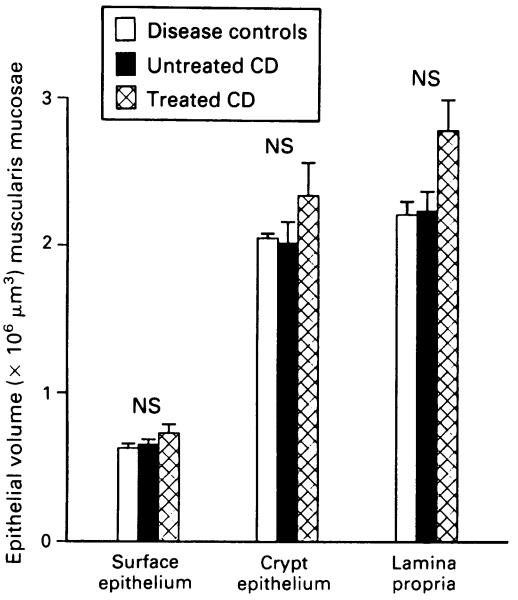


Figure 1: The volumes of each mucosal compartment of rectal mucosa (expressed as $\mu m^3 \times 10^6$ per 10^4 μm^2 test square of muscularis mucosae) showed no differences between disease control subjects and patients with untreated or treated gluten sensitivity.

were gently spread out on a flat surface, flooded with 2·5% glutaraldehyde buffered in 0·1 M sodium cacodylate solution, and fixed for four to six hours. The specimens were then progressively dehydrated in an ascending ethanol series, infiltrated with epon, sectioned at 1 μm in an OMU-3 ultramicrotome, and stained with toluidine blue for morphometric analysis in the computer.

For immunocytochemical analysis, mucosae were oriented on dental wax, embedded in OCT compound, snap frozen in melting isopentane (−160°C), and stored in a liquid nitrogen Dewar. Blocks were subsequently sectioned at 6–7 μm with a Bright cryostat and stored at −20°C. A series of monoclonal antibodies (Dako, High Wycombe, Bucks; T Cell Sciences, Cambridge, MA, USA) was used to quantitate $CD3^+$ and $\gamma\delta^+$ (TCS1) lymphocytes, neutrophils (CD15), macrophages (CD68), and the expression of MHC class 2 alloantigen and of interleukin (IL)2-R (CD25). Monoclonal antibodies were variously employed at dilutions of 1/15–1/50 in a two step indirect peroxidase method, where DAB was used as substrate and NiCl as enhancing agent[13]: nuclei were stained with Mayer's haematoxylin. Sections were mounted in Apathy's medium (BDH, Poole, UK). The APAAP technique was used for CD25 and CD15 with fast red as substrate, and then counterstained and mounted as described above. Double immunostaining was performed with CD3 and CD25, HLA-DR and CD25, CD68 and CD25 using indirect peroxidase and APAAP, as described elsewhere.[13]

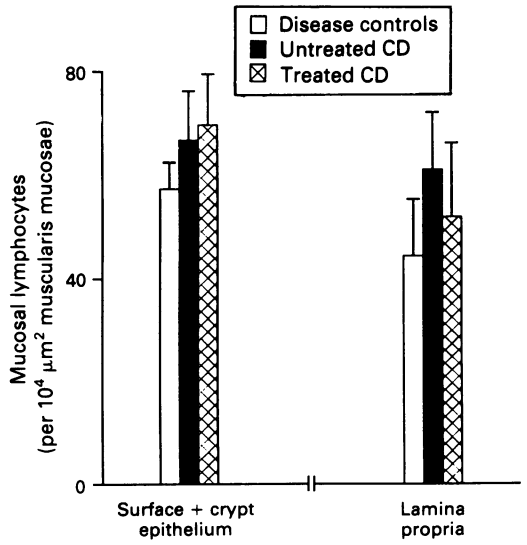


Figure 2: Absolute populations of lymphocytes (per 10^4 μm^2 muscularis mucosae). There were no statistically significant differences between populations either within the epithelium or lamina propria when assessed in toluidine blue stained sections.

ANALYSIS

Measurements were confined to the mucous membrane. The volumes of surface and crypt epithelium, and of the lamina propria, were determined with reference to a standard reference square of muscularis mucosae (10^4 μm^2)[4]: measurements were successively accumulated per specimen until a coefficient of variation <10% was secured.

