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Morphometric analysis of small intestinal mucosa

II. Determination of lamina propria volumes; plasma cell and neutrophil populations within control and coeliac disease mucosae*

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Summary. Morphometric techniques were employed to measure (i) lamina propria volumes and (ii) the absolute numbers of neutrophils and plasma cells, of A, M and G isotype, within the lamina propria of jejunal mucosa. Mucosal specimens were obtained with a Watson capsule (a) from 5 patients with untreated coeliac disease, and again at least 3 months after starting on a gluten-free diet, and (b) from 9 control individuals.

Lamina propria volume of untreated coeliac mucosa ($2.5 \pm 0.17 \times 10^6$ μm^3) was increased 2.3-fold ($p < 0.01$) above that of control mucosae ($1.35 \pm 0.08 \times 10^6$ μm^3). Compared with control mucosae, there was a 20-fold increase of neutrophils in untreated coeliac mucosae ($p < 0.005$). The total complement of all plasma cells in untreated coeliac mucosae (309) was twice that (149) of control mucosae. The populations of each isotype were also significantly increased over controls by factors of 1.6 (IgA; $p < 0.05$), 3.0 (IgM; $p < 0.01$) and 3.5 (IgG; $p < 0.01$). Their percentage distributions in untreated coeliac mucosae (A:M:G – 52:43:5) differed from those in control mucosae (A:M:G – 69:28:3) but were restored after treatment with a gluten-free diet.

However, when each isotype was expressed per unit volume of lamina propria, there was an apparent fall ($\times 1.4$) in IgA cells, while the increase in IgM and IgG cells was less marked i.e. $\times 1.4$ and $\times 1.5$ respectively. These precise measurements explain why many previous investigators found a paradoxical fall in IgA cells because the (increased) volume of distribution of these cells was not taken into account. The importance of morphometric techniques in achieving valid cell 'counts' within the intestinal mucosa is thus illustrated by this study.

Key word: Morphometry – Lamina propria – Plasma cell – Neutrophil – Coeliac disease

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There is ample reason for supposing that coeliac disease results from local immunologically-mediated reactions to gluten in genetically predisposed individuals (Booth et al. 1977; Strober 1978; Marsh 1981). The demonstration that anti-gluten antibody is actively synthesised by coeliac mucosa in vitro (Falchuk and Strober 1974) has strengthened the view that local humoral events may play an important role in pathogenesis (Booth et al. 1977), despite the fact that antibody production towards luminal antigen of dietary or microbial origin is a normal physiological function of the small intestinal mucosa.

Many studies, all employing the immunofluorescent technique, have been devoted to quantitating immunoglobulin (Ig)-producing cells within coeliac mucosa (Douglas et al. 1970; Soltoft 1970; Pettingale 1971; Savilahti 1972a; Jos et al. 1972; Gasbarini et al. 1974; Lancaster-Smith et al. 1974; Brandtzaeg and Baklien 1976). With one exception (Brandtzaeg and Baklien 1976) Ig-cells were expressed in terms of unit area, unit volume or even per high-power field of lamina propria (Douglas et al. 1970; Soltoft 1970; Pettingale 1971; Savilahti 1972a; Jos et al. 1972; Gasbarini et al. 1974; Lancaster-Smith et al. 1974). Such measurements only provide cell densities, and not 'absolute' data about the actual size of the cell population. Secondly, they fail to take account of possible alterations in the volume of lamina propria that occur in coeliac mucosae (Guix et al. 1979). Because of this the results obtained in previous studies are not strictly valid, thus probably accounting for the wide variations in the extent to which cells of each major isotype are thought to be altered in untreated coeliac disease.

