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Morphometric analysis of small intestinal mucosa

I. Methodology, epithelial volume compartments and enumeration of inter-epithelial space lymphocytes*

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Summary. Morphometric techniques for quantitating cytologic and volumetric changes in small intestinal mucosae are described: measurements were controlled with respect to a constant 'test area' of muscularis mucosae, thus allowing valid comparisons between normal and damaged mucosae. Procedures for enumerating cell populations within mucosal volume compartments are illustrated by analyzing lymphocytes within surface epithelium. These techniques serve to distinguish (i) absolute cell counts from (ii) relative cell counts ("densities") the latter being shown to be considerably affected by changes in epithelial volume. They also permit construction of mathematical models e.g. cubes of equivalent volume, volume-density graphs, proportional volumes, which are illustrated.

Use of these morphometric procedures showed that there is no major difference in epithelial lymphocyte populations between untreated coeliac disease, and control, mucosae. The data thus fail to support the widespread view that "infiltration" of coeliac disease epithelium by lymphocytes represents a local cell-mediated immune reaction to gluten.

Key words: Morphometry – Epithelium – Epithelial lymphocyte – Coeliac disease – Jejunum

Introduction

The widespread use of the peroral biopsy technique has revolutionised the investigation, description and understanding of many small intestinal diseases. The literature now contains a vast body of information gained from the application of histological (Trier 1968; Watson and Wright 1974), cyto-

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chemical (Padykula et al. 1961), transmission (Trier and Rubin 1965; Rubin et al. 1966; Shiner 1983) and scanning (Marsh et al. 1970; Marsh 1972) electron microscope techniques to human biopsy material.

Despite much descriptive literature, only a few attempts have been made to apply methods of morphometric analysis to the "measurement" of small intestinal mucosa (Aherne and Dunnill 1982). Whitehead (Guix et al. 1979) used point-counting techniques derived from Chalkley and others (1949) while others (Chapman et al. 1973; Slavin et al. 1980) have used various types of computerised technology. However interest in this kind of analysis has been slow to develop so that intestinal morphology is still often described in descriptive, or at best, semi-quantitative, terms.

Since computer-aided forms of instrumentation will assume an ever-increasing role in future histological analysis, it seems important to devise methods that are easily adaptable to such machines so that more rapid and accurate measurements can be achieved while relieving the individual of time-consuming and repetitive work at the microscope which good morphometry always demands (Marsh 1984).

The methods of analysis herein described have evolved in our laboratory over many years and are directly applicable to computerised image-analysis systems that are now commercially available. Here measurements of mucosal volume compartments within small intestinal mucosal biopsies of coeliac disease (CD) patients compared with those of control subjects are reported. Furthermore techniques necessary to enumerate the total complement of cells within any defined mucosal compartment, taking epithelial lymphocytes (EL) as an example, are described.

Materials and methods

1. Patients studied

There were 10 control subjects whose investigation required biopsy of small-intestinal mucosa but in whom gluten-sensitivity was not suspected or considered to be diagnostically relevant. All such specimens were assumed to be histologically normal by accepted criteria (Rubin et al. 1960).

Twenty CD mucosae were also studied, of which 10 specimens were obtained before treatment. These were all 'flat' and showed the characteristic histological appearances of untreated CD disease (Rubin et al. 1960). A further 10 specimens were obtained during treatment with a gluten-free diet (range 3 month–6 years) to which all patients showed objective evidence of response, either clinically, biochemically or morphologically. Several patients also showed mucosal deterioration after controlled gluten challenges.

A total of 30 biopsies was available for morphometric analysis.

2. Histological technique

Jejunal biopsies were performed with a Watson capsule located fluoroscopically just beyond the duodenal-jejunal flexure. After rapid retrieval, the mucosa was quickly orientated on dental wax, flooded with cacodylate-buffered 2.5% glutaraldehyde, embedded in epon or araldite, sectioned at 1 μm thickness with a Reichert OMU-3 ultramicrotome and stained with toluidine blue.

