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Michael N. Marsh
University of Manchester

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STUDIES OF INTESTINAL LYMPHOID TISSUE. XI - THE IMMUNOPATHOLOGY OF CELL-MEDIATED REACTIONS IN GLUTEN SENSITIVITY AND OTHER ENTEROPATHIES

Michael N. Marsh

University Department of Medicine, Hope Hospital (University of Manchester School of Medicine), Salford, Manchester, U.K.

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Abstract

Computerised image-analysis was used to quantitate small intestinal mucosae from celiac sprue and dermatitis herpetiformis patients, Gambian children with tropical-sprue-like malabsorption, first-degree celiac sprue relatives, and treated celiac sprue patients during challenge with a peptic-tryptic digest of gluten. A wide range of mucosal appearances was observed. Typically, 'flat' lesions (Type 2) revealed a reduced number of epithelial lymphocytes that were large and mitotically active. At the other extreme, mucosal architecture was relatively well preserved (Type 1) but surface epithelium contained an expanded population of small, non-mitotic lymphocytes, with or without crypt hyperplasia. Similar changes were observed in one-third of celiac relatives and following small dose gluten challenge. Larger dose challenges revealed a transition from Type 1 to Type 2 lesions over a 5-day period. Studies in a few patients over 2-4 years showed a similar type of progression. A major feature of this sequence was early appearance of crypt hypertrophy while villi persisted, indicating a role for factors other than increased loss of enterocytes from surface epithelium. These changes parallel the T lymphocyte-mediated events in graft-versus-host reactions in animals. It is thus concluded that the spectrum of immunopathologic changes observed in gluten sensitivity is fundamentally a cell-mediated effect, the degree of change being controlled by host genetic factors. In becoming flat, it appears obligatory for the mucosa to evolve through the earlier Type 1 lesion in which crypt hypertrophy is a prominent response.

KEY WORDS: Celiac Disease, Dermatitis Herpetiformis, Tropical Sprue, Graft-versus-Host Reaction, Epithelial Lymphocyte, Morphometry, Image-Analysis, Jejunal Mucosa.

Address for correspondence:
M.N. Marsh,
University Dept. Medicine, Hope Hospital, Eccles Old Road, Salford, Manchester M6 8HD, U.K.
Phone No.: 061-789-7373, x86

Introduction

The classic studies of the Dutch workers Dicke, Weijers and van der Kamer [24,52] established the central role of gluten protein in the pathogenesis of celiac sprue (CS). Although CS was considered to be a disease of children, it became evident, by 1960 [99,105] that some adults previously lumped together in an amorphous diagnostic group termed "idiopathic steatorrhoea", had an identical condition by virtue of a similar lesion of upper jejunal mucosa ("subtotal villous atrophy"). Thus, CS came to be regarded as a life-long disorder [84], while the combined use of jejunal biopsy and a gluten-free diet subsequently showed that after a few years of treatment, complete morphological restoration of jejunal mucosal architecture could be expected [2,122].

The term "subtotal villous atrophy" embraced a lesion comprising loss of surface villi associated with shortened, cuboidal epithelial cell profiles, marked hypertrophy of the crypts, and a chronic inflammatory cell infiltrate of the lamina propria. Another less severe lesion, termed "partial villous atrophy" was originally [26,118] considered to be a distinct entity intermediate in appearance between normal and the more severe "flat" lesion. Although this terminology has unfortunately become engrained in the literature, later studies by SEM [63,74,75] and kinetic analyses of epithelial cell turnover [120,121] showed conclusively that there is a progressive continuum of mucosal change between the two extremes.

The abnormal epithelial structure in the severe lesion seemed compatible with a major theory of pathogenesis envisaged by Frazer [37]. This proposal saw epithelial cells, congenitally lacking a "peptidase" and hence unable to complete the digestion of gluten protein, damaged by the direct action of an incomplete breakdown product of gluten. This idea is no longer consistent with recent concepts of digestion, transport and absorption of proteins by mammalian intestine [17,56,83,94,111]: not surprisingly, there has been little success in identifying the missing peptidase [13,95]. Furthermore, the gradual realisation that CS

patients (i) have antibodies to gluten and lymphocytes reactive to gluten protein in vitro (ii) exhibit splenic atrophy and mesenteric lymph node cavitation (iii) may develop T cell lymphomas and (iv) are genetically-predisposed by virtue of certain HLA Class II D-locus allospecificities, indicated widespread involvement of the immune system in this condition [3,21,22,42,45,46,47,48,49,53,55,57,82,92,93,103, 112,115]. More specifically, the demonstration of a rise in the relative density of interepithelial cell space lymphocytes (EL) in untreated CS patients [33] was one of the first major studies indicating that CS is primarily an immune-mediated condition.

Interest in the importance of EL was stimulated by their close proximity to the intestinal lumen, suggesting that they might play a major role in "immune surveillance" at this strategic host-environment frontier [5,25,29,31,72]. With others [18,64,100] we initially discovered the presence of blast-transformed EL within murine small intestine and that these lymphocytes have a high mitotic rate [46,47,65,67,69,97]. At that time it seemed reasonable to suppose that EL were reacting to antigen traversing the intestinal epithelium. It thus followed that if CS were due to local immunisation to gluten peptides, then similar changes in the EL population might be expected in the target of immunologically-sustained damage - the upper jejunal mucosa [68].

The purpose of this review is to summarise our principal findings regarding EL populations in CS in relation to the background morphological appearances referred to above. In studying the entire range of "gluten sensitivity", which includes patients with overt CS [66] and dermatitis herpetiformis (DH) [8], first-degree relatives of known CS patients [80], together with the effects of graded oral challenges of gluten peptides on treated CS patients [58], a much wider spectrum of immunopathologic changes has emerged. While such studies are still being pursued, the results closely parallel events accompanying various models of graft-versus-host reaction (GVHR) and allograft rejection in experimental animals [32]. It is on this latter background that the varied features of the celiac lesion, as well as those associated with other presumptive 'immune' disorders of the small intestine, such as tropical sprue and giardiasis, may be more effectively evaluated. Together, they suggest that in all these conditions, the various degrees of intestinal involvement are (i) reflections of T cell lymphocyte-mediated attack and (ii) dependent on a specific (genetically-mediated) host response for their expression.

Materials and Methods

Subjects Studied

Controls. These were either healthy young volunteers (HV) (age range: 20-26); family member controls of known CS subjects (FMC) (age range: 24-58); disease-controls (DC) (age range: 18-65) comprising patients referred with gastrointestinal symptoms but in whom jejunal morphology was judged to be normal and CS not considered to be the diagnosis; a group of miscellaneous patients (age range: 28-72) accompanied by a 'flat' mucosa (FDC) that was not considered due to gluten sensitivity [76]: among this group were patients with jejunal Crohn's disease, α-chain disease, small intestinal lymphoma and common variable immunodeficiency.

Celiac Sprue. Mucosae were obtained from among 25 patients (age range: 21-68) with diarrhea, malabsorption or anemia as a diagnostic procedure, and who were shown to have a flat mucosa in which the percentage mitotic index of EL > 0.2% [69]: note that this index in the non-CS flat disease-control group was <0.1% [76].

Mucosae were also obtained from these patients at varying times during treatment with a gluten-free diet. At that stage, patients were in good health, without malabsorption or in need of nutritional supplements, and in whom there was histological evidence of improved mucosal architecture.

Dermatitis Herpetiformis. These comprised a group of 30 subjects with pruritic, eruptive skin vesicles appearing on face, shoulders, elbows and knees, and in whom immunofluorescence of uninvolved skin revealed linear/granular deposits of IgA [54]. They were referred by physicians at the Manchester Hospital for Diseases of the Skin. Treatment was with oral dapsone and gluten-free diet: gluten-sensitivity was demonstrated either by marked histological changes in jejunal mucosa and/or by subsequent challenge with gluten, or derivatives thereof.

First-Degree CS Relatives. A sample of 52 first-degree CS relatives was studied in terms of HLA status, mucosal morphology, EL populations, and permeability to the probe ^{51}Cr-EDTA (ethylene diamino tetra-acetate) [13,14]. Relatives (symptomatic or asymptomatic) with pre-existing flat jejunal mucosae (prevalence 15.5% of 200 relatives) were excluded from this particular study [8].

Immunogens

Gluten. Peroral challenges in healthy volunteers and treated CS patients were performed with a peptic-tryptic digest of crude commercial gliadin (Sigma) as originally described by Frazer and colleagues [38] and modified by Jos et al [51]. Dose ranges were 0.1, 0.5, 1, 1.5, 3.0, 6gm.

Control. 500mg of β-lactoglobulin (BDH) was used as a control challenge immunogen.

Challenge Protocol

Patients and controls were admitted to a metabolic ward for 5 days [58]. On the morning of day 1, a control jejunal biopsy was performed and at 10pm the selected dose of immunogen was given by mouth. The first post-challenge biopsy was performed at 10am on the morning of day 2, i.e., 12h after the challenge immunogen was ingested. Subsequent biopsies were obtained on

the morning of the 3 remaining days, at 36, 60 and 84h after challenge.

Histologic Technique

Mucosal tissue was obtained under fluoroscopic control from the first loop of jejunum by Watson, or Quinton hydaulic multiple-biopsy, instruments. Specimens were quickly retrieved within 0.5 min, gently spread on cards and fixed in either Dalton's chrome-osmium fixative [20] or (more recently) with 2.5% ultrapure glutaraldehyde buffered with 0.1M sodium cacodylate to pH 7.2: these specimens were post-fixed in 1% osmium tetroxide for 1 h.

All specimens were routinely processed into Araldite plastic, sectioned at 1µm on Sorvall MT2-B or Reichert OMU-3 ultramicrotomes and stained with toluidine blue. Six consecutive 1µm sections were mounted per slide and 10µm steps were discarded between successive slides. Only one section per slide was analyzed, the one chosen being least subject to preparative or technical artefact. The remaining sections served for verification of cytologic detail if required, while the intervening 10µm step ensured that no cell was counted twice. With some analyses, several parts of the chosen section were used, but no observations were duplicated on the same part of individual sections. Care was taken to ensure that the bulk of the specimen was sectioned vertical to the surface mucosal plane. Appropriate micrographs were recorded on Ilford FP3 high grain film through either Zeiss "Photomicroscope III" or Olympus BH2-S research microscopes.

Morphometric Analysis of Mucosal Specimens

Sections were examined through an oil-immersion objective (x100) and analyzed with a MOP-Videoplan (Kontron-Reichert) image-analysis system. All measurements as detailed below, were related, directly or indirectly, to a constant test area ($10^4 \mu m^2$) of muscularis mucosae [71,91].