The cellularity of each specimen was determined in absolute terms for (a) total (crypt+surface) epithelial lymphocytes and (b) lamina propria plasma cells, lymphocytes, mast cells and neutrophils which, from earlier work,[4 14 15] were all assumed to have a mean nuclear diameter of 5 μm. Thus, the total number of sectioned profiles of each cell type observed within the tissue volume overlying 10^4 μm^2 muscularis mucosae was divided by effective section thickness (mean diameter of nuclei plus actual section thickness, or 5+1=6 μm) to obtain the absolute number of cells present within each tissue volume-compartment. Similarly, for immunostained cells, absolute populations were determined with reference to a similar standard reference area of muscularis mucosae (10^4 μm^2): however, these counts were performed at the microscope with a calibrated ocular graticule aligned parallel to the muscularis mucosae.

STATISTICS

Group means for structural components of mucosae were compared by unpaired *t* tests. For cell populations, data were all log transformed and expressed as geometric means (SD) and analysed parametrically. p Values ≤0·05 were taken as significant.

Results

COMPUTER ASSISTED MORPHOMETRIC ANALYSIS

There were no statistically significant differences in volume compartments (volumes of surface epithelium, crypt epithelium, lamina propria) between disease control and gluten sensitive (treated or untreated) patients (Fig 1). Total intraepithelial lymphocytes (surface and crypt epithelium combined) as assessed in 1 μm toluidine blue stained epon sections differed insignificantly from treated patients or disease controls in the untreated gluten sensitivity groups (Fig 2). Populations of plasma cells, increased 10 fold above those of lymphocytes, were responsive to gluten restriction (p=0·02) but mast cell populations remained unchanged (Fig 3). Neutrophils, eosinophils, and basophils

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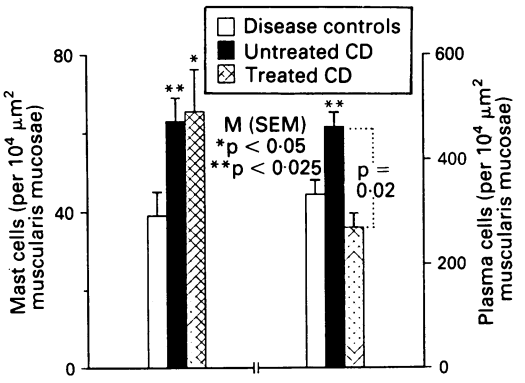


Figure 3: Lamina propria mast cell and plasma cell populations (per 10^4 μm^2 muscularis mucosae). Both showed significant increases in untreated gluten sensitive patients in comparison with disease controls. In the treated patients, mast cells seemed to remain high while plasma cell numbers fell.

were too few to count and could not be analysed further.

With regard to intraepithelial lymphocytes, it should be noted that in absolute numbers there were fewer surface than crypt intraepithelial lymphocytes, but their densities – that is, intraepithelial lymphocytes per 100 epithelial cells – were higher in surface than in crypt epithelium.[16]

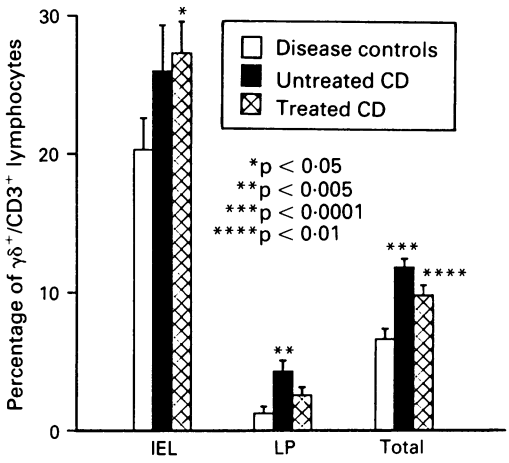


Figure 5: Percentage of $\gamma\delta^+$ lymphocyte populations for intraepithelial (IEL) (surface+crypt), lamina propria (LP), and total, $CD3^+$ lymphocytes per 10^4 μm^2 muscularis mucosae. The percentage of intraepithelial $\gamma\delta^+$ lymphocytes is increased significantly in treated patients, while that of lamina propria $\gamma\delta^+$ lymphocytes is significantly raised in untreated gluten sensitive patients, compared with disease controls. There was a significantly increased percentage of $\gamma\delta^+/CD3^+$ lymphocytes combined for both groups of gluten sensitive patients.