Five to six sections were mounted per slide and 10 μm steps of tissue were discarded between successive sections. Selected sections (one only per slide) were observed through a × 100

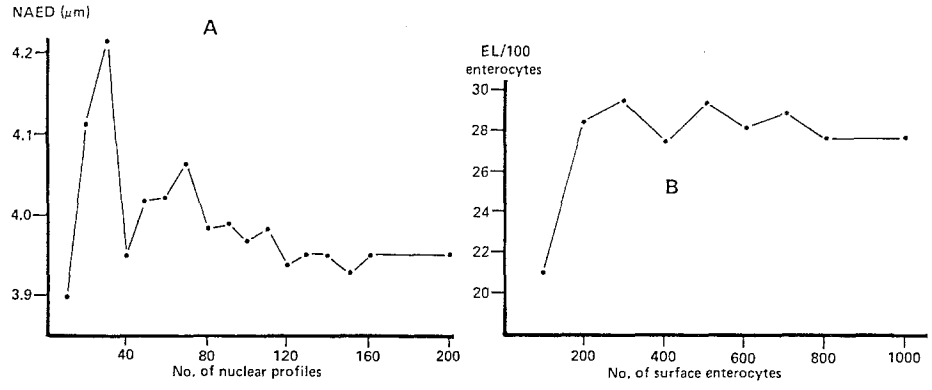

Fig. 1 A, B. The mean ±SD of successive cohorts of measurements is determined until constant. In **A**, at least 200 lymphocyte nuclear "profile" diameters (NAED) must be measured, while in **B**, 1000 enterocytes are required to yield a constant value for %EL.

oil-immersion objective with an Olympus BHS-2 microscope. Photographs were taken on Ilford PanF 35 mm film through either Zeiss Photomicroscope III or Olympus BHS-2 research light microscopes. Photographic montages of the original 35 mm frames (final magnification ×300) were used for analysis.

3. Quantitative methods

A. Volume compartments of mucosa (Surface Epithelium, V_E: and Crypt Volume, V_{CR}).

All measurements were carried out relative to a standard test area i.e. $(100\ \mu m)^2$ of muscularis mucosae: thus total length of photographic montage required to determine epithelial volumes was $(100)^2$ multiplied by magnification factor (×300), or 3×10^6 μm (300 cm). Photographic paper was assumed equivalent to 1 μm section thickness (t). Areal weights (M_p) of profiles of surface, and crypt, epithelium were compared with those (M_0) of known standard control areas (So) cut from the same montage per specimen, based on the original method of Hammar (1926). In this way V_E, and V_{CR}, were determined for each specimen.

B. Enumeration of inter-epithelial space lymphocytes (N)

I. Mean nuclear area equivalent diameter (NAED). It is more convenient to measure nuclear, rather than cell, diameters since they are more nearly circular in sectioned profile: sectioned "profile" diameters of nuclei were measured directly with a calibrated ocular graticule through a ×100 oil-immersion objective. Imperfect (noncircular) nuclear profiles were approximated by eye, which can be achieved with considerable accuracy (Miles and Davey 1977). It follows that only lymphocytes displaying part of a nuclear profile were counted.

The mean values of successive cohorts of measurements were continuously monitored until constant (Fig. 1A); measurement of at least 200 lymphocyte profile diameters was necessary to obtain a constant mean value, termed "mean nuclear areal equivalent diameter" (NAED).

II. Mean actual nuclear diameter ($\bar{D}$). Mean NAED is smaller than the true diameter, ($\bar{D}$). One reason is that the NAED distribution is deficient in its lower one-third, partly because smaller profiles are sectioned less often than larger ones, and because smaller profiles tend to be lost from tissue sections (Elias et al. 1971). The data are thus displayed as a histogram and a smooth distribution curve drawn (Fig. 2): a point half-way between the peak value is identified and joined to the origin: the 'lost' profiles are then added and scaled up to meet that line and the new 'mean' diameter (d) calculated (Giger and Riedwyl 1970).