Mucosal Compartment Volumes (V_{SE}, V_{CR}, V_{LP}). For the determination of surface epithelial volume (V_{SE}), crypt epithelial volume (V_{CR}) and lamina propria volume (V_{LP}) per specimen [91,79,23], the appropriate sectioned profiles in 1µm epon sections were outlined with the scribing cursor, and individually cumulated relative to a total length of 100 x 100µm (10mm) muscularis mucosae, thus approximating the test area. Coefficients of variation for successive measurements per 100mm muscularis per specimen were <10%: these calculations are automatically included in the print-out by the manufacturer's soft-ware programme.

Grouped values for V_{SE}, V_{CR} and V_{LP} were expressed in terms of their equivalent cube volumes [71], and depicted graphically by drawing their areal faces to scale (Fig. 1).

Mean Epithelial Diameters (D_N). The nuclear profiles of 100 EL were traced with the cursor per specimen: in previous work it was shown that this population size was sufficient to achieve a constant mean ± SD. The initial crude distribution for nuclear diameters was subsequently modified to include "lost profiles"

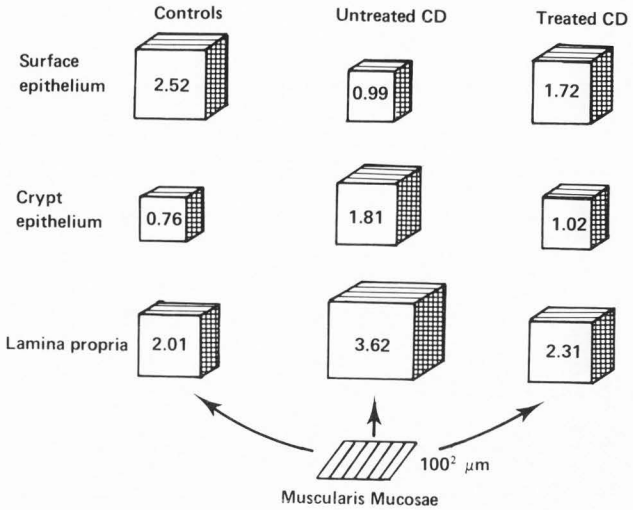

Figure 1. This diagram represents mucosal components for lamina propria, surface and crypt epithelium in control subjects and patients with untreated, and treated celiac sprue, expressed as equivalent cube volumes. The means for each group of measurements ($x10^6 \mu m^3$) accompany each cube, the faces of which are drawn to scale to permit quantitative comparison. All measurements are controlled by reference to the constant test area of muscularis mucosae.

[41] by adding the smallest profiles (left hand 25% of distribution) determined by scaling existing values up to a line joining the zero origin with the half-value of the mode. The mean of this distribution was multiplied by $4/\pi$ to correct for imperfect (non-sagittal) sectioning [117]. In this way, the true mean nuclear diameter (D_N) of EL (surface or crypt epithelium) per specimen was obtained.

From each corrected distribution, the percentage of nuclei with $D_N > 6\mu m$ (% 'immunoblastoid' lymphocytes) was obtained.

Calculation of Absolute EL population (Nv) in V_{SE} or V_{CR}). In attempting to determine the absolute number of a population of cells contained within a defined tissue component, of volume V, the true diameter of those cells must be known [77], since each sectioned "profile" of a cell observed in a section of known thickness (t µm) represents only a fragment of the whole cell. It has been shown [117] that all particles (diameter, D) whose profiles appear in any finite section of t µm thickness are contained in a superslice whose overall thickness (or 'effective section thickness' EST) is given by the relationship:

$$EST = (t + D)\mu m \qquad (1)$$

Since D_N for surface or crypt EL was calculated, the total number of EL (N) occupying V_{SE} or V_{CR} per specimen was obtained by counting EL profiles relative to 100 x 100µm muscularis mucosae per specimen: thus $N_V = [100 \times (100/EST)]$ units of tissue volume. Nuclear profiles were used in calculating EST because they are more nearly circular in sectioned

profile and hence more accurately measured.

Percentage Mitotic Index (%MI) of EL. The percentage mitotic index was calculated from the number of metaphases observed in a total sample of 3000 EL in surface epithelium only per specimen [66], thus avoiding confusion with mitotic precursors in the generative crypt epithelium. Furthermore, it has been shown [113] that once ascended onto the villi mature enterocytes, goblet cells and argentaffin cells cease mitotic activity. In flat mucosae, proliferative epithelial cells may occur throughout the length of the hypertrophied crypts. With such specimens, scoring of mitotic figures was strictly confined to surface epithelium, crypt mouths being avoided.

In order to determine rates of EL mitosis in vivo [66], four intramuscular injections of 1.2mg colchicine at hourly intervals were given to selected controls, and CS patients, both before and during treatment with a gluten-free diet. Jejunal biopsies were performed before and at variable time-points during the 4h period of mitotic blockade. The total dose (4.8mg) administered was considered suitable for this purpose and was controlled by being shown to be effective in arresting crypt epithelial cell mitotic activity during the study period [66].

Results and Discussion

Untreated CS Disease

In untreated CS (n = 14), mean corrected nuclear diameter (D_N) of surface EL was 5.6 ± 0.2, compared with 5.0 ± 0.1, 5.0 ± 0.2 and 4.9 ± 0.1 for healthy volunteers (HV, n = 10), family members (FMC, n = 10) and disease controls (DC, n = 10) with normal mucosal structure, respectively ($p < 0.001$). With remission on a gluten-free diet, D_N for treated CS patients returned to the normal range. These data clearly show that the increased proportion of immunoblastoid cells (Fig. 2) in untreated CS shifts the distribution to the right, returning to overlap the normal distribution for D_N once a singular change in diet, removal of gluten, had occurred [66].

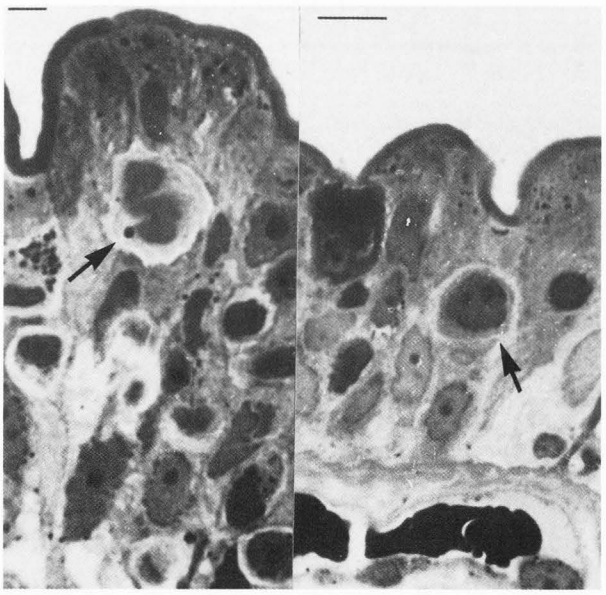

Figure 2. In the established CS lesion, EL reveal an increase in mitotic activity (LH panel, arrow) and increase in size: some have an 'immunoblastoid' appearance (RH panel, arrow). Bars = 10μm.

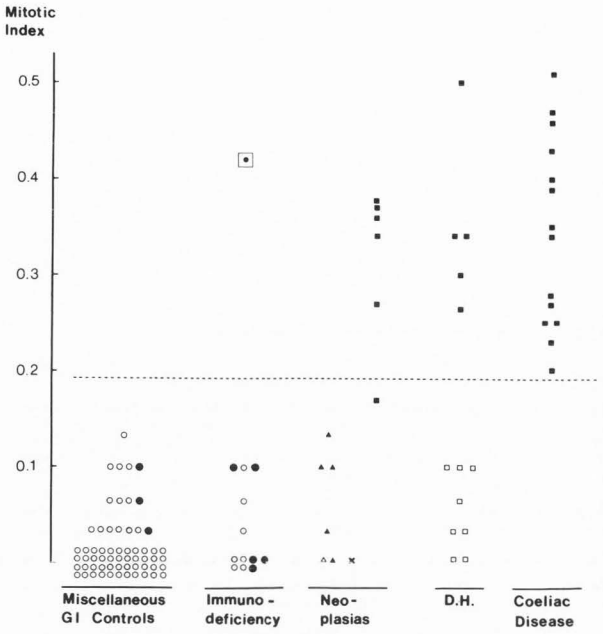

Figure 3. Percentage mitotic indices for EL in various categories of human small intestinal biopsies are illustrated. In the majority of control biopsies mitotic EL are rarely seen, while in gluten-sensitised patients (coeliac disease, dermatitis herpetiformis DH and celiac-associated lymphomas) with Type 2 lesions (■) the index usually exceeds 0.2%. DH patients with Type 1 lesions (□), immunodeficiency (●), non-gluten-induced lymphoma (▲) or carcinomas (✖), differ insignificantly from controls: solid symbols denote 'flat' biopsies. EL in the patient with combined CS and immunodeficiency (◉) behaved like other CS lymphocytes. Given a flat biopsy, an elevated index > 0.2% among EL is a useful presumptive marker for CS disease.

In determining the % mitotic index (MI) of EL, values greatly exceeding 0.2% (Figs. 2,3) were usually observed in untreated CS patients [66,69], while a variety of control subjects, including some with flat-mucosae had lower MI < 0.1% [76]. In treated CS patients, MI for EL in surface epithelium fell below the arbitrary cut-off point (0.2%). Thus it was concluded that activity of EL was reduced by the removal of a single antigen only from the patients' diet.

The use of colchicine served to highlight both the high basal mitotic activity of EL (Fig. 4) and the increased rate of arrival of new cells into metaphase with time (Fig. 5). The

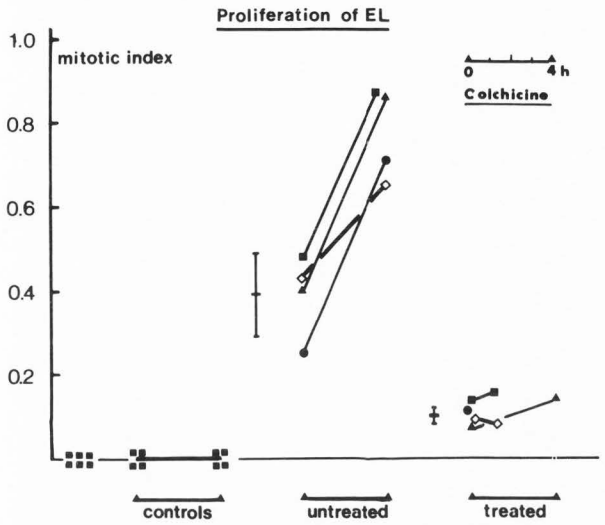

Figure 4. This figure illustrates mitotic indices (vertical axis) for EL in control and CS patients, before and after treatment. Basal activity in 10 controls was zero, and no additional metaphases were observed after a 4h colchicine blockade. In contrast, mitotic indices in CS are raised, with a marked increase with colchicine: after treatment, mitotic indices fall almost to control values.

dynamics of such changes were markedly reversed by gluten-restriction [66].

