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ORIGINAL ARTICLE

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Morphometric analysis of intestinal mucosa. VI – Principles in enumerating intra-epithelial lymphocytes

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Abstract The mucosal response by intra-epithelial lymphocytes (IEL) to antigenic challenge is a useful monitor of local immune activity. Conventional counts of IEL (determined as profile ratios of IEL to enterocyte nuclei) are inaccurate, and over-estimate values by a factor of two, both for disease-control mucosae and untreated 'flat' gluten-sensitized mucosae. Two further proofs are advanced in this paper which expose the inaccuracy of conventional profile-density IEL counts. New ranges (log-transformed data) indicate a disease-control mean of 11 IEL per 100 enterocytes (95% confidence limits 5–27) and 29 IEL per 100 enterocytes (95% confidence limits 14–61) for untreated flat gluten-sensitive mucosae. For simplicity, if conventional IEL "counts" are halved, correct values (based on precise morphometric analyses) are easily obtained for comparative and other purposes.

Key words Intra-epithelial lymphocytes · Morphometry · Coeliac · Quantitation · Reference range

Introduction

Several mechanisms provide defensive functions at the intestinal mucosal surface, some of which are non-immunological in nature (Udall and Walker 1987), while others, like sIgA production, are clearly antigen-driven (Kagnoff 1987). In addition to mechanisms of local humoral defence, T cell-mediated responses also provide some degree of protection at this interface, although they are the least well-characterised (Ferguson 1987; MacDonald 1992). Nevertheless, in this respect, the functional role of intra-epithelial lymphocytes (IEL) has excited considerable interest over the last two decades, but despite such intensive activity and research into this unique, yet heterogeneous, collection of lymphocytes their presence and actual function at the interface between lumen and mucosa still eludes precise definition.

IEL are certainly influenced by intra-luminal antigens, as is evident from observations of the fetal intestine at weaning, and in adult enteropathies such as gluten-sensitivity, giardiasis, cryptosporidiosis, tropical enteric diseases, AIDS-associated enteropathy or graft-versus-host disease (Marsh and Cummins 1993). The issues have become more complex in recent years by the discovery that IEL expressing the γδ form of T-cell receptor (TCR) are also influenced by, and possibly involved in, these pathological processes (Halstensen et al. 1989; Spencer et al. 1989; Viney et al. 1990). In many studies the functional activity of IEL is inferred from alterations in number or subset, this quantity invariably being expressed numerically, as "counts" of IEL per 100 enterocytes (Ferguson and Murray 1971; Marsh 1980; Corrazza et al. 1985).

The question posed by this paper is whether such counting techniques are justified, and hence, whether the conclusions and speculations about function drawn from such counts are useful or valid.

The reason for such a question is based on several considerations. First, in certain forms of enteropathy associated with loss of villi, the total number of surface enterocytes compared with normal is markedly reduced. Since the total number of enterocytes, to which IEL are related in any given specimen, is variable it is difficult to see how this yardstick can be used as a basis for reliable estimates of the lymphocyte population, particularly as comparisons are invariably drawn between normal and abnormal mucosae (Ferguson and Murray 1971). Second, although such observations are designated cell counts they are count-densities of sectioned cell profiles (Marsh 1980). Great care has to be taken in counting sectioned profiles of cells (i.e. 2-dimensional data) and extrapolating back to real cells (i.e. 3-dimensional relationships) as would have existed in the original material (Marsh 1980; Niazi et al. 1984). Thirdly, in a recent morphometric analysis of enterocyte volumes (Crowe and Marsh 1993), we discovered a marked difference be-

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tween values of "profile-density counts" of IEL per 100 enterocytes as usually practised, and the absolute relationship between IEL and enterocytes, when based on a defined volume of epithelium, and the actual volume ratio between enterocytes and IEL. The explanations for such discrepancies are detailed here in two further "proofs", thus revealing the extent to which data based on IEL "counts"/per 100 enterocytes per mucosal specimen are inaccurate and how these may be simply rectified.