The second reason is that only a small proportion of nuclei are sectioned through their

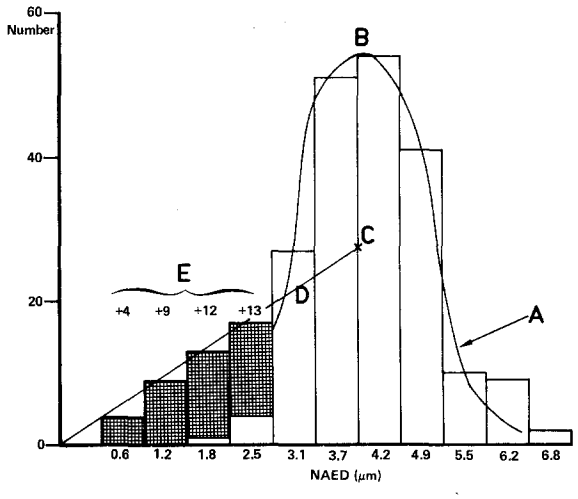

Fig. 2. In correcting for small, and "lost" profiles, a histogram of values for a measured sample (200 lymphocyte nuclear profile diameters) is drawn together with distribution curve, *B*. The halfway value (*C*) of the mode (*B*) is joined to zero, and the deficient part of the distribution (*shaded blocks*) is added. The new mean, which is thereby calculated, is subsequently multiplied by $4/\pi$ to correct for sectioning errors.

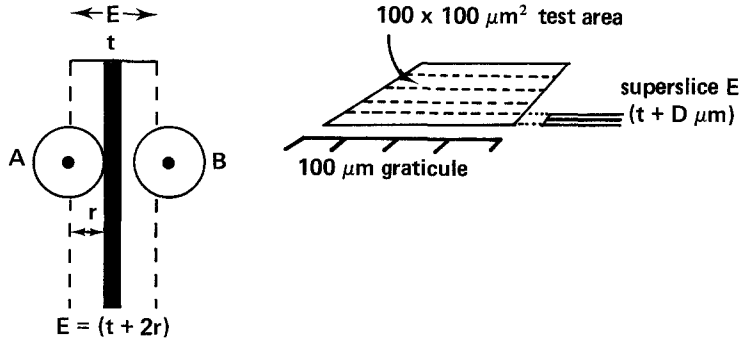

Fig. 3. Profiles of spheres (i.e. nuclei) of radius r (*A*) will appear in tissue section t if their centers lie within superslice t+2r: spheres (*B*) whose centers lie outside this superslice (*E*) will not have profiles in section t. In order to determine correct number of cells in tissue compartment overlying 100×100 μm^2 test area, width of test area is divided by E (100/E) to obtain the total number of applications of the 100 μm grid required to effect the count

equators, which also underestimates real nuclear diameter, $\bar{D}$. $\bar{D}$ is obtained by multiplying d by $4/\pi$ (Weibel 1979).

III. Effective section thickness. Once $\bar{D}$ has been obtained, the number of cells contained within a defined volume compartment of the mucosa, relative to the 10^4 μm^2 test area of muscularis mucosae, is determined. In practice, an ocular graticule (100 μm in length) is orientated parallel to the muscularis mucosae, and the number of nuclear profiles overlying the grid length is counted. By re-aligning the grid in another part or the same, or succeeding sections, the count is repeated until the total number of cells contained within that volume compartment is obtained.

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. It has been shown that all the particles (i.e. nuclei) of radius r, whose profiles appear in a section of finite thickness, t (i.e. 1 μm) are contained within a 'superslice' of thickness t+2r. In other words, all nuclear profiles observed in a section thickness t μm have their centers within the superslice and conversely, those profiles whose centers lie outside it do not exhibit profiles in section t (Fig. 3). The thickness

of the superslice within which all the observed profiles lie is termed the *effective section thickness* (EST). Thus $EST = t + 2r\ (t + \bar{D})$. By these procedures the absolute number of lymphocytes overlying the test area was obtained; this value is independent of epithelial volume, V_E, (Fig. 3).

C. Lymphocyte density

In addition to estimating the epithelial lymphocytes as a finite count (N) within an epithelial volume, V_E, they were also expressed in terms of density by the following two methods:

I. Numerical density (N_V). This was obtained mathematically from the relation $N_V = N/V_E$. Since V_E and N were previously determined for each specimen, no additional measurements were necessary.

II. Lymphocytes per 100 Epithelial cells (%EL). This was performed as previously described (Ferguson and Murray 1971; Otto 1972): by the method of cumulative means (Fig. 1B) it was necessary to count epithelial lymphocytes relative to 1000 enterocyte nuclei to obtain a constant ratio; results were expressed as EL/100 enterocytes (%EL).