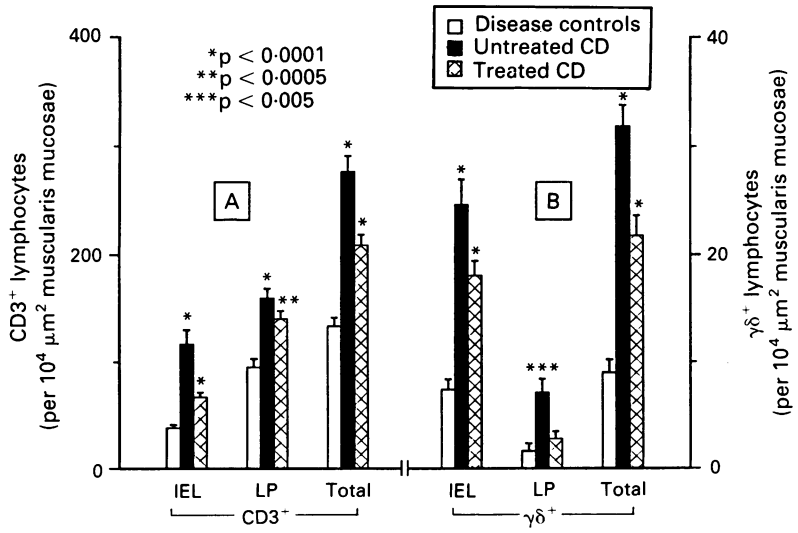
IMMUNOHISTOCHEMISTRY

In contrast to the histological analysis, as described above, populations of $CD3^+$ lymphocytes (per 10^4 μm^2 muscularis mucosae) were all significantly increased in untreated gluten sensitive patients within the epithelium ($p<0·0001$), the lamina propria ($p<0·0001$), and when both compartments were combined ($p<0·0001$) (Fig 4A): similar findings also held for treated patients too when compared with disease control subjects (Fig 4A).

The $\gamma\delta^+$ lymphocytes (per 10^4 μm^2 muscularis mucosae) were all significantly increased in untreated gluten sensitive patients in the epithelium ($p<0·0001$), lamina propria ($p<0·005$), and when both compartments were combined ($p<0·0001$). In treated gluten sensitive patients epithelial $\gamma\delta^+$ lymphocytes but not those in the lamina propria (Fig 4B) were significantly raised ($p<0·0001$) when compared with the disease control group. The percentage of intraepithelial $\gamma\delta^+$ to $CD3^+$ lymphocytes was increased only in treated gluten sensitive patients ($p<0·05$), while that for lamina propria was increased only in the untreated group ($p<0·005$) (Fig 5). Nevertheless, there was a significantly increased percentage of $\gamma\delta^+/CD3^+$ lymphocytes, when combined, for both untreated ($p<0·0001$) and treated ($p<0·01$) gluten sensitive patients (Fig 5).

Only a few $CD15^+$ cells (neutrophils) were observed in lamina propria throughout and populations did not differ significantly between either group. $CD25^+$ (IL-2R expressing) cells were mainly located subjacent to basal lamina, with occasional cells distributed singly throughout the deeper parts of the lamina. Intraepithelial lymphocytes rarely expressd CD25, although when found this was more common in the crypt than surface epithelium. Double staining with CD25 and CD3 showed that many of the T lymphocytes were activated, though it was not technically possible to count these. MHC class 2 positivity was largely confined to subepithelial clusters of macrophage-like cells ($CD68^+$) with foamy cytoplasm: double labelling of these clusters with anti-IL2R (CD25) confirmed the presence of activated macrophages. These appearances, while making enumeration of individual cell types impossible, allowed some estimate of the strength and extent of expression.

Figure 4: (A) $CD3^+$ lymphocytes (per 10^4 μm^2 muscularis mucosae) in the epithelium (IEL), lamina propria (LP), and combined were increased in both compartments in untreated gluten sensitive patients compared with disease controls. (B) $\gamma\delta^+$ lymphocytes (per 10^4 μm^2 muscularis mucosae) showed similar increases in the epithelial compartment for gluten sensitive patients, although only the untreated patients showed significantly raised lamina propria $\gamma\delta^+$ populations when compared with disease control subjects.



Discussion

This study reaffirms our previous work[7 8 17] indicating that the rectal mucosa is abnormal in gluten sensitivity and, in particular, displays features of a lymphocytic proctitis that is responsive to gluten restriction. This proctitis is a gluten driven phenomenon, therefore, and not the result of idiopathic inflammation or, in other words, distal inflammatory bowel disease. The data also show that immunohistochemical techniques were much better than histological analysis, despite measurements performed on 1 μm epon sections observed under oil-immersion optics. No statistically significant differences in lymphocyte populations were

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apparent histologically. This discrepancy arises from the ever present difficulty of deciding whether a particular cell could be either a lymphocyte or part of a macrophage, neutrophil, or fibroblast. In our laboratory the rule adopted is 'ignore if uncertain'. This always results in numerical underestimating, a factor that is clearly not a problem when a specific cell type is identified by a specific marker: then, morphological distinctions become irrelevant.