Materials and methods

Mucosal biopsies

Jejunal biopsies, obtained by Watson capsule located fluoroscopically at the duodeno-jejunal flexure, were taken from patients attending the Gastroenterological Out-Patient service of the University Department of Medicine, Hope Hospital. Patients are divided into two major groups: those with proven gluten-sensitivity and those without: these latter are, for the purposes of this paper, designated disease-controls.

After retrieval of the capsule, each mucosal sample was quickly spread out, orientated and flooded with ice-cold, ultrapure 2.5% glutaraldehyde in 0.1 M cacodylate buffer. Specimens were then washed in fresh buffer, dehydrated in an ascending ethanol series and embedded in plastic resin. Sections, 1 μ m thick, were cut on a Reichert OMU-3 ultramicrotome, stained with toluidine blue, and quantitated by means of computer-assisted image-analysis.

Computerised image-analysis

Our method of computerised image-analysis of intestinal mucosae relates all measurements, whether of tissue compartments (e.g. surface epithelial volume; crypt epithelial volume; lamina propria volume) or concerned with determination of cell populations within those compartments, to a constant test square of muscularis mucosae (10^4 μm^2) as detailed elsewhere (Dhesi et al. 1984; Niazi et al. 1984; Marsh and Hinde 1986; Crowe and Marsh 1993; Ensari et al. 1993).

Briefly, the outline of surface compartment is recorded by the light pen as it is manually traced around the appropriate boundary, together with the length of muscularis mucosae underlying that compartment. Similar measurements are performed in succession, the results (determined as volume of compartment per 10^4 μm^2 muscularis mucosae) being constantly updated by the computer, until sufficient items of data yield a constant mean value.

In order to determine absolute cell populations, the light pen is traced around 100 nuclear profiles, from which the computer calculates a crude diameter. Corrections for lost (small) sections, and for non-sagittal sectioning are applied (as extensively detailed elsewhere (Marsh et al. 1983; Niazi et al. 1984) in order to obtain a true value: the inter-observer variation in this laboratory for mean IEL nuclear diameter is 0.1 μm. Once the true mean diameter (D) has been determined, the number of profiles counted per tissue compartment is divided by the sum of D, and section thickness (t). ($D+t$) is termed "effective section thickness", and defines the total thickness of tissue occupied by each nucleus.

Results and Discussion

In a previous companion paper (Crowe and Marsh 1993) the volumes of individual small intestinal surface enterocytes in disease-control and untreated gluten sensitive mucosa were measured. Since our computerised image-analysis technique allows measurement of surface epithelial volume (V_{SE}) per 10^4 μm^2 muscularis mucosae, the mean number of cells within V_{SE}, as defined for both sets of mucosae, could therefore be calculated. Since we had also calculated the mean (absolute) number of IEL contained in V_{SE}, we were able to deduce the ratio of IEL to enterocytes. These ratios (disease control, and gluten-sensitive mucosae) were less by a factor of two, the values obtained by conventional IEL profile density counts (Ferguson and Murray 1971). This paper is an attempt to understand and explain this marked

Table 1 Morphometric data. Note: IEL have virtually circular (spherical) nuclei; widths of enterocyte nuclei are equivalent to 'diameter', and have been determined by direct measurement; data are obtained from precisely oriented 1 μm epon sections: the sections have a very accurate thickness since they are cut with a high-precision EM-type ultramicrotome