A further problem concerns sampling error. Plasma cells are focally, and not uniformly, distributed throughout the lamina propria so that the use of a calibrated grid or projection technique may not provide a sufficiently large, or representative sample of plasma cells to overcome this important source of error. Furthermore, direct use of a graticule at the microscope may make it difficult, or even impossible, to obtain a completely unbiased sample of lamina propria because the frame overlaps other unwanted structures, i.e. crypt epithelium or lumen (Marsh 1984)

We have therefore re-investigated this problem by using a valid, comparative morphometric technique (Marsh 1980; Niazi 1982) applied to peroxidase-labelled preparations for each major Ig isotype. In addition, the volume of lamina propria per specimen was measured, thus permitting comparison between absolute cell counts, and counts calculated per unit volume of lamina propria. The opportunity was also taken to evaluate populations of acute (neutrophil) inflammatory cells within coeliac mucosae compared with controls. Finally, our observations have provided base-line data for current studies evaluating the effect of acute gluten challenge on Ig-producing cells; similar studies, to date, have only involved gluten-sensitive children (Scott et al. 1980).

Materials and methods

Normal jejunal mucosa was obtained from 4 healthy volunteers and from 5 patients with non-specific gastrointestinal symptoms. Five patients with untreated coeliac disease were stud-

ied, and again at least 3 months after commencing a gluten free diet. The initial diagnosis was made by accepted criteria (Booth 1970) in the presence of a flat mucosa, in which the mitotic index of epithelial lymphocytes exceeded 0.2% (Marsh 1982); furthermore, all mucosae were subsequently shown to deteriorate after a controlled gluten challenge.

Mucosal specimens were obtained with a Watson capsule located fluoroscopically at the duodeno-jejunal flexure. After rapid retrieval and orientation, they were processed either in plastic or paraffin wax, as described previously (Marsh 1980).

1 μm plastic sections stained with toluidine blue were used for the measurement of mean cell diameters and for the calculation of lamina propria volumes. 5 μm wax sections were hydrated and reacted with rabbit anti-human IgA, IgM or IgG (1:5,000; 1:2,000; 1:5,000 respectively) for 30 min followed by swine anti-rabbit antibody and rabbit anti-peroxidase (PAP) complexes; peroxidase was subsequently localised by incubation with DAB and H_2O_2. All immunoreagents were obtained from Dakopatts (Denmark).

Quantitative morphometry

All measurements were carried out relative to a standard test area (10^4 μm^2) of muscularis mucosae (Marsh 1980; Marsh et al. 1983).

1. Volume of lamina propria. From 1 μm toluidine-blue stained sections photographic montages at ×300 magnification were constructed for each specimen. The total length of muscularis mucosae required to determine the volume of lamina propria overlying the test area was thus $10^4 \times 300$ μm, or 300 cm, assuming photographic paper to be equivalent to 1 μm thickness. The weight of all lamina propria tissue above this length of muscularis was cut from the montage, and compared with that of known areas of paper, cut from the same montage. In this way volumes of lamina propria for each specimen were individually determined.

2. Enumeration of cell types within lamina propria. A. Measurement of mean nuclear profile diameter (MNPD): It is more convenient to measure nuclear, rather than overall cell diameters, since the former are more nearly circular in sectioned profile. Imperfect (non-circular) nuclear profiles were approximated by eye which can be achieved with considerable accuracy (Miles and Davy 1977). Profile diameters were measured directly through ×100 oil-immersion objective with a calibrated graticule. Successive cohorts were continuously monitored until a constant mean value was obtained; at least 200 nuclear profiles required measurement to achieve this end.

B. Calculation of "true" nuclear diameter: The MNPD, as determined above, is always smaller than the true diameter (D) of the actual particles:

One reason is that the MNPD distribution is deficient in its lower one-third (Weibel, 1979) partly because smaller profiles are sectioned less often than larger ones, and thus tend to be lost from tissue sections. To correct for these losses, a histogram is constructed and a smooth distribution curve drawn (Weibel 1979); the point half-way between the baseline and its peak value is joined to the origin, to which the smaller 'missing' profiles are added. The new mean of the corrected distribution (d) is calculated.

A second reason is that only a small proportion of the total profiles measured is actually sectioned through their equators, which also results in an underestimate of true nuclear diameter: the apparent (d) and real diameter (D) are related by: $D = 4/\pi \cdot d$ (Weibel 1979).