An investigation into the EL of crypt epithelium [79] was prompted by an observed increase in the flattened mucosa of endemic tropical sprue patients [77,98]. Despite a 3-5 fold increase in V_{CR} compared with various control mucosae (1.7 x 10 μm vs 0.5-0.6 x $10^6 \mu m^3$), a considerably elevated population of EL (~ 180) was observed (Fig. 6), confined predominantly to their upper regions (Fig. 7). Even allowing for the scaling-up of control crypt volumes to those of untreated CS crypts, the latter still contain an excess of EL over controls that is considered to be gluten-induced. In parallel with their counterparts in surface epithelium, D_N for crypt EL was 5.7± 0.2, compared with controls (4.9 ±0.1, $p < 0.001$), reverting to the control range with dietary gluten restriction. Note also the marked increase in large immunoblastoid cells ($D_N > 6\mu m$) (Fig. 8).

Paradoxically, although the population of crypt EL was considerably elevated irrespective of hypertrophy of crypt epithelium and fell on treatment [79], the results for surface epithelium were different, the total population of EL in untreated CS mucosae being somewhat lower (250) than the means (300-350) for each of the various control groups [66]. Furthermore, on treatment, the population size for EL in surface epithelium increased to a mean of 370, while that in crypts fell (180 to 60).

Dermatitis Herpetiformis

In this condition, a wider spectrum of

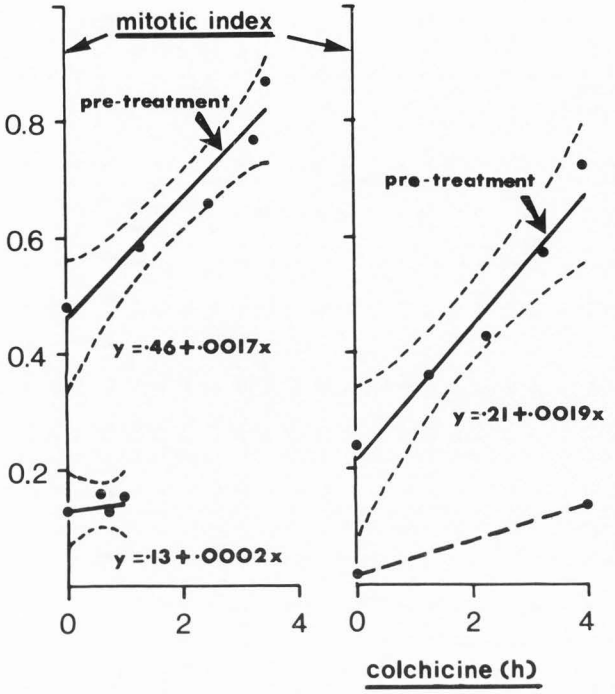

Figure 5. This illustrates use of multiple biopsies during a 4h period of colchicine blockade in establishing rates of EL mitosis (measured by % mitotic index) in two CS patients, before (upper curves), and after (lower curves), treatment with dietary gluten restriction: Note alterations in rates of mitotic activity. Dotted lines represent 95% confidence limits.

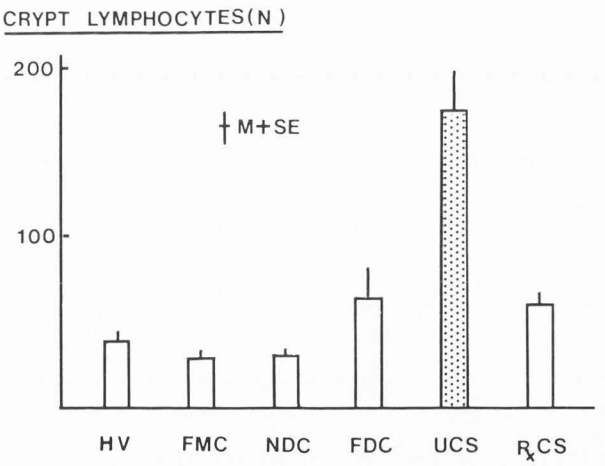

Figure 6. Crypt lymphocytes (N) in untreated CS (UCS) are markedly elevated over all other control groups (HV - human volunteers: FMC - family member controls: NDC - normal disease-controls: FDC - flat-disease controls) and fall after treatment (R_xCS). Data represent absolute population sizes related to $10^4 \mu m^2$ test area of muscularis mucosae.

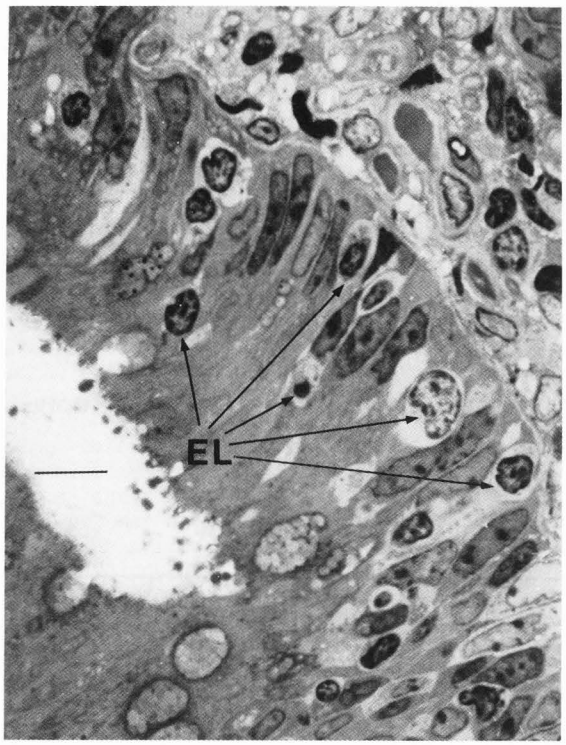

Figure 7. This representative 1μm toluidine blue-stained micrograph illustrates infiltrated crypt epithelium in untreated CS. Note marked heterogeneity in size and appearances of lymphocytes (EL). Bar = 10μm. Note that EL occupy upper zones of crypt epithelium.

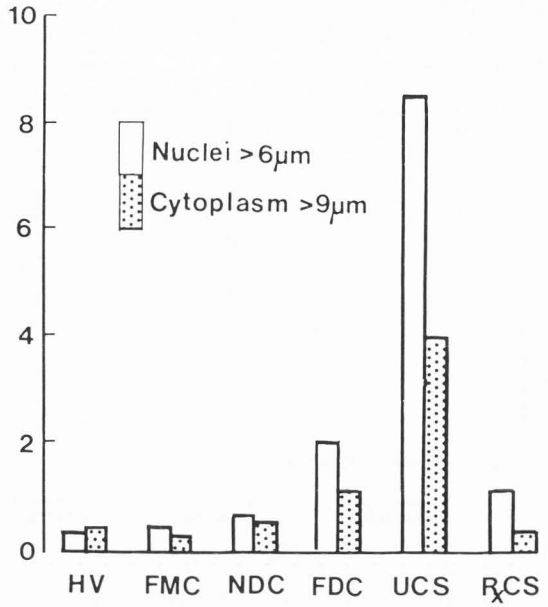

Figure 8. Like surface epithelium in CS, crypt epithelium also contains a high proportion (~8%) of immunoblastoid EL over all other groups (see Figure 6). Note that EL nuclear diameters (> 6μm) provide a more accurate assessment of cell size than cytoplasmic diameters (> 9μm) which are subject to greater sectioning errors as overall size increases. In treated (R_xCS) cases, size of lymphocytes rapidly returns to control values.

morphologic changes were wrought on jejunal mucosa by gluten compared with CS. Previous investigators [12], especially Fry et al [39,40] had emphasised that the majority of DH patients have a virtually normal villous structure bearing an increased population of EL.

It was instructive to apply image-analysis techniques to the DH lesion in patients before gluten restriction in order to compare background morphologic changes with those involving the EL pool within surface (villus) and crypt epithelium (Fig. 9). The data are ranked according to V_{SE}, thus demonstrating the progressive flattening of villous structure to a degree identical to that seen in untreated CS disease.

It is interesting to note that crypt hypertrophy appears early in the series and is present in some specimens whose surface epithelial volumes (V_{SE}) had not fallen below the lower 95% confidence limits for control mucosae. This raises the intriguing possibility that crypt hypertrophy is not a compensatory response to an exaggerated rate of migration and desquamation of surface enterocytes that characterises severe villous flattening, but rather is due to other mechanisms, as yet unidentified. Thus, the validity of the analogy between enterocyte loss followed by crypt hypertrophy and excess erythrocyte breakdown (haemolysis) leading to compensatory bone marrow growth, may be incorrect.

In terms of EL, it can be seen that the villi to the left hand margin of the diagram contain markedly increased populations of EL, although these are small and non-mitotic. As V_{SE} falls, the absolute number of EL drops, so that for flat mucosae (right-hand margin), values lie around the low-normal range. In this setting, however, EL are large and highly mitotic and thus identical in behaviour to those in the classic CS lesion, as detailed above. Conversely, note that as the crypts hypertrophy, their content of EL progressively rises: furthermore, with severe lesions, crypt EL become larger, as in untreated CS crypts [79].

Malabsorption Syndrome in Gambian Children

In a collaborative study organised by the Dunn Nutrition Laboratory, Cambridge, UK, we have been analyzing jejunal mucosae from young children from villages in the Fajara region of Gambia. These children have a severe malabsorption syndrome (not apparently due either to dietary causes, giardiasis or an acute infectious enteritis) in whom a pilot study revealed a jejunal lesion reminiscent of coeliac disease: they might therefore provisionally be deemed to have a chronic 'tropical sprue-like' illness.

Our preliminary data are displayed as for DH patients (Fig. 10) with villous volumes (V_{SE})

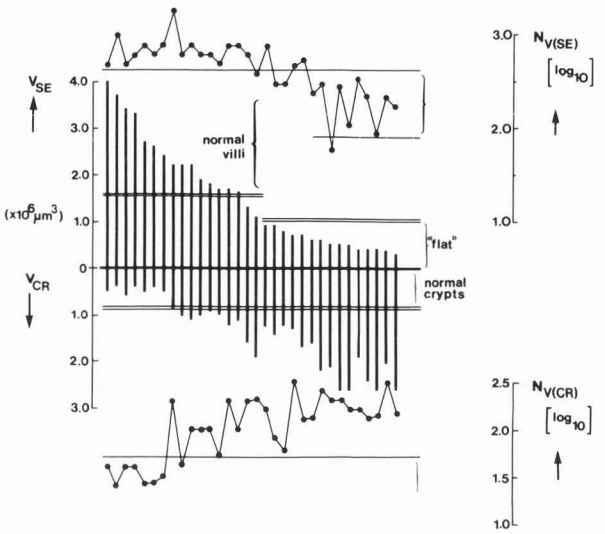

Figure 9. Mucosae from 32 untreated DH patients are arranged in decreasing order of surface epithelial volumes (V_{SE}), together with their corresponding crypt epithelial volumes (V_{CR}), as indicated on LH axis ($x10^6\mu m^3$). The three sets of horizontal paired lines represent 95% confidence limits for lowest control V_{SE} and highest CS V_{SE}, and uppermost crypt epithelial volume (V_{SE}) for control mucosae. RH axes are logarithmic scales for absolute populations of EL in surface $[N_{V(SE)}]$, and crypt $[N_{V(CR)}]$ epithelium.