Quantity	Disease-control mucosae	Untreated gluten-sensitivity
Volume of epithelium, V_{SE} (per 10^4 μm^2 muscularis mucosae)	2.3×10^6 μm^3	0.4×10^6 μm^3
Mean volume of enterocyte	800 μm^3	610 μm^3
Number enterocytes in V_{SE}	3000	600
Number IEL in V_{SE}	350	190
Absolute ratio IEL: enterocytes (%)	12	32
Ratio IEL: enterocytes (profile density-count) (%)	24	61
Mean width of enterocyte	5.1 μm	4.7 μm
Mean width enterocyte nucleus (i.e. diameter of cylinder)	3.3 μm	3.5 μm
Mean nuclear diameter (IEL)	4.9 μm	5.2 μm
Effective section thickness (1 μm section)		
(i) enterocytes	4.3 μm	4.5 μm
(ii) IEL	5.9 μm	6.2 μm

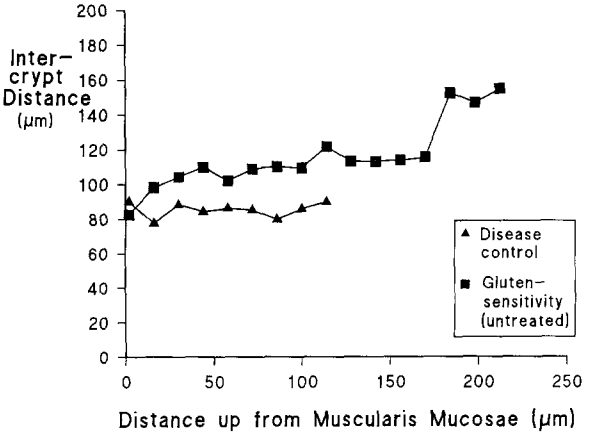

Fig. 1 Use of muscularis mucosae as invariant third-party reference is only valid if mucosal relationships remain identical in the presence of villous effacement. This diagram shows that inter-crypt distances remain constant (and virtually identical) in specimen mucosae cut at successively higher planes above the muscularis

difference in results. In order to clarify the arguments which follow, relevant data are set out in Table 1.

Previous morphometric analyses of IEL populations in human small intestinal mucosae (Guix et al. 1979; Marsh 1980; Niazi et al. 1984; Crowe and Marsh 1993) also produced values at variance with previous data which asserted that IEL are raised in untreated gluten sensitivity. In each of these studies (Marsh 1980; Niazi et al. 1984) an invariant 'third-party' reference structure (i.e. muscularis mucosae) was used to provide comparative data independent of mucosal shape, based on the critical assertion that mean inter-crypt distances do not alter, or diverge, between disease-control and flat mucosae: this assertion has now been completely validated (Fig. 1).

When thin sections are quantitated by microscopy, it has to be remembered that all structures observed are profiles of their actual 3-dimensional reality. The number of (cellular) profiles actually observed depends on three variables: (i) section thickness, (which for quantitative work should be constant and hence irrelevant) (ii) the actual number of cells present in the tissue (the quantity to be determined) and (iii) importantly, the size (i.e. diameter) of those cells (Weibel 1979).

The size (diameter) of cells influences the number of profile discs present in any unselected tissue section when viewed in the microscope (Fig. 2): thus, larger cells create larger profiles and these may appear more frequently in a given section compared with those of smaller cells whose profiles in section will appear less often (Fig. 2). Thus in quantitating unknown tissues, it is essential (i) to know mean cell size (diameter) and (ii) to control for tissue volume into which each cell type under investigation is distributed. Cell size can be derived either from measurements of cytoplasmic, or nuclear, membranes: nuclei, in general, are easier to work with because they stain well, lend themselves more effectively to accurate quantitation because their profiles are more regular in outline: thirdly, all cell profiles, as far as this study is concerned, are deemed to be nucleated cellular profiles.