D. Density-volume curves

The relationship $N_v = N/V_E$ was explored for control and untreated CD mucosae by plotting values for V_E (other than that derived from the actual specimen) against corresponding calculated values of N_v, assuming N to be constant. In addition, for five paired CD mucosae, values of V_E and N_v for treated specimens were related to density-volume curves derived from measuring the corresponding untreated specimen.

E. Proportional volumes

The proportion of total surface epithelial volume (overlying 10^4 μm² muscularis mucosae), V_E, that was occupied by 100 enterocytes (V_{100}) was calculated:

V_{100} = h.w. 100 t where t = section thickness (1 μm), and h and w represent mean height and width of enterocytes. Thus, proportional volume = V_{100}/V_E.

A similar value for the proportional volume of enterocytes overlying 100 μm length of basement membrane (V_{bm}/V_E) was calculated:

V_{bm} = 100 ht where h is mean height of enterocytes per specimen and t = section thickness (1 μm).

Results

1. Epithelial volumes ($V_E + V_{CR}$). There were highly significant differences between the volume of surface epithelium and crypt epithelium, relative to 10^4 μm² muscularis mucosae, between control and untreated CD mucosae ($P < 0.001$) (Fig. 4). Values for V_E and V_{CR} during treatment occupied intermediate positions, but were significantly different from corresponding volumes of untreated mucosa ($P < 0.005$). V_E for untreated CD was 2.5 times less than V_E for controls, and V_{CR} for untreated CD crypts was 2.4 times greater than V_{CR} for controls.

2. EL/10^4 μm² muscularis mucosae (N).

Values for N in each group of subjects did not differ significantly (Fig. 4).

Figure 4 also contains two additional control columns (A, B). Those in A and formerly published (Marsh 1980) were recalculated taking effective

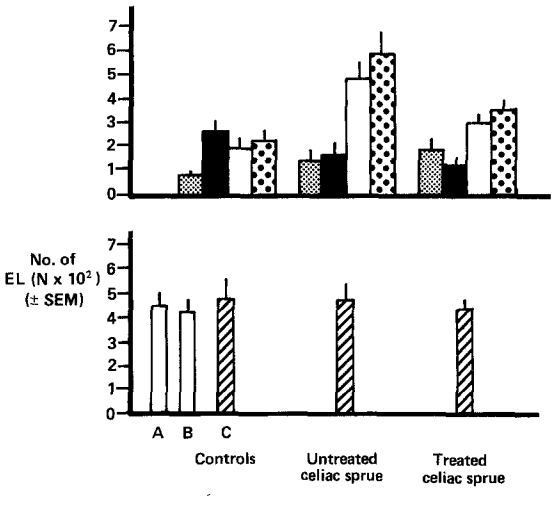

Fig. 4. In the upper panel crypt, and surface, epithelial volumes are shown for controls, and treated and untreated CD patients. Also shown are results of EL densities (expressed both as numerical density, N_v, and %EL) which vary inversely with surface epithelial volume. However the absolute number of lymphocytes (N) within each group of subjects *(lower panel, cross-hatched bars)* per $10^4 \mu m^2$ muscularis mucosae is constant ▨ = Crypt volume ($\times 10^6$ μm^3); ■ = Surface epithelial volume ($\times 10^6$ μm^3); ▭ = Numerical density ($\times 10^{-4}$ μm^{-3}); ⊡ = IEL/100 enterocytes ($\times 10$)

section thickness into account, and related to the revised standard test area of 10^4 μm^2 muscularis mucosae. The other set of data (B) refer to a group of 8 South Indian controls in a study of tropical sprue (Marsh et al. 1983); this shows that surface epithelium of 'normal' tropical controls does not contain an increased number of EL, compared with Western control subjects.

3. Epithelial lymphocyte densities

A. Numerical density (N_v). Numerical densities were derived from the foregoing values for V_E and N. In untreated CD mucosae, N_v was increased compared with control mucosae (Fig. 4).

B. Lymphocytes/100 enterocytes. %EL were also raised in untreated CD, and paralleled values for N_v (Fig. 4).

For control mucosae, correlations of N vs %EL ($r = 0.963$; $P < 0.05$) and N vs N_v ($r = 0.944$; $P < 0.05$) were highly significant, whereas similar correlations for untreated CD mucosae did not achieve statistical significance ($r = 0.659$ and 0.679, respectively).