Left hand villi that are within normal range contain raised EL populations: population size for surface epithelial EL falls as mucosae flatten (RH side). In contrast, crypt EL abruptly rise above upper reference range (horizontal single line) in at least seven mucosae whose villous volumes still lie within the lower 95% confidence limit, but whose crypt volumes exceed normal control values. As V_{SE} gradually falls, there is a slight progressive increase in $N_{V(CR)}$.

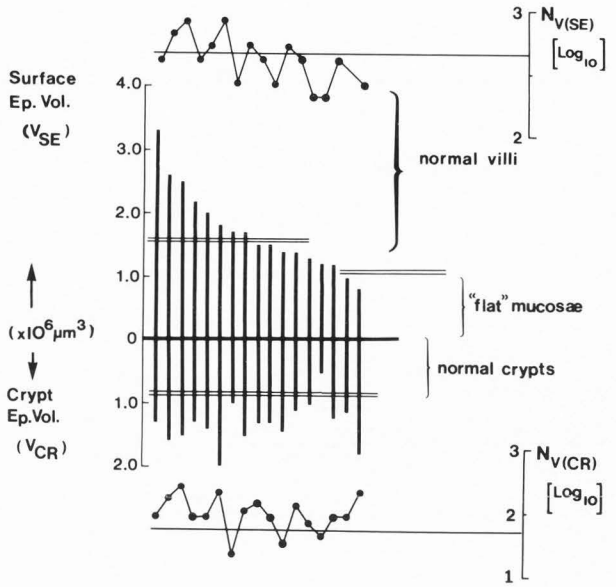

Figure 10. The format of the diagram is identical to Figure 9, but illustrates data from 17 Gambian children with "tropical sprue" type malabsorption. Several left hand villi are of normal volume, contain a high number of EL and are associated with markedly hypertrophic crypts bearing their own increased population of lymphocytes. While crypt EL $[N_{V(CR)}]$ remain above upper reference range (horizontal line), note that surface epithelium lymphocytes $[N_{V(SE)}]$ tend to drift downwards into control range (below horizontal line) as mucosae flatten. There is a striking resemblance between these data and the immunopathological features in untreated DH mucosae (Figure 9).

ranked from left to right: note the surprising similarity between both sets of data. Notice also the hypertrophy of the crypts in many biopsies in the absence of major reductions in V_{SE}, and that they have high EL populations. Likewise, villi on left of diagram contain a markedly increased population of small, non-mitotic lymphocytes while with more severe lesions a fall in $N_{V(SE)}$ occurs.

The similarity in the drift of the mucosal changes (from L to R in both conditions) hints at a common mode of progression, and hence pathogenesis. However, the difficulty in interpretation is that each 'framework' is static and fails to answer the most important question arising from these data, i.e., in becoming flat is it obligatory that the mucosa passes through an identical series of changes in each patient. There is currently no answer to this important question: the natural in vivo evolution of a celiac flat mucosa, nor the time period over which it occurs, has never been observed. Note, however, that the reverse process during dietary gluten exclusion, is surprisingly long and requires 3-12 months on average for complete resolution in children [2], and between 1-5 years in adult coeliac patients [107]. One way of elucidating this problem directly is by gluten-challenge.

The Immunopathologic Effect of Gluten Challenge

Although much has been published on gluten challenge [19], all studies when evaluated together can be seen to lack uniformity of approach, since much of the impetus has been purely diagnostic, i.e., to flatten the mucosa in order to prove gluten-sensitivity.

In our laboratory it was thought desirable to approach the problem in a more systematic way, since it was argued that if the celiac lesion is due to cell-mediated immune mechanisms then a series of graded challenges (analogous to performing a Mantoux test) might reveal detailed information about the sequence of changes reflecting the evolution from normal villous architecture to that of avillous flattening.

We therefore set out to challenge small groups (~6) of well-treated CS patients with a

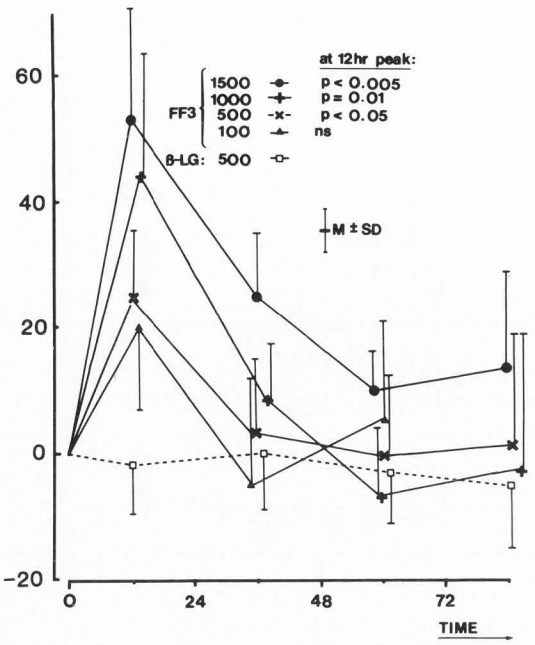

Figure 11. These are group mean data for treated CS patients challenged orally with Frazer fraction 3 (peptic-tryptic digest of gliadin) at doses of 100-1500mg per challenge. There is a dose-dependent, time-related increment (shown on vertical axis as net percentage change over pre-challenge control values) in surface epithelial lymphocytes. The lymphocytic infiltrate comprised small, non-mitotic cells that had virtually ceased by 36h post-challenge. There was no response to the control protein β-lactoglobulin (horizontal dotted line).

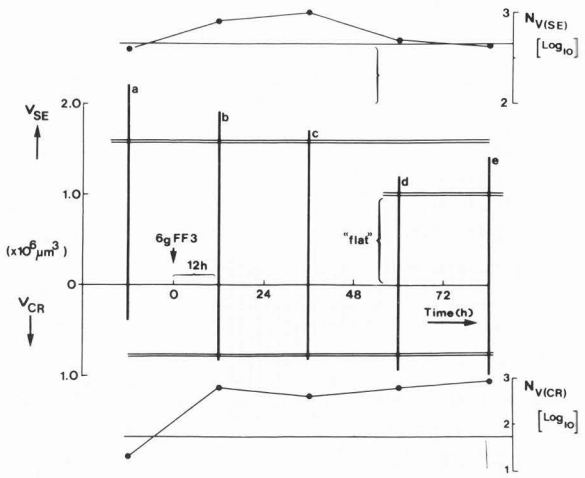

Figure 12. This diagram illustrates sequential immunopathologic changes in small intestinal mucosa in a gluten-sensitive patient challenged orally with 6g Frazer fraction 3 (peptic-tryptic digest). Format as described in Figure 9. The control (pre-challenge) biopsy (a) is normal. At 12h and 36h post-challenge (b,c) there is already marked crypt hypertrophy exceeding upper 95% confidence limit for control crypt volumes (V_{CR}) while villi are still in control range. Nevertheless both compartments are subject to major rises in EL content, but whereas crypt EL population [$N_{V(CR)}$] remains elevated, that in villi [$N_{V(SE)}$] falls as villi approach 'flat' range (d,e). EL in specimen e revealed an increase in size and mitotic activity.

peptic-tryptic digest (PTD) of gliadin [58] and to observe the effects of each dose of PTD over a 5d period, as described in Methods. These studies are still progressing.

In the smaller challenges (Fig. 11), in which oral doses of either 100, 500, 1000 or 1500mg of PTD were employed, a dose-dependent, time-related accumulation of small, non-mitotic lymphocytes into surface epithelium was observed [58]. Despite the influx of EL, there was no reduction in V_{SE}, rise in V_{CR}, nor change in total length of basement membrane (per $10^4 \mu m^2$ muscularis mucosae) in any specimen analyzed. It should also be noted that CS patients did not react to β-LG, while control subjects showed no response to either immunogen. The maximal increment in EL was observed at 12h post-challenge, the effect having largely disappeared when the next biopsy was obtained at 36h post-challenge.

With a 6gm PTD oral challenge, a considerably more interesting set of data was obtained, as exemplified by one of the patients in this group. The data are displayed as for DH (Fig. 12), although the difference now is that they represent a sequential series of mucosal changes occurring with time in one individual.

At 12 and 36h post-challenge there is no statistical change in villous height (V_{SE} remains constant), but there is a doubling of crypt volumes (V_{CR}) which persists throughout the challenge. At 60-84h, there is considerable loss of villous height although true flattening fails to occur. The events occurring within the EL pool are striking. At 12h the absolute population of EL ($N_{V(SE)}$) in villous epithelium has almost doubled (note log scale) although the lymphocytes are small and non-mitotic while at later times, they gradually fall in number in parallel with the progressive reduction in V_{SE}. In the crypts, EL rise throughout the challenge and remain elevated at 84h. At 84h, their size has increased and the rate of mitosis has also begun to accelerate.

In dynamic terms, we see for the first time the temporal evolution of events leading to mucosal flattening and which also precisely recapitulate the static picture suggested by grouping mucosae as in DH (Fig. 9) and the Gambian children with sprue-like malabsorption (Fig. 10). Although these challenge studies are continuing, there is already strong evidence to show that the evolution of a flat mucosa requires progression through a series of specific changes. Most notable is the observation that crypt hypertrophy is an early event, and not primarily a compensatory response to villous flattening with its implied increased

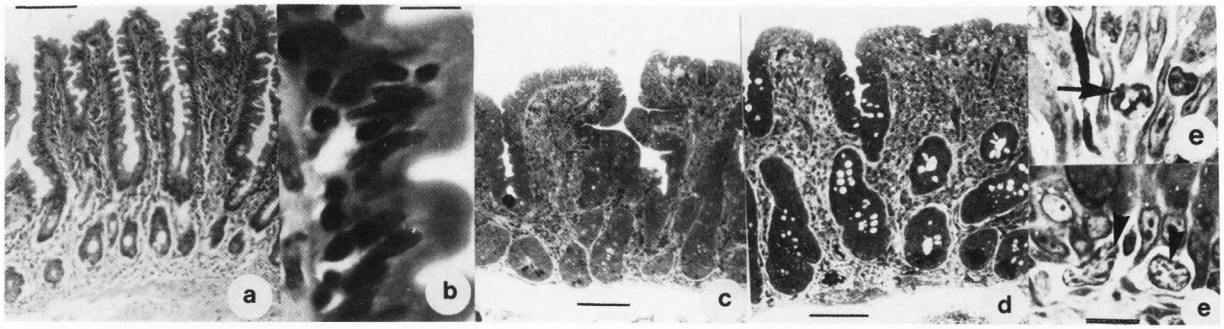

Figure 13. Initial biopsy (panel a) shows normal villi with considerable infiltration of epithelium by small, non-mitotic EL (panel b). Second biopsy (panel c) reveals intermediate lesion with "low" villi. Final biopsy (panel d) is characteristically flat with epithelium containing both mitotic (panel e, upper segment) and immunoblastoid (lower segment) EL. This sequence, somewhat similar to those depicted in Figure 12, evolved over a period of ~4 years. Magnification bars: a,c,d = 100μm: b,e = 10μm.

desquamation and loss of surface enterocytes.