Effect of effective section thickness on conventional profile-density IEL counts

Any cell (nuclear) profile appearing in a thin section represents in reality, a superslice of tissue whose thickness is the sum of actual cut section thickness (t μm) plus mean (nuclear) diameter of cell (Dμm), relationship $(D+t)$μm termed effective section thickness (EST) (Weibel 1979; Niazi et al. 1984). Thus, in considering raw "counts" of IEL profiles/100 enterocyte nuclei profiles, both for disease-control and untreated gluten-sensitive mucosae, four EST have to be considered (Table 1: Fig. 3) since each derive from structures of different diameter, i.e. 5.9 and 6.2 μm for IEL nuclei, and 4.3 and 4.5 μm for enterocyte nuclei, respectively (Crowe and Marsh 1993). Each raw profile count therefore must be divided by its individual EST, in order to deduce the real number of cells present within the original (3-dimensional) tissue block. It is the failure to calculate and

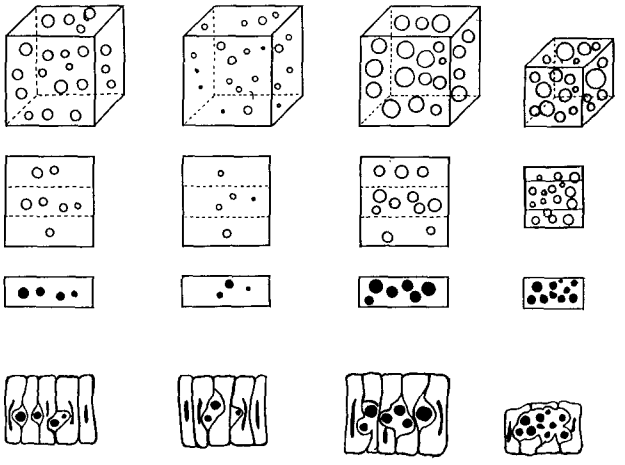

Fig. 2 This diagram illustrates (i) the effect of cell size and (ii) volume of distribution, in determining the number of profiles that appear in random sections. In each box (representing a cube of surface epithelium, V_{SE}) there are twenty cells of differing size (diameter). In second and third columns, fewer, and additional profiles are seen, due to size differences alone. In the fourth, a reduction in surface epithelium further acts to increase the number of sectioned profiles seen. A strip taken from each 'thin section' of each of the four cubes is further elaborated (bottom line) to simulate epithelium embracing infiltrating cell profiles. Clearly it would be erroneous to conclude that "infiltration" increases progressively from left to right since, as the initial premise stated, the actual number of cells in each cube is identical

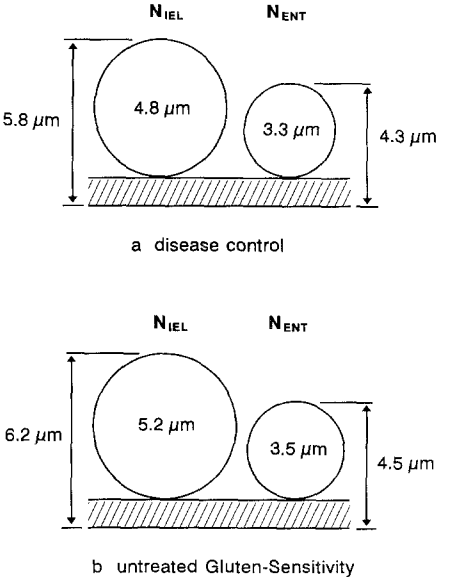

Fig. 3 In performing simple intra-epithelial lymphocyte (IEL) "counts", there is an "effective section thickness" (EST) which must be respected for all structures concerned, i.e., IEL and enterocyte nuclei in disease-control (a) and untreated gluten-sensitive (b) mucosae. EST is that superslice of tissue in which part of any profile of any structure of interest would appear, and is approximate to sum of mean particle diameter (D) and nominal section thickness (t)μm

apply EST which accounts, in part, for the high IEL/100 enterocyte density-counts for disease-control (24%) and untreated gluten-sensitive (61%) mucosae, compared with corresponding 'absolute' values of 12% and 32%, respectively (Table 1, Crowe and Marsh 1993).