Contrary to the finding in other types of inflammatory colitis or proctitis,[18] the rectal mucosa in gluten sensitivity lacks any predominance of neutrophils when assessed by either histological staining[7 8 14] or monoclonal antibody recognition (CD15),[19] indicating that the epitheliotropic T cell responses are probably caused by residues of specific antigen present within luminal colonic contents, and hence dependent on the circulatory presence in and recruitment of gluten sensitive T cells to the rectal mucosa. This viewpoint is also corroborated by the brisk response of rectal mucosa to local gluten infusion.[16 20-22] Clearly the coeliac type lymphocytic proctitis, as defined in this study, is an entity entirely unrelated to those (as yet undetermined) mechanisms operative in ulcerative colitis or Crohn's disease, except in the rare concurrence of either condition in the same patient.[9 23-31]

Diarrhoea continues to be one of the most common presenting symptoms of untreated gluten sensitivity.[32] The loose stools are invariably considered to be osmotic in type, due to the passage of unabsorbed solute delivered to the large bowel. It would be difficult to distinguish osmotic effect (jejunal malabsorption) from diarrhoea generated entirely from within the colon, as implied by other workers.[9] Thus, despite the striking familial association between gluten sensitivity and ulcerative colitis,[11 12] it is clear from this study that the gluten driven proctitis is not part of a continuum of inflammatory change which culminates in some individuals in full blown ulcerative colitis. The computerised image analysis showed that there is no architectural difference between gluten sensitivity and disease control rectal mucosae. This is important since it has been shown that in inflammatory bowel disease, architectural disturbances in the mucosa, manifest especially in crypt distortion, are well established by the time of the first clinical episode.[33]

Our failure to show architectural deformity, lamina propria swelling, or neutrophilic infiltrates cautions against too ready acceptance of the gluten induced proctitis as a major contributor to diarrhoea. It is not uncommon, however, for these patients to present initially with watery diarrhoea and lower abdominal pain which together may suggest a colonic based pathology, especially if other clues like iron deficiency, folate deficiency, or features of splenic atrophy are either not immediately evident, or overlooked. Our observations also indicate that in the presence of a lymphocytic colitis[10 34] despite its continuing uncertain nosology,[35-37] gluten sensitivity should always be excluded.[38] Conversely, it is also important to realise that if diarrhoea starts again or even occurs for the first time in a patient treated by gluten restriction, the patient should be investigated for colonic disorders,[24 28] since the answer may not simply or necessarily rest with dietary lapses, an unresponsive phase, or lymphoma. Finally, in this regard it should be recalled that although gluten sensitivity never presents with rectal bleeding, patients were seen to develop an acute erythematous/haemorrhagic proctitis[8] after massive rectal challenge with gluten or its derivatives.

Apart from our own prospective studies[16 20] there seems to have been only one other approach to rectal mucosa in gluten sensitivity[39] based on a retrospective analysis of intraepithelial lymphocytes in material obtained 25 years previously.[8] These data, as presented, are difficult to interpret because (i) fewer crypt intraepithelial lymphocytes than surface intraepithelial lymphocytes (per 100 enterocyte nuclei) were found; (ii) no detectable response to gluten/gliadin challenge, contrary to other findings,[17 20] was observed; and (iii) data were analysed and presented in terms of total specimens counted rather than numbers of patients from whom all such specimens derived. Thus, all this study[39] seems to tell us is that it is rather dangerous to evaluate rectal intraepithelial lymphocytes in terms of enterocyte nuclei.

In line with various studies of jejunal tissue,[40] our data have shown a predominance of $\gamma\delta^+$ TCR intraepithelial lymphocytes within the expanded population of $CD3^+$ T lymphoctyes distributed throughout surface and crypt epithelium of rectal mucosa.[19 21 22] It was not easy, however, to show whether, and to what extent, these cells were 'activated'. Certainly some $\gamma\delta^+$ intraepithelial lymphocytes in rectal crypts were $IL2R^+$ but within the lamina propria the MHC class 2^+ collections of cells, most probably ($CD68^+$) macrophages,[41] made identification difficult. Since surface enterocytes were positive for MHC class 2 display, it is possible to infer that T cells in gluten sensitive mucosae were activated in order to bring about this effect, possibly via IFN-γ,[42] and because surface cells in normal colon, are generally negative for these alloantigens.[43] However, these aspects of the immune response to gluten will be addressed in depth in a subsequent paper in which the immunopathological responses in rectal mucosa to local antigenic challenge will be further detailed.

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