Natural Evolution of the CS Lesion

The above studies still invite the criticism that the dynamic spectrum of mucosal changes brought about by graded challenges with gluten, however interesting, represents an artificial model of flattening. However, we have observed certain patients in the University Department of Medicine at Hope Hospital, in whom a similarly evolving series of mucosal changes was observed over several years. An example is shown (Fig. 13) of mucosal biopsies obtained from a 60-yr-old lady who presented us with a 20-yr history of steatorrhea and nutritional deficiencies, with an initial biopsy revealing normal villous architecture (subsequent review in our laboratory showed epithelium full of small, non-mitotic (%MI = zero) lymphocytes (Figs. 13a,b). No specific diagnosis had been made, but gluten restriction caused prompt improvement. She relapsed six months later and pancreatic extracts were added. Two years later (Fig. 13c) a further biopsy showed 'ridged' villi (%MI = 0.03) and like the first, was thought to be normal: an unrestricted diet was advised. This led to a rapid increase in diarrhea within six months, and after another year she was referred to our Unit, where a third biopsy (Figs. 13d,e) now revealed complete flattening, and large, mitotic EL (%MI = 0.5). Nutritional supplements, anti-diarrheals and strict gluten restriction swiftly brought her into remission, with improved well-being and subsequent weight gain. She remained well until lost to follow-up, but refused additional investigative procedures during that time.

The presumptive diagnosis was celiac disease. Nevertheless, her mucosal biopsies (if considered to be representative) showed a hitherto unrecorded progression from villous pattern with lymphoid infiltration of epithelium to a classic flat lesion containing mitotic, 'immunoblastoid' EL, evolving over a period of 3-4 years. A similar mucosal progression, evolving more rapidly within two years, was observed in biopsies submitted to us for review on another lady with long-standing malabsorption: her last biopsy, before gluten restriction, was flat and again showed characteristic proliferative activity of EL.

Both cases invite the suggestion that the temporal evolution of the classic CS lesion may involve an initial phase similar to (i) that evoked by small-dose gluten challenges and (ii) appearances noted in some first-degree CS relatives.

First-Degree Celiac Sprue Relatives

Several extensive family studies have revealed a prevalence rate of either symptomatic or asymptomatic ('latent') celiac sprue with classical 'flat' biopsies among 10-15% first-degree relatives [62,90,108,30]. We have recently completed a study of 52 first-degree CS relatives [80] in terms of HLA status and intestinal morphology, epithelial lymphocyte populations and permeability to ^{51}Cr-EDTA. That an increase in intestinal permeability predisposes to CS disease was suggested by its persistence in several people whose mucosal biopsies were morphologically normal as a result of many years of gluten restriction [80,6]. Given the hereditary-genetic background to CS disease, it was argued that first-degree CS relatives might be expected to show defects in permeability if the latter is an important risk-factor in pathogenesis. We were careful to exclude from study all relatives who had already been shown by jejunal biopsy to have a flat biopsy, the prevalence rate in our local population (15.5%) being similar to that elsewhere in the UK and world.

None of the 52 relatives studied had any abnormality in villous morphology, and permeability was within normal limits (24h excretion of ^{51}Cr < 3%). Unexpectedly, 38% of these individuals revealed villous epithelium containing an increased population of small, non-mitotic lymphocytes: the numerical range (413-1002) exceeded the 95% upper confidence limits for control biopsies, within which the EL populations of the remaining CS relatives fell

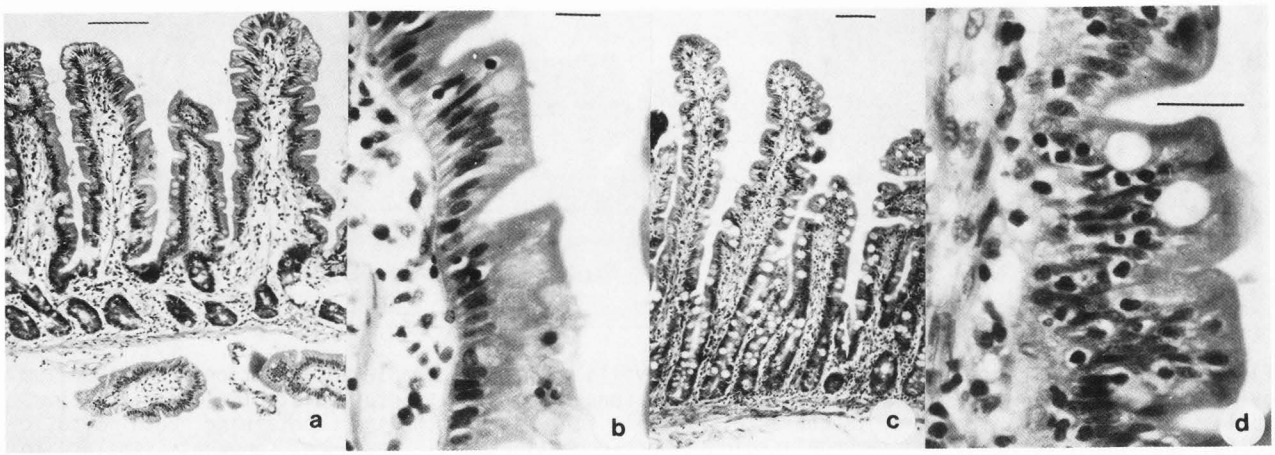

Figure 14. In a study of first-degree CS relatives, some were found to have a normal villus-bearing mucosa (panel c) in which epithelium was heavily infiltrated by small, non-mitotic lymphocytes (panel d).

Mucosal appearances in the remainder were within normal limits (panels a,b). Bars: panels a,c = 100μm: panels b,d = 10μm.

(78-395) (Fig. 14).

The lymphoid infiltrate did not appear related to HLA status, since the frequency of $DR3^+$ individuals in the normal 'group' of relatives was 50% thus providing an adequate control for the second group with increased numbers of villous EL. Furthermore, it was notable that two individuals in the latter group were $DR3^-$. Since a large proportion (15.5%) of CS relatives with flat mucosae were excluded from this study, it is unclear whether the high lymphocytic infiltrates could be a progressive lesion, or at least a "marker" indicating development of a flat lesion at some future date. Such a view is also suggested by the case-histories recorded in the previous section. What is evident from this study is that a lymphocytic infiltrate into villous epithelium does not necessarily cause either structural abnormalities nor alterations in mucosal permeability. Other factors may thus be required for full disease expression: the nature of such factors is at present unknown.

Extended Discussion

Morphometric Considerations

The results in all these studies derive from a computerised morphometric system of analyzing intestinal mucosa, in which x,y,z coordinates are projected into the tissue sections. The x,y coordinates define a constant test area ($10^4 \mu m^2$) that overlies the muscularis mucosae, while the z scalar controls structural measurements in the vertical component of the mucosa. All data, obtained by drawing around profiles observed in the 2-dimensional plane of the section, are always related to a known length of muscularis mucosa while repeated measurements permit a statistical reconstruction of the original (3-dimensional) structure. A relative coefficient of variation < 10% indicates that sufficient measurements have been made for any tissue component. The three structural components of interest are (i) surface epithelial volume (V_{SE}) (ii) crypt epithelial volume (V_{CR}) and (iii) volume of lamina propria (V_{LP}). Thus, irrespective of the degree of abnormality (z) present in any specimen, its component volumes are calculable, valid and comparative, since they are always related to the same test area (x,y) of muscularis mucosae (Fig. 1). This diagram also shows, despite the irregular shape of those components, that they may be simply displayed, quantitatively, as cubes of equivalent volume. A similar approach was used by Guix et al [43].

The use of cubes provides a convenient model for evaluating cell types within a given structural component. Component volume, V, number of cells (N), and their resultant density (D) per unit volume is given by the relation $D = N/V$ (Fig. 15). This simple relationship permits construction of volume-density curves [91] and evaluation of the effects of varying one component in relation to the other quantities. For example, it was shown [43,66] that the (absolute) EL population in untreated CS mucosa lies at the lower limit of normal. When EL are 'counted' by reference to epithelial cells [33], a measure of density is obtained, which is not the same as absolute number, i.e., $D \neq N$. With our mathematical model, values of V_{SE} and N_{SE} for a normal mucosa can be calculated: if V is then progressively reduced to values commensurate with a flat mucosa while N is held constant, the effect of volume reductions on D can be examined (Fig. 16): this illustrates how density rapidly rises as the mucosa flattens although the actual size of the lymphocyte population has not altered: similar events clearly occur in celiac sprue.

In contrast, crypt EL populations in CS are increased presumably because crypt epithelium does not exfoliate. The decrease in surface EL

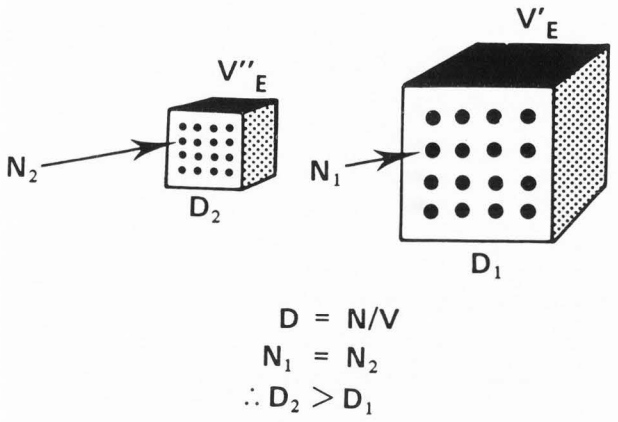

Figure 15. In addition to displaying mucosal volumes in terms of equivalent cube volumes (Fig. 1), this model more effectively illustrates the relationship between epithelial volume (V_E), the absolute number of contained cells (N) and their resultant density D, in terms of the relationship $D = {}^{N}/V$.