Thus, using our data for disease-control mucosae, EST = 3.3+1 = 4.3 μm for enterocyte nuclei (Fig. 3): so, for a cumulative count of 100 enterocyte nuclear profiles, only 100/4.3 or 23 actual enterocytes are, in reality, counted, while for gluten-sensitive mucosae, the number of enterocytes is 100/(3.5+1), or 22.

Likewise, the actual count of lymphocyte profiles (Table 1) must also be divided by EST. Thus for disease-control mucosae, 23/5.9 gives 4 lymphocytes, which by proportion, yields a true value of 17 IEL per 100 enterocytes (100/24 × 4). Similarly for untreated gluten-sensitive mucosae, actual IEL would be 61/6.2 or 10, yielding a true value of 46 IEL per 100 enterocytes (100/22 × 10).

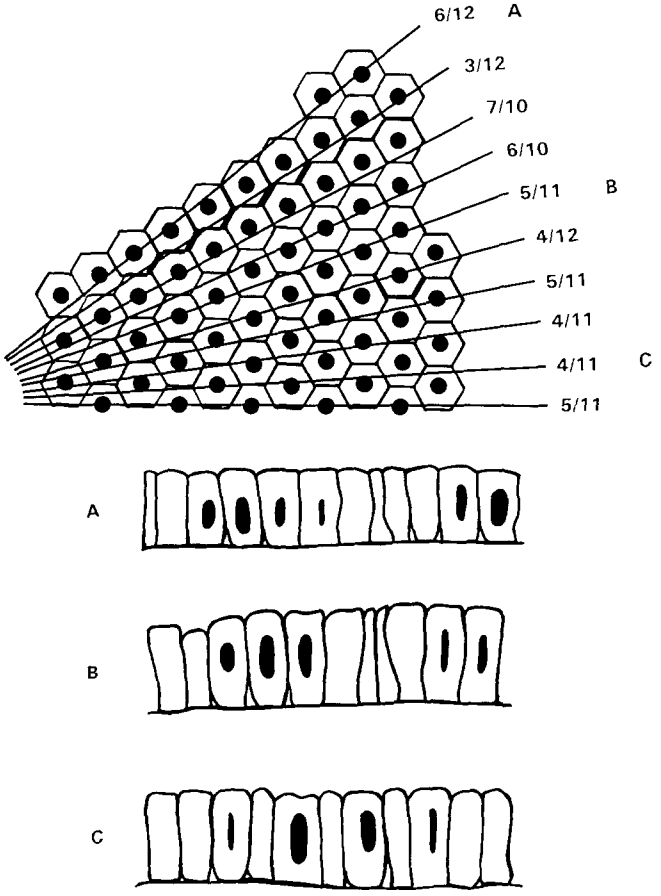

Fig. 4 The upper region is a scale drawing of enterocyte packing, as seen in horizontal sections (or by surface scanning electron microscopy). Random lines represent imagined section cuts, and the ratios at the right hand margin of each indicate number of nuclear cuts relative to the number of cycloplasmic (cell) cuts, which approximate 50%. *A*, *B*, and *C* are representations of the epithelial profile that might be expected to be observed along the corresponding cuts, when observed in vertically-sectioned material. This emphasises the way in which cells may be lost when only enterocyte nuclei are counted, and partly explains why IEL "counts" performed in this way are significantly overestimated

The effect of "lost" enterocytes

However, it can be seen that these values are still higher than 'absolute' values (Table 1). A second problem, sustained by counting enterocyte nuclei alone, must also be addressed. Because of their complex geometry and packing, villous enterocytes are not aligned as individual, parallel rows of cells (as is often assumed from the sectioned appearance of villi) but in short range hexagonal array (Fig. 4). Thus, despite vertical mucosal sectioning, several enterocytes will always be "lost" in any extensive count of enterocyte nuclei: in passing, note than 700–1000 enterocytes must be counted (Niazi et al. 1984) if a constant value for IEL/100 enterocytes is to be secured. For 1 μm sections, this loss or undercounting of enterocytes amounts to approximately 50 cells in every hundred nuclei counted.