This final value (D) for mean nuclear diameter of plasma cells was found to be 4.89, 5.18 and 5.11 μm for each of one control; untreated; and treated coeliac mucosal specimen, respectively. A nominal value of 5 μm was therefore taken to represent actual plasma cell nuclear diameter for every specimen analyzed. This figure was also used for neutrophils, since their lobulated nuclei are not available for direct profile measurement.

C. Effective section thickness (T): The number of cells contained within any volume compartment of the mucosa is not simply the number of profiles observed in a series of histological sections, since each profile represents a fraction of the whole structure. A nucleus of radius r will occupy a greater width of tissue than that of the actual section in which it appears (Weibel, 1979); indeed, all particles (nuclei) of radius r are contained within a 'superslice'

determined by the relationship $T = D + t$, where D is true particle diameter (5 μm) and t is actual section thickness (5 μm): thus for this study, T was 10 μm.

Hence, in order to quantitate selectively-stained plasma cells overlying the 10^4 μm^2 test area of muscularis mucosae, the side of the test area (100 μm) must be divided by T (=10 μm) so that the correct number of applications of the 100 μm graticule to the sections can be determined. While it is realised (McIntyre and Piris, 1981) that the peroxidase developed on the surface of a section alters its thickness relative to T, this effect could not be measured and was ignored throughout the analysis; its effect on the final enumeration of cells is likely to be small.

With this approach, the total number of plasma cells of each isotype, together with neutrophils, within the lamina propria was determined for each specimen.

Results

1. Lamina propria volumes

For 9 control specimens, mean volume of lamina propria was 1.35 ± 0.08 μm^3 which differed significantly ($p < 0.01$) from that of untreated coeliac mucosa (3.12 ± 0.16) which was increased more than two-fold ($\times 2.3$). The volume for the untreated coeliac group (2.50 ± 0.17) was intermediate but significantly reduced compared with the untreated mucosa ($p < 0.05$).

2. Lamina propria cell populations

A. *Plasma cells.* The total complement of plasma cells (A+G+M) in untreated coeliac mucosae ($309/10^4$ μm^2 muscularis mucosae) was increased two-fold over control specimens ($149/10^4$ μm^2 muscularis mucosae) ($p < 0.01$).

When considered separately in terms of isotype, each population of plasma cells was significantly increased in untreated patients compared with controls (Fig. 1). For IgA, IgM and IgG cells the absolute increases were $\times 1.6$ ($p < 0.05$), $\times 3.0$ ($p < 0.01$) and $\times 3.5$ ($p < 0.01$) respectively.

For control mucosae, the percentage distribution A:M:G was 69:28:3; for untreated coeliac mucosae 52:43:5 and for treated specimens 68:29:3. Thus, after at least 3 months treatment with gluten restriction, the percentage distribution had already normalized to that of control mucosae.

Although there were significant increases in the absolute numbers of each isotype in coeliac mucosae, the increase in IgM and IgG cells was considerably greater than for IgA cells.

When the individual figures were re-expressed in terms of unit volume of lamina propria, the results showed surprising differences. Although the same percentage distribution (A:M:G – 52:42:5) was retained, there was an apparent *fall* in IgA cells (reduced by 1.4 times) while the rises in IgM cells ($\times 1.4$) and IgG cells ($\times 1.5$) were considerably *smaller* than those resulting from measurement of absolute numbers of plasma cells.

B. *Neutrophils.* These were recognised in 1 μm plastic sections by their densely-staining nuclear chromatin and dark greenish-grey cytoplasm containing numerous small, green-staining granules.