4. Density-volume curves

These curves are illustrated in Fig. 5a,b. In Fig. 5a paired values of N_v against V_E for 5 CD patients before and during dietary treatment fell within the mean ± 1 SD for 10 control mucosae. For the same CD patients (5b) points for N_v and V_E, obtained by analyzing biopsies after treatment had begun, were related to those predicted by the density-volume curve projected from values of N and V_E obtained for each of the original untreated specimens. There was close agreement between predicted and actual values, thus

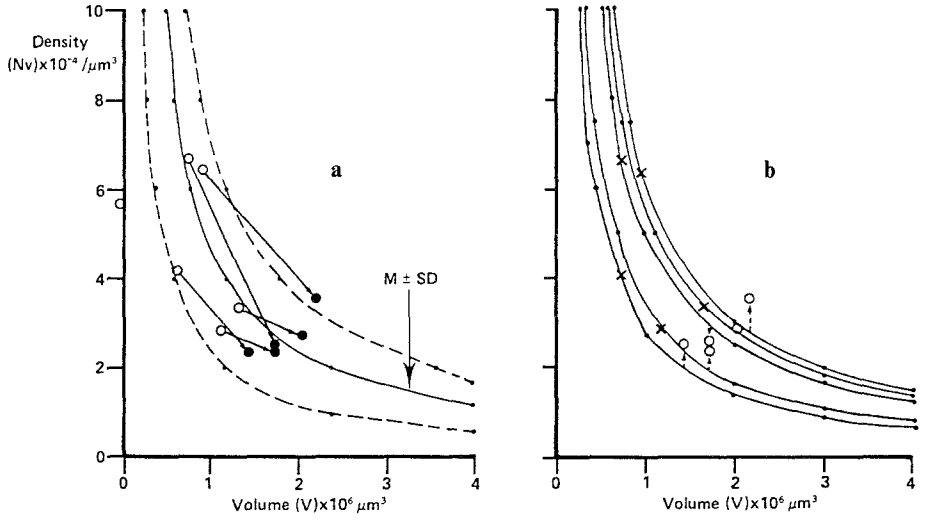

Fig. 5a. Relationship between epithelial lymphocyte density (N_V) and surface epithelial volume (V) (Fig. 7) can be explored by means of density-volume curves, assuming N to be constant. Here pre- and post-treatment points for 5 CD mucosal specimens fall close to the group mean (±SD) density-volume curves for 10 control subjects. Note sharp rise in density (N_V) as V falls (o = Pre-treatment; • = Post-treatment). **5b** Here volume-density curves are plotted for each of the 5 untreated CD specimens: x represents actual values for N_v and V for each specimen, the remaining points being determined by taking other values for V and calculating N_v from relationship $N_v = N/V$. Subsequent points (O), obtained for biopsies taken sometime after gluten restriction, deviate little from values predicted by the original curves, thus emphasising that N does not change during mucosal recovery (x = Pre-treatment; o = Post-treatment; ----→ = Deviation from expected density)

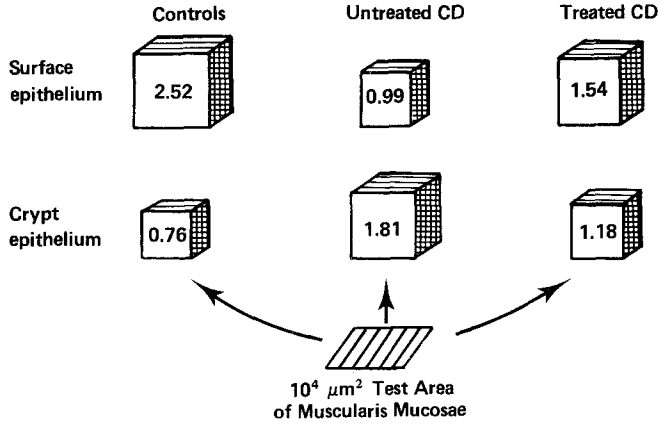

Fig. 6. The epithelial volume data shown in Fig. 4 are expressed here as cubes of equivalent volume: the group mean volume ($\times 10^6$ μm^3) accompanies each cube, the faces of which are drawn to scale. Clearly once volume (L^3) is known, L^2 and L may be determined thus permitting valid, comparative areal or lineal measurements within the 2-dimensional plane of any tissue section