Thus instead of 24 disease-control enterocytes (see above) the actual revised number should be 50% higher, or 36, providing a final correct IEL/enterocyte ratio of 4/36, or approximately 11%. Likewise, for gluten-sensitive mucosae, instead of 10 IEL/22 enterocytes, the ratio would be nearer 10/33, or 30 IEL per 100 enterocytes. These compare favourably with our previously determined mean absolute ratios (Table 1) of 12 and 32%, respectively (Crowe and Marsh 1993). In general, it can be seen that profile-density counts (and not "lymphocyte counts" as is commonly misconstrued in papers on this subject) over-estimate true values by two-fold, both for disease-control and gluten-sensitised tissues. Thus, as a simple rule-of-thumb, real values can be quickly and accurately obtained by halving conventional "profile-density" counts.

How many enterocytes are necessary for comparative counts?

The problem of counting IEL can be approached from yet another angle which provides further strength to the argument. Here we now consider volume of tissue in which any desired cell count is to be undertaken. As emphasised in Fig. 2, a difference between the actual number of cells within a defined volume of tissue and the number of profiles apparent within any corresponding thin section, occurs when the cells under consideration are of different size (diameter).

Since we know values for epithelial volume (V_{SE}) and total enterocytes contained with reference to 10^4 μm^2 muscularis mucosae (Table 1: Fig. 5), it follows that 100 disease-control enterocytes are not proportional to 100 gluten-sensitive enterocytes. For the former, there are 3000 enterocytes in V_{SE}, and hence 15 (or $\sqrt[3]{3000}$) cells which would occupy one edge of cube (i.e. which could be envisaged as applied to basement membrane). Similarly for gluten-sensitive mucosae, V_{SE} contains 600 enterocytes, that is, $\sqrt[3]{600}$, or 8, cells per side of cube.

Thus, if IEL profile counts are related to 100 entero-

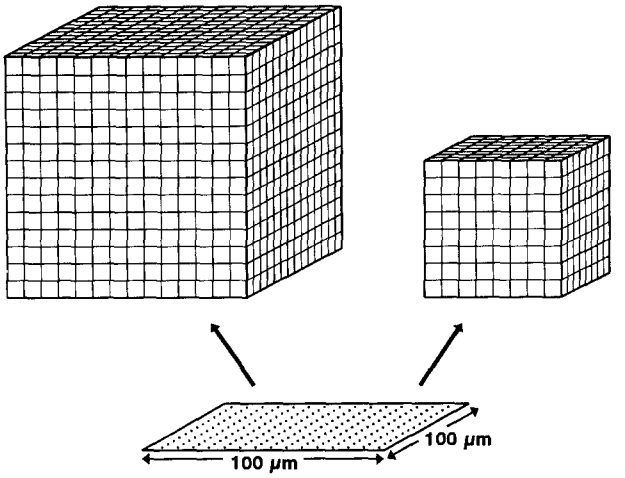

Fig. 5 Surface epithelial volume, when determined relative to a constant test area of muscularis mucosae, can be re-expressed (and drawn) as an equivalent cube volume. Since these volumes for disease-control and flat gluten-sensitive mucosae are known, as well as the mean volumes of their respective enterocytes, the average number of enterocytes lying alongside one side of each cube can be calculated. Clearly, 100 disease-control enterocytes are not equivalent to 100 gluten-sensitive enterocytes: the ratio is, in reality, approximately 2:1

Table 2

Gluten-sensitive subjects	N_{ENT}[a]	$\sqrt[3]{N_{ENT}}$[b]	Enterocytes required for IEL count[c]	Revised IEL count[d]	Conventional profile density count (IEL/100 enterocytes)[e]
n=20	619±234	8±2	53±7	32±17	65±27

[a] Number of enterocytes contained within surface epithelial volume (V_{SE}) with reference to test area (10^4 µm^2) of muscularis mucosae

[b] Number of enterocytes lining one edge of cube (V_{SE}). See Fig. 5.