Although CS epithelial volume (V''_E) is decreased relative to control mucosae (V'_E) their populations of EL are approximately equivalent ($N_1 = N_2$), such that the density of CS cells D_2 exceed D_1, the density in control mucosae: these concepts can be further exploited as demonstrated in Fig. 16.

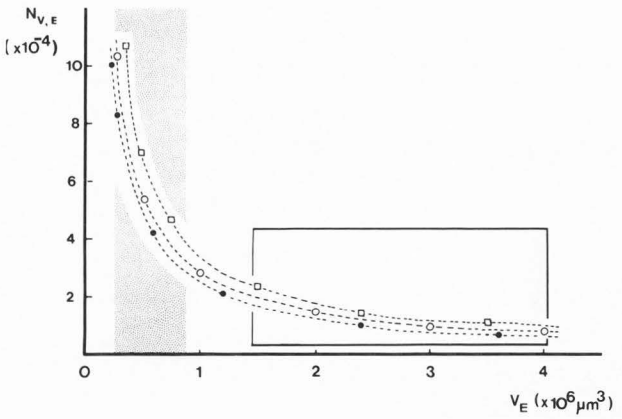

Figure 16. These graphs illustrate the important relationship between cell (EL) density (expressed as $N_{V,E}$ on vertical axis) and epithelial volume (V_E) (shown on horizontal axis) while N remains constant. As V_E is progressively reduced, the density of EL changes little through the range for control values (V_E) (open box) but rises acutely and disproportionately when V_E moves through the range typical of flat mucosae (shaded area). This model emphasises the dangers inherent in expressing cell population sizes in terms of densities rather than absolute numbers.

populations as flattening occurs is probably a reflection of the rapid desquamation of surface epithelium which removes EL at a rate greater than they can be replaced. This is, of course, a speculative deduction and no true data exist to support or refute it.

Type I and Type II Mucosal Lesions in CS

It is now evident that two distinct extremes of mucosal change exist. The type I comprises normal villi whose epithelium is filled with numerous, small, non-mitotic lymphocytes. This occurs in (i) many DH cases (Fig. 9), in some 1° CS family relatives (Fig. 14), and in treated CS patients challenged orally with FF3 in low dose (0.1-1.5g) (Fig. 11). Conversely the type II lesion is seen in untreated CS, and a small proportion of DH patients who may have a severe lesion with, or without, accompanying malabsorption (Fig. 9). In Type II lesions, the mitotic activity and mean size of surface, and crypt, EL are increased (Fig. 17).

Some preliminary evidence is presented to suggest that the evolution of a type II 'flat' lesion requires transition through an earlier type I lesion as demonstrated (i) by our sequential challenge data (Figs. 11,12) (ii) in some patients spontaneously (Fig. 13) and as hinted at (iii) by the graphical representation of mucosae in DH (Fig. 9) or the malabsorption syndrome in Gambian children (Fig. 10). Clearly, although many details require further documentation or substantiation, the framework upon which these changes occurs is becoming evident.

Mechanisms of Villous Flattening

Although we are still not in possession of all the factors that result in villous flattening, several important leads have been established. Firstly, the T cell dependence of villous flattening was demonstrated several years ago in immunologically-driven models involving worm infestations of rats (Nippostrongylus brasiliensis) [34,35]. Further studies have elaborated on these earlier experiments, involving either allograft rejection or graft-versus-host reaction (GVHR), and have confirmed the effects on mucosal architecture [60,61]. In these studies, the occurrence of crypt hypertrophy before any villous shortening is apparent, was carefully documented and emphasised as a major early event in these reactions.

Allograft Rejection. Heterotopic grafts of fetal intestine are sterile and lack their normal complement of lymphoid cells: thus while the graft stroma is of donor origin, the lymphoid infiltrates during rejection are derived from the host [32]. In these experiments the early onset of crypt hypertrophy was initiated by increases in crypt cell proliferation from day 3 onwards. A statistically significant reduction in villous height was generally only evident during the second week after engraftment [60,61]. Accompanying the early crypt changes was an

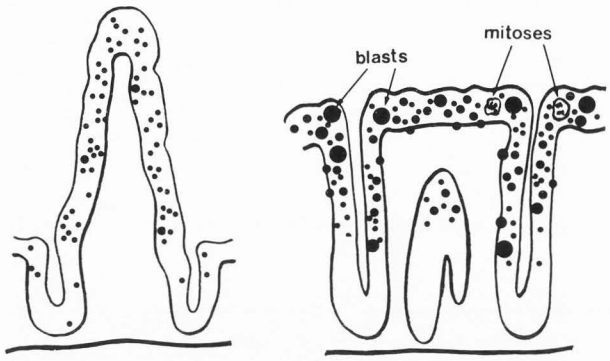

Figure 17. Two distinctive patterns occur at each end of the gluten-induced spectrum of mucosal insults.

The Type 1 lesion, comprising normal villi containing a large population of non-mitotic, small lymphocytes is seen in CS patients after small dose challenges; a large proportion of DH patients; and in some 1° CS relatives. The established Type 2 'flat' lesion, comprising large, highly mitotic EL is seen in CS disease and some DH patients: the other feature of this lesion is that while the size of the EL population in crypt epithelium is markedly increased, that in surface epithelium lies within the control range: these differences may be due to increased rates of enterocyte exfoliation from the surface epithelium only.

increase in lamina propria lymphocytes, closely followed by an infiltrate of lymphocytes into surface epithelium [34]. These appearances are identical to the type 1 CS lesion.

Allograft rejection represents a phenomenon that is (i) thymus- dependent and (ii) in which the extent of tissue damage is related to the degree of histocompatibility differences between host and graft [110]. Analogically, it could be stated that as the "challenge dose" increases, progressively more severe intestinal damage is produced. Anti-graft (cytotoxic) antibody is not involved [28].

GVHR. Intestinal damage is produced, along with other tissue effects, by injections of alloreactive T lymphocytes and is conveniently measured by the spleen index [106]. Avoiding recognition of donor lymphocytes as foreign by the host-recipient can be achieved with the use of neonatal animals ("immunodeficient"), irradiated hosts or semi-allogeneic chimaeras (F_1 hybrid host) [32].

Although in adults (CBA x BALB/c) the effects on intestine are not marked, in neonatal mice crypt hypertrophy and increased crypt cell production (which correlate directly with spleen index) occur without evident effect on villus size. However EL populations rise significantly within 24h: later on, some EL assume a blast-like appearance and mitotic cells are also present [86,87,32]. Again, these events recall the type 1 CS lesion.

Other experiments have shown that enterocyte damage in GVHR is not due to T cell-mediated cytotoxicity [32,11,36]. During GVHR there is a boosting of NK cell activity and recruitment of such cells into intestinal tissues [88]: however depletion of NK cell activity, either in host or donor, fails to prevent the anticipated immunopathologic effects [116]. Thus it seems that the latter are due to soluble mediators released by activated graft T cells coming into contact with host-derived lymphocytes and macrophages [27,85]. The cells initiating the reaction are Lyt 1+ (T helper) cells restricted by I-A (MHC Class II) gene products: these are DTH effector T cells [89,44].

IFN-γ, a lymphokine secreted by activated T lymphocytes, is one agent known to stimulate the constitutive expression of I-A (class II) antigen by crypt and surface epithelial cells [4,16]. Thus I-A expression by enterocytes is probably a good 'immunohistologic marker' of a cell-mediated (DTH) reaction in intestinal tissues [101,102,104], although the secondary role of such expression by enterocytes (e.g., in local antigen presentation [9,10]) is not entirely straightforward and awaits further detailed clarification. However, it seems reasonable to conclude from observations based on GVHR models that class II antigen expression neither (i) implies nor (ii) inevitably results in, enterocyte damage.

Despite such a brief description of an extensive field of study, important lessons to be drawn are (i) that intestinal damage is a dose-related effect proportional to the degree of disparity at the MHC locus (ii) that crypt hypertrophy is an early feature of damage in mild lesions, in which normal-appearing villi may be infiltrated by small, non-mitotic lymphocytes (iii) that villous flattening occurs late and is T-cell dependent (iv) that the delayed appearance of blast-like, mitotic EL is delayed and (v) that lesion development is dependent on DTH effector lymphocytes restricted by MHC class II antigens.

The Immunopathologic Spectrum of Cell-Mediated Intestinal Damage

In the above summary, we can recognise the structural features already recognised in various human enteropathies, especially CS and dermatitis herpetiformis. The parallelism not only suggests a common immunopathogenic mechanism, but also that there are varied and progressive structural facets of cell-mediated immune damage to the small intestinal mucosa.

There are additional features common to both, such as (i) the constitutive expression by villous and crypt enterocytes of MHC Class II antigens, (ii) the presence of expanded populations of mast cells [78,109] and basophils [78] within the lamina propria, (iii) alterations in fluid and ion transport by epithelium [14,15] and (iv) secondary changes in

permeability [8,6] that are probably due to the generation of inflammation within the lamina [7]. Another feature of altered pathophysiology involves changes in the activity of brush border enzymes, such as disaccharidases, but the mechanisms governing these changes are more subtle than the mere presence of immature surface enterocytes resulting from an increased rate of cell migration [32]. For example, it has been shown that varying rates of cell migration influence the enzyme maturation profile within individual enterocytes [59]. This is clearly a field ripe for further explorations. Parenthetically, clinicians might also be wary of jumping to the obvious conclusion that gluten is directly damaging to surface enterocytes in CS : there is likely to be a less facile explanation for such events.

In considering enterocyte damage, it is noteworthy that even in the most severe GVHR reactions, such damage is not always conspicuous, despite (i) the presence of increased numbers of EL and (ii) the boosting of non-specific T cell cytotoxicity and NK cell activity. In this context, the fact that CS crypts contain a 5-6 fold increase in EL should not be forgotten [19]. These EL are identical to those in surface epithelium by a variety of criteria [104,73,81]: yet if they are cytotoxic to surface enterocytes, it is strange that crypt epithelium remains completely unscathed, and is neither inhibited in its vast hypertrophic response nor ability to generate argentaffin, goblet and Paneth cells. Furthermore, the constitutive expression of MHC Class II antigen by crypt cells clearly does not lead to their 'autoreactive' destruction.

The role that changes in microvascular perfusion of the mucosa may play in regulating the functional and structural integrity of enterocytes must also be considered. These aspects of pathophysiology have not been well explored, although ultrastructural lesions documented within CS enterocytes are highly reminiscent of those evoked by short-term ischemia/anoxia in experimental animals [70,71].

CS occurs in genetically-predisposed individuals (A1, B8, DR3/7, DQw2) as a result of wheat ingestion. Extensive studies of the target tissue of injury - the upper jejunal mucosa - have provided important insights into the immunopathology of the lesion. Analogous studies of other presumptive "immunological" enteropathies such as tropical sprue [77,98], giardiasis [1,119], and short-lived reactions to various food components (cow's milk protein, soya, eggs and fish) in young infants have revealed similar patterns of intestinal injury which, in part, are consistent with a cell-mediated form of intestinal injury [50].