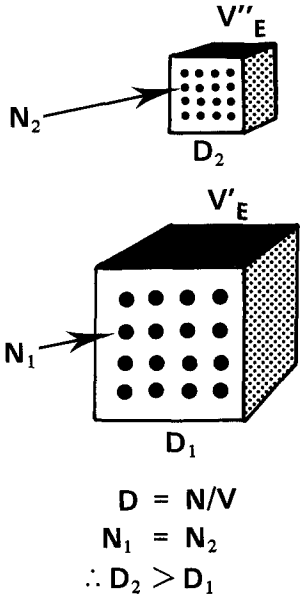

Fig. 7. Visualising mucosal volume compartments ($V'_E + V''_E$) in terms of equivalent cube volume helps to emphasise differences between absolute numbers of particles or cells ($N_1 N_2$) and their density (D). If $N_1 = N_2$, $D_2 > D_1$ merely because V falls: thus, measuring cell densities may tell us nothing about changes, if any, in N. Morphometrically, we must be sure which quantity (D or N) has been measured

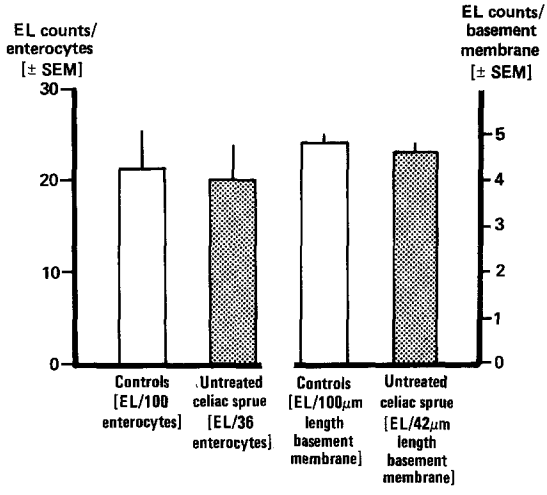

Fig. 8. By use of proportional volumes, the appropriate number of enterocytes, or length of basement membrane, necessary to obtain correct counts of epithelial lymphocytes in "flat" coeliac sprue mucosae was determined: counts performed in this way are now identical to those for control mucosae

emphasising that there is no apparent change in N as the mucosal contour, and hence V_E, increases in response to gluten restriction.

5. Equivalent cube volumes and proportional volumes

The volumes of surface (V_E) and crypt (V_{CR}) epithelium for control and CD mucosae, calculated with reference to 10^4 μm^2 test area of muscularis mucosae, may be expressed as *equivalent cube volume* (Fig. 6). This kind of display (i) provides an easy conceptual way of visualising these quantities; (ii) emphasises the inter-relationship between V, N and density N_v (Fig. 7), and (iii) provides a means of understanding proportional volumes.

The proportional volume ratio $[V_{100}/V_E]$ for CD mucosae ($21.2 \pm 2.3 \times 10^{-3}$ μm^3) was 3 times greater than that ($6.9 \pm 0.5 \times 10^{-3}$ μm^3) for control mucosae. The proportional volume ratio $[V_{bm}/V_E]$ for CD mucosae ($4.12 \pm 0.41 \times 10^{-3} \mu m^3$) was 2.6 times greater than that ($1.57 \pm 0.12 \times 10^{-3} \mu m^3$) for control mucosae. By reducing both ratios obtained for CD mucosae to those of control mucosae, new volumes of epithelium were calculated, which were "equivalent" to 36 enterocytes, or 42 μm basement membrane, respectively. For each untreated CD specimen, those latter values were used for re-calculating the number of epithelial lymphocytes (Fig. 8) which now differed insignificantly from control values.

Discussion

These results provide a new quantitative approach for evaluating mucosal specimens in objective, numerical terms. These data therefore supersede subjective descriptions of mucosal morphology adopted over thirty years ago (Rubin et al. 1960; Shiner and Doniach 1960) when the histological evaluation of peroral biopsies was in its infancy.