[c] Based on 100 cells for disease-control mucosae

[d] Revised IEL count (based on proportionally/reduced number of enterocytes[c]) compared with conventional profile-density count[e]. When transformed logarithmically, mean ±SD is 34 (23–51), while 95% confidence limits are 16–76. These values are slightly different from those derived by applying corrections for estimated section thickness (Fig. 3) and "lost" enterocytes (Fig. 4), as given in Table 3. Nevertheless they are markedly different from those given originally by Ferguson and Murray (1971)

cytes in disease-control mucosae, then for gluten-sensitive mucosae, the appropriate corresponding number of enterocytes would be 100/15 × 8, or 53 enterocytes, in order to obtain a correct estimate of coeliac IEL. It can be seen that evaluating gluten-sensitive IEL/per 53, rather than 100, enterocytes would now virtually reduce the count by a factor of two, and hence bring it into line with the morphometric data (Table 2), i.e. the spurious profile-density count (65±27) when properly revised, becomes 32±17 IEL.

What length of basement membrane is necessary for comparative counts?

Finally, use of standard lengths (e.g. 1 mm) of basement membrane have also been used as a reference point for counting IEL. From the above, it likewise follows that 1 mm length of basement membrane for control and diseased mucosae are not equivalent reference lengths. The length (cubed root of V_{SE}) of epithelium for disease-control mucosae, equivalent to 15 enterocytes of mean width 5.1 µm, is 76 µm of basement membrane, but only 38 µm (8 × 4.7) for gluten-sensitive mucosae. Thus, if IEL in disease-control mucosae are related to 1 mm (1000 µm) basement membrane, then for a flat, gluten-sensitised tissue mucosa, the appropriate length of basement membrane used should only be 500 µm. This would also halve the profile-density count. However, as this is not a widely-used technique, the data derived from its use are not so widely known or applied as those related to the widely-used IEL/100 enterocyte technique.

Conclusions

Conventional profile-density counts (Ferguson and Murray 1971) are inaccurate and over estimate the true value of IEL in flat mucosae by a factor of two. This error has been demonstrated by three independent ways: (i) by actually determining the relationship of IEL to enterocytes from individual measurements of cell volumes with reference to a defined tissue volume (Crowe and Marsh 1993), and in this study by (ii) correcting for effective section thickness, and for "lost" enterocytes incurred by counting their nuclei alone and (iii) determining the putative number of enterocytes lying along the edge of a cube of epithelium (V_{SE}) (determined by computer in respect of a test area (10^4 µm^2) of muscularis mucosae) for disease-control, and flat, mucosae.

In practical terms, given a flat mucosae, the simplest

Table 3 Human small intestinal IEL: ranges (±95% confidence limits)

	Numerical	Log-transformed
Disease-controls ($n=50$)	(i) Raw profile density counts: 24 (6–42)	22 (9–57)
	(ii) Corrected counts: 12 (3–21)	11 (5–27)[a]
Untreated gluten-sensitivity ($n=20$)	(i) Raw profile density counts: 65 (19–111)	61 (31–124)
	(ii) Corrected counts: 32 (8–56)	29 (14–61)[a]

[a] Logarithmic-transformed data provide the most reliable mean values and reference ranges for IEL, both for disease-control, and flat, untreated gluten-sensitive mucosae. Log transforms are preferred because the raw IEL data are skewed

way to obtain a correct ratio of IEL to enterocytes is to divide the conventional profile density-count by two: such procedure corrects both for estimated section thickness and for lost enterocytes incurred in cumulative counts of enterocyte nuclei. Revised (logarithmic) ranges for IEL in disease-control and untreated gluten-sensitive mucosae are appended (Table 3).

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