It also appears that each of these conditions requires a specific genetically-determined host response to the relevant environmental immunogen, whether that be antigen(s) of microbial/parasitic origin, food-derived, or resulting from discordances at the MHC class I/II loci in allograft rejection or GVHR. The role of genetic factors in CS/DH has been

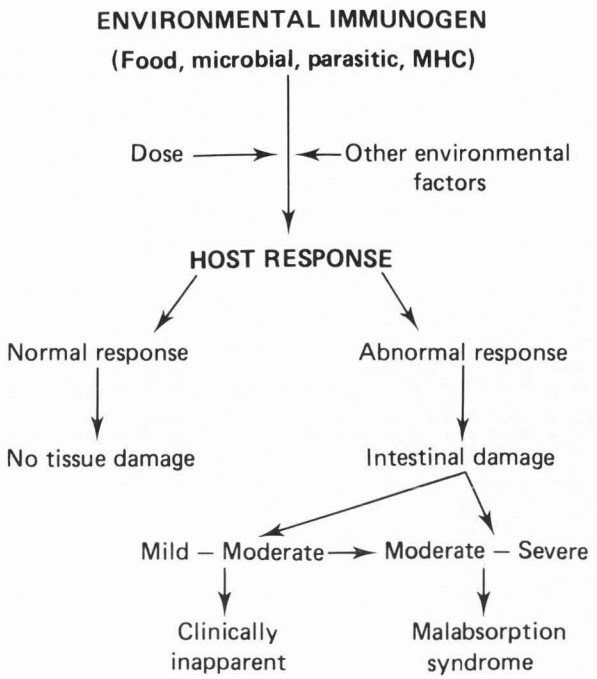

Figure 18. The degree of intestinal damage caused by presumptive cell-mediated immune responses to a variety of unrelated environmental stimuli is likely to be regulated by host responses. Despite these varied stimuli, the actual immunopathology observed in affected mucosae is stereotyped and appears to evolve through a series of recognisable changes and patterns of injury.

reasonably well documented, but we also know that giardiasis is dependent on genetic factors, both in humans where HLA-B12 may be relevant [96], and in animals since BALB/c mice rapidly clear the parasite while C3H/He mice (like T cell deficient animals) have defective elimination mechanism(s) [114]. Conversely, there is no available evidence for tropical sprue, but since this condition does not affect all those wholly exposed to the same environment, some restrictive element must be operative and thus presumed to be host-derived (Fig. 18).

The various immunopathologic studies referred to above have helped to bring such apparently diverse conditions within a unitary model of pathogenesis. This framework seems now to be well established and provides the data required for recognizing either the "early" or "late" phases of such reactions within human and non-human intestinal tissue. For the future, work concerned with the elaboration of lymphokines, interferons and leukotrienes, as well as other cell-derived factors from mast cells, basophils, macrophages and lymphoid cells, and the role of the neuroendocrine system, will need to be unravelled in order to further promote our understanding of these complicated tissue reactions.

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Discussion with Reviewers

J.R. Poley: Does age have an influence on morphometric indices, particularly on mucosal compartment volumes and on lymphocyte populations? In untreated CS, and after challenge?

Author: In an extensive study of various control groups, whose ages range over five decades of adult life, no effect of age on either mucosal compartment volumes, or on their contained EL populations, has been apparent. We find that the same indices in childrens' mucosae also are commensurate with those of adults.

J.R. Poley: Was there adequate sampling of biopsy specimens to exclude the presence of so-called patchy lesions?

Author: Patchiness of the mucosal lesion only occurs in about 20% mucosae from either untreated DH, or CS patients. My opinion is that far too much emphasis on mucosal patchiness has been made than is warranted by the facts, which have been consistent in three independent published studies [123,128,129]: it does not appear to have had much effect on the consistency of our results, as described above.

J.R. Poley: What is the nature of the lymphocytes - cytotoxic, NK, CD4 or CD8 cells?

Author: I assume that this query relates both to EL (i) normally present within surface and crypt epithelium and (ii) to those induced by gluten challenge. In both circumstances [text refs. 29, 104] the majority of EL bear the CD8 antigen. Whether more subtle changes in EL populations occur after challenge has not been investigated in depth. Furthermore, the availability of newer sub-subset markers provides an opportunity for further detailed study on this point.

A. Ferguson: You have used your technique to scale up crypt volumes from normal mucosae to the level seen in CS and shown that the size of the revised EL population still falls short of that in the untreated CS crypts, indicating that the marked increase of EL in the latter is not merely an effect of crypt hypertrophy. In other words there is an 'excess' of EL that is truly gluten-induced [text ref. 79]. If you were to scale up the reduced surface epithelial volumes of untreated CS mucosae to values approximating V_{SE} for control mucosae, is the discrepancy in the low values for surface EL obliterated?

Author: This is an interesting point which our model of quantitating mucosal biopsies permits. This specific intervention had not been carried out, but the results sought by this question, both for 14 untreated CS patients, and for the 15 DH patients with flat mucosae (Fig. 9), are shown (Fig. 19). Each mucosa has been scaled up

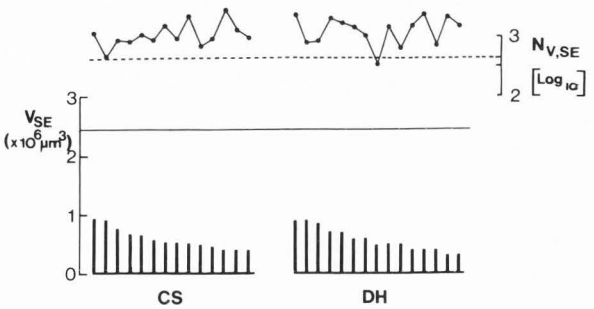

Figure 19. Values of V_{SE} obtained for 14 untreated CS flat mucosae, and 15 untreated flat DH mucosae were individually scaled up to a mean control value of 2.5 x $10^6\mu m^3$ (horizontal solid line). The new proportional values for EL populations ($N_{V,SE}$) obtained by these procedures lie above the upper 95% confidence limit (dotted line) for control mucosae. These derived data are reminiscent of the Type 1 lesion.

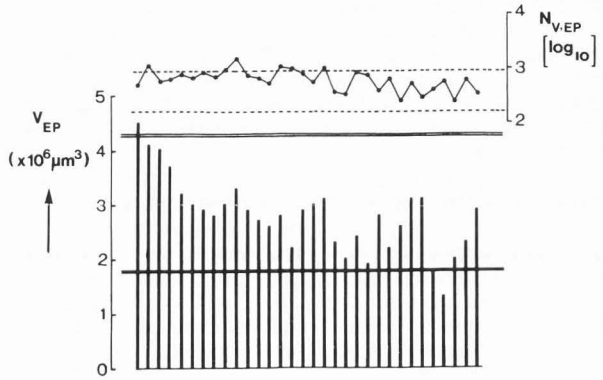

Figure 20. For all 32 untreated DH patients, the total volume of epithelium (V_{EP} x 10^6 μm^3) obtained by summating V_{SE} + V_{CR} per specimen, has been plotted. The majority fall within the ± 95% confidence limits (horizontal paired solid lines) for 30 control mucosal specimens. The corresponding total EL populations ($N_{V,EP}$), representing the sum [$N_{V,SE}$ + $N_{V,CR}$], lie between ±95% confidence limits (horizontal dotted lines) for the same control mucosae. Although V_{EP} is fairly uniform throughout the entire series, values for $N_{V,EP}$ corresponding to the right-hand 15 flat mucosae, are lower than those of the left-hand specimens, thus suggesting a greater loss of EL from the epithelium when flat.

to a value for V_{SE} of 2.5 x 10 μm , which is the mean for 30 controls (10 young healthy volunteers + 10 healthy CS relatives + 10 disease-control patients without mucosal abnormality), and the corresponding new values for $N_{V,SE}$ displayed on a logarithmic scale. The majority of values for $N_{V,SE}$ lie above the 95% upper confidence limit for control EL populations in villous surface epithelium. We are back to the Type 1 lesion!

D. Moffitt: From Fig. 9, the impression is also gained that there is no overall change in total epithelial volume ($V_{SE} + V_{CR} = V_{EP}$) and that the total EL population ($N_{V,SE} + N_{V,CR}$) may also not vary. If so, this suggests that the conformation of the mucosa (villous to avillous) and type of EL present (small, non-mitotic, to large and mitotic) are the basic changes present. Furthermore, high EL mitotic activity with little change in absolute count implies either increased losses into the intestinal lumen or increased trafficking back into the lamina propria. Does this conflict with the conclusion that the density of EL increases with mucosal flattening?

Author: By summating the values ($V_{SE} + V_{CR}$) and ($N_{V,SE} + N_{V,CR}$), it can be seen (Fig. 20) that total epithelial volume in the 32 untreated DH patients (text Fig. 9) falls within the upper and lower 95% confidence limits for the 30 varied control mucosae referred to in the answer to the preceding question. Furthermore, the total EL population tends to lie about the upper 95% confidence limits establishd for the 30 control mucosae, although for the flat mucosae (right-hand 15 values) $N_{V,EP}$ drifts downwards, again suggesting a net loss of EL in the most severely damaged mucosae. The fall in the absolute number of EL ($N_{V,SE}$) in surface epithelium is a real phenomenon and does not conflict with the fact that their density is increased - it depends on what is being measured and how those measurements are interpreted (see Fig. 16): my data suggest a considerable loss of EL occasioned by the increased dynamic state of epithelial cell turnover when the mucosa is flattened.

Note that although total epithelial volume is unchanged, the ratio of crypt to surface epithelial cells rises. Since the functions of these two populations of cells are different, the digestive-absorptive capacity of a flattened mucosa is considerably disturbed because of (i) mucosal flattening per se (ii) damage to the surface enterocytes and (iii) rapid migration of cells, leading to (iv) a higher proportion of 'immature' cells lining the luminal surface. Teleologically it can be seen that in the process of becoming flat we have an adaptive response that reduces antigenic exposure and uptake, as possibly limits the surface available for colonization, for example, by worms or parasites.

A. Ferguson: The proliferative compartment in CS mucosae is greatly expanded relative to the overall size of the crypts. Is there any particular pattern in the distribution of EL between the proliferative, and maturational, compartments?

Author: This distinction has not been formally evaluated. EL occur more in the upper one-half of the crypts and therefore would extend right across the maturational zone from the surface downwards and into the proliferative zone. I think there is a closer relationship between the presence of EL and the expression of MHC class II antigen by crypt enterocytes - although which comes first and to what degree there is a functional connection [124], requires further analysis.