The method of analysis described is based on an arbitrary $10^4 \mu m^2$ test area of muscularis mucosae; the latter provides a convenient tissue structure that is not involved with other processes damaging to the mucous membrane proper. All quantitative measurements, whether volumes or cell counts are related either directly, or indirectly, to that constant test area, thus providing a uniform approach to the interpretation of every mucosal specimen. Thus, irrespective of the overlying shape of the mucosa, whether villus-bearing or "flat", the volumes of surface epithelium, crypt epithelium and lamina propria can be accurately obtained and compared directly with those of other specimens.

These volumes may also be considered in terms of their *equivalent cube volumes* (Fig. 6): this (i) allows the essential 3-dimensional nature of the mucosa, and of its individual epithelial compartments, to be easily visualised and (ii) permits the relationship between the number of cellular particles (N) contained within any volume compartment (V) and their resultant density (N_v) to be more vividly appreciated, since $N_v = N/V$ (Fig. 7). The difference between N_v and N should be clearly grasped so that there is no doubt as to which quantity is actually being measured when morphometric techniques are applied to 2-dimensional tissue sections.

When determining the number of cells (N) within any volume compartment of the mucosa, it is necessary to convert the raw 'profile' diameters to 'true' diameters (Figs. 1, 2, and 3). The importance of relating counts to the *effective section thickness* has also been stressed and we have corrected previous values (Marsh 1980) to bring them into line with data obtained in this study: furthermore, there is no apparent difference in the number of EL between mucosae of Western and Eastern controls (Marsh et al. 1983) (Fig. 4). Although V_E was not measured in the latter mucosae, villous shortening is a feature of non-specific tropical enteropathy and so probably contributes to the visual impression that surface epithelium is "infiltrated"

by numerous lymphocytes, as stated in several early publications (Chaudhuri and Saha 1963; Gangarosa et al. 1960; Montgomery and Shearer 1974; Sprinz et al. 1962); these conclusions now appear less certain.

The present study illustrates the effect of V_E, and particularly changes in V_E, on EL counts when these are expressed, not in real terms, but as relative densities (N_v, or %IEL) (Fig. 4). That changes in *density* of EL are significantly affected by changes in V_E is confirmed by the density-volume curves which were constructed on the premise that N remains constant (Fig. 5). If this were not so, the CD curves would not have approximated those obtained for control mucosae (Fig. 5a) and neither would the data on treated mucosae have corresponded to values predicted from the untreated curves (Fig. 5b). Thus the widely-used techniques employed for "counting" EL (either in terms of 100 epithelial cells (Ferguson and Murray 1971; Otto 1972) or unit length of basement membrane (Holmes et al. 1974) are shown, on these grounds, to be morphometrically suspect. It is presumably for this reason that correlations of either N_v, or %IEL, against N lack significance for CD mucosae, as distinct from those in control mucosae.

The problem can also be viewed by considering proportional volumes, i.e. the ratio of either the volume of 100 enterocytes, or the volume of enterocytes dependent on unit length of basement membrane, to V_E; ratios for CD mucosae were 3×, and 2.6×, respectively, greater than their corresponding control ratios. In other words, previous methods (Ferguson and Murray 1971; Holmes et al. 1974) sample up to 3× more epithelium (and hence their contained lymphocytes) than is strictly necessary to obtain true comparative counts of EL. By reducing the CD ratios in proportion to those obtained for control mucosae, the number of celiac enterocytes corresponding to the new, reduced volumes of epithelium were calculated (equivalent to 36 enterocytes or 42 μm basement membrane, respectively). Estimation of the *number* of EL in terms of these new figures for CD mucosae produced values identical to those obtained for control mucosae (Fig. 8).

From all this, it is not difficult to understand why count-densities obtained by other methods (Ferguson and Murray 1971; Holmes et al. 1974) are invariably raised in every instance in which villous flattening occurs, not only in CD disease, but also cow's milk protein intolerance (Philips et al. 1979) and tropical sprue (Ross et al. 1981). It is not evident, therefore, that the conclusions based on such methods permit the widely-held interpretation that a local cell-mediated immunologic reaction occurs within the jejunal epithelium in each of these conditions.

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