There was a highly significant rise ($p < 0.005$) in the absolute number

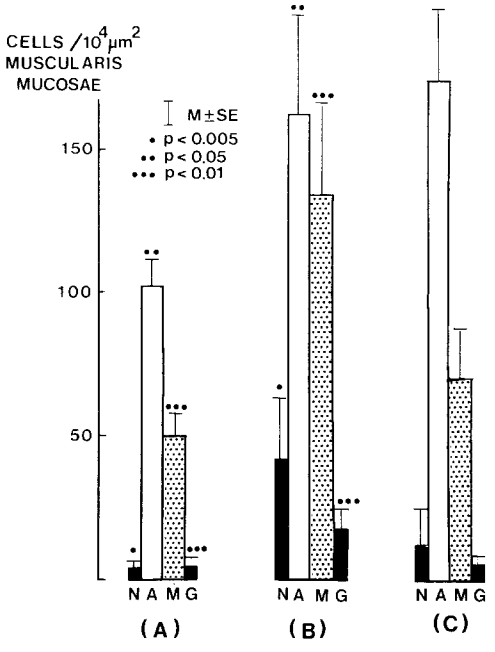

Fig. 1 A–C. This diagram illustrates absolute counts of neutrophils (*N*), and of plasma cells of IgA (A), IgG (*G*) and IgM (*M*) isotype within the lamina propria of 9 control (**A**), and 5 untreated (**B**) and treated (**C**) coeliac patients. The number of cells in each population was obtained morphometrically with respect to a constant test area (10^4 µm^2) of muscularis mucosae per specimen

of neutrophils in untreated coeliac mucosae compared with control ($p < 0.005$), and post-treatment ($p < 0.0025$), specimens (Fig. 1). In real terms, the total population of neutrophils, relative to 10^4 µm^2 muscularis mucosae, was increased 20-fold in untreated coeliac mucosae.

Discussion

This study illustrates application of a morphometric technique to the measurement of lamina propria volumes, and analysis of cell populations contained therein, with reference to a constant test area (10^4 µm^2) of muscularis mucosae.

In untreated coeliac mucosae, lamina propria volumes were increased two-fold ($\times 2.3$) over control mucosae, thus confirming previous results obtained by point-counting (Guix et al. 1979). These volumes may be more easily understood if represented as *cubes of equivalent volume* (Marsh 1984) thus permitting their areal faces to be drawn accurately to scale (Fig. 2). Shown in this way, it is immediately apparent that the area of a planar grid (A, or L^2) should be proportional to the volume (V, or L^3) of tissue to be analyzed.

Most previous workers have failed to make allowance for possible changes in lamina propria volume when studying plasma cell populations in coeliac mucosae, relative to controls. Hence, by comparing identical areas, or volumes, they have exposed themselves to the risk of underestimating the actual numbers of each isotype within coeliac mucosa. As shown in Fig. 2, the proportional difference in areas is almost two-fold ($\times 1.7$) which clearly emphasises the degree to which cell numbers will be underestimated. In addition, the 'dilution' of cells per unit volume of tissue by edema fluid

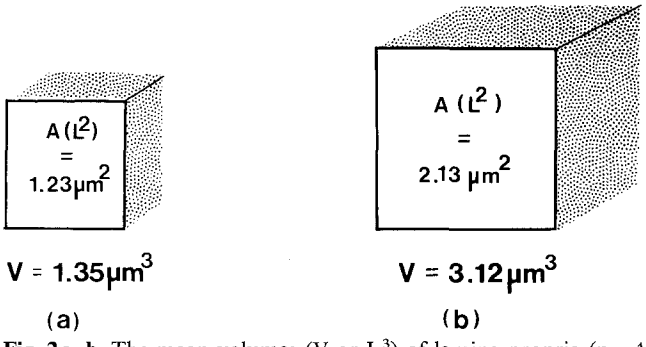

Fig. 2a, b. The mean volumes (V or L^3) of lamina propria (per 10^4 μm^2 muscularis mucosae) for control (**a**) and untreated coeliac mucosae (**b**) are here illustrated as *cubes of equivalent volume* i.e. 1.35 μm^3 and 3.12 μm^3 respectively. The areal faces (A or L^2) of each cube are drawn precisely to scale i.e. 1.23 μm^3 and 2.13 μm^2 respectively. In this way it is evident how lamina propria cells in untreated coeliac mucosa could be grossly underestimated when either grids, or projected planimetric areas, of identical size are used for counting purposes. These areas should, in fact, be proportional to their respective volumes

(which in part accounts for the increased lamina propria volume in coeliac mucosae) provides another additive, although neglected, factor that also depresses cell counts when measured planimetrically.