A. Ferguson: In untreated DH patients and in the first degree CS relatives without enteropathy, have you considered whether these represent one end of a bimodal distribution in comparison with all others who form a continuum from marginal to unequivocal enteropathy together with their abnormalities of EL populations?

Author: I doubt whether we are seeing a bimodal population. The DH cases you refer to (first seven cases on left-hand side of text, Fig. 9) and the subgroup of first-degree CS relatives, have entirely normal morphology except for the singular presence of an increased population of small, non-mitotic EL in surface (villous) epithelium. Identical changes were induced with oral challenge (0.1-1.5g FF3) (Fig. 11) but since a larger dose oral challenge evoked the sequential appearances of type 1 and then type 2 lesions during a 5-day period of observation (Fig. 12), it seems evident that all three distinct pictures are successive phases in the evolution of a flat mucosa. However, in our challenge studies we were careful to use only patients previously known to have a flat biopsy. Whether those particular DH patients and CS relatives could be stimulated on challenge to produce a more severe type of lesion is not known. It is conceivable that such individuals are genetically restricted from proceeding further along the evolutionary pathway described. This is an important point which needs to be tested.

A. Ferguson: In the gluten-challenge series, it is presumed that the controls were ingesting a normal diet containing substantial amounts of gluten, and that the treated coeliac patients were taking milk providing a substantial amount of β-lactoglobulin. Although I do not think this is likely, presentation of antigen not encountered for many years and in the absence of blocking antibody might theoretically induce the effects observed. Were levels of local secretory, or systemic, anti-gliadin antibodies by any chance measured in these patients before challenge?

J.R. Poley: Were any of the CS patients followed by serologic tests (anti-gliadin and anti-endomysial antibodies) to parallel morphologic findings?

Author: Such antibody levels were not studied before challenge. However, by challenging controls and CS patients with gluten and β-lactoglobulin (on separate occasions) and showing that only CS patients responded to gluten, it was felt that a specific, immunologic

response had been obtained. In subsequent work, we have tried to impose a milk-free diet on controls before challenge, but they have not been at all keen to do that, so that in practise, it is not easy to induce patients to confirm to what, admittedly, is a more ideal experimental setting for these challenges.

A. Ferguson: Another very critical point concerns whether all the effects, particularly the later ones, can be attributed to the gluten challenge itself, or whether an initial abnormality produced by the immunopathologic effect of gluten leads to the evolution of a range of secondary, non-specific immune and other reactions that extend, and amplify, the effects of the gluten-targetted immune response. Although difficult for the patient, it would be interesting to maintain the patient on an elemental diet following challenge for the remainder of the study: I predict that the enteropathy would resolve rapidly within 24-48h.

Author: We have not done this, although the point you raise is of considerable theoretical interest. It might be possible to incorporate this modification into our challenge protocol and compare results with our 'standard' challenge at each dose level of gluten employed. Incidentally, it may not have been apparent, but all CS patients remained 'gluten-free' throughout each series, except for the single challenge dose of FF3.

A. Ferguson: The late appearance of lymphocytes with mitotic activity in the infiltrates in various situations is fascinating. This could be explained by different stimuli producing mitosis in the population of precursor ELs and allowing enhanced migration/accumulation of EL within the epithelial microenvironment, irrespective of mitotic activity. If humans are analogous to mice, one would expect a 2-3 day delay between antigen re-exposure and the appearance of cells within the gut which had been stimulated within Peyer's patches migrating via lymph and bloodstream.

Author: It was previously shown [text ref. 66] that the ratio of EL traversing the basement membrane to those within epithelium ("Flux Ratio") was elevated, suggesting increased lymphocytic trafficking across the basement membrane in untreated CS mucosae, compared with controls. My own feeling is that because of the extremely accelerated loss of surface enterocytes from flat untreated CS mucosae, the tempo of movement of lymphocytes into epithelium is increased to "make good" their continuing loss into the lumen: hence also the raised flux ratios. In this regard, I believe that the large EL that appear at this time are not immunoblasts, but may be large immature cells (i.e. lymphoblasts) reflecting the high proliferative rate of precursors necessary to maintain the size of the EL population (analogous to the presence of large nucleated precursors of erythroid and myeloid cell lines in haemolysis, or leucocytosis, respectively). The absence of activation markers [127] on these large EL (i.e. Tac or transferrin) also suggests that they may not be immunoblasts. Furthermore, if they did reflect gluten-triggering of gluten-sensitised EL, then they should also be seen in the earlier 'proliferative' (Type 1) lesion, but here they are conspicuously absent, despite the fact that these infiltrates are gluten-dependent. In this respect, it is correct to suggest that these high infiltrates may be due to antigen-induced lymphocyte retention within the epithelium. It therefore follows that, if the epithelium was not subject to destructive influences (Type 2 lesion), the established flat CS surface epithelium would also contain an elevated EL population (Fig. 19): the postulated high turnover of EL into epithelium in this situation might therefore be geared up to attempt this - although in reality this aim is hardly achieved.

A. Ferguson: I completely agree with the concept that there is an early phase of coeliac-like enteropathy in which EL infiltration and crypt hyperplasia are predominant and that villus atrophy is a distinct and separate later phenomenon. It is only in the villus atrophy phase that there is obvious enterocyte damage. I think I am right in the assumption that there are no features of EL to differentiate between these two states, and that you would agree that some factor other than T cell mediated damage is likely to be the explanation for the villus enterocyte abnormalities. You published some years ago on the sub-epithelial fibroblast sheath, and you have made many observations on the connective tissue and vascular components of the lamina propria, in addition to the infiltrating cells. Have you any comments on abnormalities of these structures, or of the basement membrane, which may be relevant to the enterocyte damage? Might there be relative ischaemia at villus tips? What about immune complexes?

Author: The only differences in EL between the proliferative (Type 1) and destructive (Type 2) lesion are the increased size of population of EL in the former, and the relative increase in their diameters in the latter: additional TEM studies would not provide differential information. With regard to the mechanism of enterocyte damage, it is clear that EL do not appear to be relevant factors, since they evidently cause no obvious damage to the mucosa in the Type 1 lesion. Furthermore their sustained increase in the crypts of Type 2 lesions without apparently affecting the high rate of crypt cell division; crypt hypertrophy; migratory capacity; ability to cytodifferentiate into Paneth cells, argentaffin and goblet cells and their continued ability to synthesise and express MHC class II glycoproteins, is also indicative that EL are not agents of mucosal destruction, as I have consistently argued elsewhere [text ref. 73,79]. My own view is that ischemia may be a very relevant factor that leads to impaired surface enterocyte structure and function. Another second factor of importance may be the release of connective tissue and basement membrane-degrading enzymes from neutrophils and mast cells that constitute some of the major cell infiltrates within lamina

propria [text ref. 23,78]. The effect of these enzymes is probably to weaken the attachment of surface enterocytes to the basement membrane and thus impair their viability. These points have been explored, together with the role of antigen-antibody complexes, elsewhere [text ref. 70,71], but I have no firm data at this time.

A. Ferguson: Were there differences in lamina propria cell density when the coeliac relatives with abnormalities of EL were compared with the others?

Author: Unfortunately this was not done. Since these specimens were formalin-fixed/wax-embedded, the chances of doing good image-analysis on such material, especially within the lamina, would be very low.

A. Ferguson: In discussing these coeliac relatives and also the DH patients, the important variable, of dietary gluten intake, has not been considered. There either may be a variable tissue reaction, which is pathological, to standard dietary gluten in about one third of coeliac relatives and the majority of DH patients; or within a fairly uniform population of gluten sensitive individuals, tissue changes reflect fairly accurately the dietary gluten intake of the previous couple of weeks. I may say that we are in the process of analysing jejunal biopsy data from a large number of DH patients and have found not only minor abnormalities of histology, particularly of EL, but also a very high proportion with abnormal intestinal permeability even when jejunal biopsy morphology is essentially normal.

Author: This raises a very important point, but is an aspect of research in this field that is rarely considered. Nevertheless, it would be very difficult (a) to control and (b) to determine the actual daily dietary intake of gluten per person. Dose of gluten may be important, as revealed for the first time in our carefully-monitored, sequential challenges with FF3. Having said that, I am certain that on average, each person's daily intake of gluten is fairly constant and it may also be true for the whole population that person-to-person variations in daily gluten intake may not vary to any great extent. This has never been investigated, to my knowledge. Furthermore I doubt, for example, whether the intake of gluten varied much in the patient (Fig. 13) whose mucosa was observed to slowly flatten over the course of about 3 years. This raises another new question as to what factor(s) controlled the slow evolution of her mucosal lesion.

I think you are right about DH patients - all our DH patients had increased mucosal permeability to Cr-EDTA [text ref. 8] even after they had been on a gluten-free diet for a reasonable period (in some cases, several years). It is also my impression that the inflammatory response in DH mucosae is considerably more vigorous than in CS patients, even though their degree of mucosal architectural distortion is considerably less. This also suggests a multiplicitiy of operative pathogenetic mechanisms, ie, a basic T-cell mediated response that determines the degree of mucosal architectural disruption (? determined by host MHC class II) upon which other secondary inflammatory- or cytokine-driven responses may be enacted.

A. Ferguson: It is conceivable that dietary gluten induces a state of suppressed mucosal immunity. Thus, if normal people (or people incorrectly diagnosed to have celiac disease) were maintained on a gluten-free diet for a prolonged period, an altered capacity to subsequently respond to any antigen, including gluten, might be observed.

Author: We have not challenged any person falling into the category suggested. The proposal, however, is interesting in terms of orally-induced tolerance and the influence of prolonged dietary antigenic restriction on mechanisms of local immune suppression.

J.R. Poley: Not mentioned are possibilities of lectin-mediated damage of small bowel mucosa, which has been postulated by several groups as an important pathogenic mechanism, and this reviewer believes that such hypothesis should be included in the discussion.

Author: I note your use of the word 'postulated'! The theory that gluten might act as a "lectin" (in other words, attach itself to some kind of cell receptor) was proposed over ten years ago [130]. It was supported by the isolation of a glycopeptide shown to damage CS mucosa [125], that damage being caused by gluten attachment to an abnormal receptor expressed by surface enterocytes in such patients. Another group implied that crypt enterocytes (admittedly from rats - if this is at all relevant) bind gluten via oligomannosyl residues [126]. It is my guess that we all suspect there are receptors on cells that bind gluten, although I am not clear as to why such receptors have to be aberrant. But if this is so, why glorify that principle into a new theory of pathogenesis? Surely we just have to get on and determine the characteristics of the relevant receptor. Nor does it follow that attachment of gluten to the membrane should necessarily cause that cell to be killed, which is a corollary of the proposal. Finally, these notions have to be squared with all the immunopathologic features of gluten sensitivity described in this paper. The latter fit more easily into the scheme of a cell-mediated immune response rather than to some unproven ideas about an abnormal cell receptor.

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