Conversely, population sizes may be overestimated if effective section thickness (that tissue 'superslice' containing the entire (nuclear) structure seen in profile within actual tissue section) is not determined. Furthermore, attention must be paid to the way in which 'true' nuclear diameters are determined, by correcting for lost profiles and non-sagittal (i.e. non-equatorial) sectioning. It is difficult to elucidate just how the balance between the effects of these opposing factors finally influences the results of previous authors' studies as recently tabulated and recalculated by Brandtzaeg and Baklien (1976).

However, when cells are enumerated within known tissue units, as defined by measurements of muscularis mucosae (this study; Brandtzaeg and Baklien 1976; Scott et al. 1980), the need to correct for volume changes is avoided, since 'absolute' figures are obtained. With the technique herein described, a 20-fold rise in neutrophils and a 2-fold increase in the total plasma cell population of untreated coeliac disease mucosa was demonstrated, while differential counts revealed significant increases in each Ig-isotype.

Since lamina propria volumes were measured in this study, the data could be recalculated as number of Ig-cells per unit volume. Expressed in this way, there was a marked *decrease* in the number of IgA cells, while the apparent rises in M and G cells were much less than the absolute rises obtained when correct morphometric procedures were used. Mathematical manipulations of the results in this manner illustrates and explains clearly why previous investigators have found a paradoxical fall in IgA cells in coeliac mucosae (Douglas et al. 1970; Soltoft 1970a; Pettingale 1970; Gasbarini et al. 1974; Lancaster-Smith et al. 1974).

On the other hand, it is difficult to explain the divergence between our results and those of Brandtzaeg and Baklien (1976) whose technique is

also independent of tissue volume, and probably also of section thickness. Although their overall totals for plasma cells, like those of Scott et al. (1980), both for controls and coeliac patients are virtually identical to ours, the percentage isotype distributions differ widely, such that our percent rise in IgG cells is only one-half that described by these workers.

Again, while their work (Brandtzaeg and Baklien 1976; Scott et al. 1980) suggests a role for IgG immunoglobulin in coeliac disease, our observations showed that IgG cells fell to normal within 3 months of gluten restriction, although IgA and IgM cells remained somewhat elevated. Although antigluten antibody of A and M isotype is produced in vitro (Falchuk and Strober 1974), it is not known whether IgG class antigluten antibody is synthesised or could play a role in phlogistic mechanisms damaging to the mucosa. That classical coeliac disease may apparently occur in the presence of marked immunodeficiency (Webster et al. 1981), although the mucosal lymphocytes in such patients behave like those of other coeliacs (Marsh and Haeney 1983), argues against a major pathogenetic role for any isotype, including IgG, in the production of the 'flat' coeliac lesion.

It is also important to realise that the major proportion of the increased number of plasma cells is probably polyclonal in origin, and hence non-specific. Similar qualitative rises occur in gastroenteritis (Soltoft and Soeberg 1972), cows milk protein intolerance (Savilahti 1972b) as well as in ulcerative colitis and Crohn's disease (Soltoft 1969). The number of specific antiglobulin cells within the mucosa after antigen challenge is always surprisingly small, as originally shown in mice following oral immunization with ferritin (Crabbe et al. 1969).

Clearly, much more needs to be learned of the specificity of local humoral antibody production in coeliac disease, particularly regarding antigliadin activity. Until such knowledge is obtained, views about the pathogenesis of coeliac disease (Booth et al. 1977; Strober 1978; Marsh 1981), and of mechanisms concerned in the production of mucosal flattening (Marsh 1983), must remain tentative. The methods described here provide a sound basis for further accurate quantitative studies of Ig-cells in coeliac mucosa.

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