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# Studies of intestinal lymphoid tissue

## X-observations on granular epithelial lymphocytes (gEL) in normal and diseased human jejunum

**M.N. Marsh*[,1], R.J. Leigh*[,1], D.E. Loft*[,1], G.V. Garner[2], and D.B. Gordon[2]**

[1] University Department of Medicine, Hope Hospital, University of Manchester School of Medicine, Eccles Old Road, Salford M6 8HD, UK
[2] John Dalton Institute of Technology, Oxford Road, Manchester, UK

**Summary.** A proportion of epithelial lymphocytes in various mammalian species is characterised by cells containing cytoplasmic granules. We have studied the total number of granular lymphocytes within surface and crypt epithelium of jejunal mucosae (per $10^4$ $\mu m^2$ muscularis mucosae) from six groups of subjects, comprising (i) young healthy volunteers (ii) family relatives of known coeliac patients, patients with gastrointestinal disorders associated with either (iii) normal or (iv) "flat" mucosae, and groups of (v) untreated and (vi) treated patients with coeliac disease. There was no difference in the absolute number of gEL between the three control groups with normal mucosal architecture, the proportion of granular to total EL per unit of tissue varying between 30–40%. In untreated coeliac mucosae, there was a significantly increased population of gEL, compared with the same control groups ($p < 0.001$): the ratio of granular to total EL approximated 65%, and did not differ from flat-control mucosae in which the proportion of gEL was 55%. On withdrawal of gluten, the absolute number of gEL fell significantly in comparison with the untreated coeliac group ($p < 0.05$). To further evaluate the effect of gluten challenge, granular lymphocytes were monitored during a five-day period in groups of treated coeliac patients orally challenged with increasing doses (500–3000 mg) of a peptic-tryptic digest of gluten. A significant rise in the absolute number of granular lymphocytes occurred at 12 h, but without any deterioration in mucosal architecture.

**Key words:** Jejunum – Granular epithelial lymphocyte – Coeliac disease – Morphometry

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*Offprint requests to:* M.N. Marsh

### Introduction

Despite considerable investigation over many years, the functional role of epithelial lymphocytes is still poorly understood. Nevertheless, in many studies it has been shown that some EL in a variety of species, including man, comprise a markedly heterogeneous population based on structural, cytochemical and immunohistological criteria as well as by in vivo and in vitro functional assays (Collan 1972; Ferguson 1977; Marsh 1985; Ernst et al. 1985).

One striking morphological characteristic of EL is their granular cytoplasm. These granules vary in size (0.1–1.0 μm diameter) and number (1–20) per cell, are bounded by a single membrane, are osmiophilic and exhibit metachromasia when stained with alcian blue or toluidine blue (Toner and Ferguson 1971; Rudzik and Bienenstock 1979; Marsh 1975). The origin and nature of granular epithelial lymphocytes (gEL) is still uncertain.

More recently, the demonstration of the cytotoxic potential of EL separated from guinea pig jejunum (Arnaud-Battandier et al. 1978) was followed by confirmatory data that gEL subserve local NK activity (Tagliabue et al. 1981, 1982), analogous to that exhibited by circulating large granular lymphocytes (Timonen et al. 1981). This view is strengthened by the observation that in both populations of cells, cytotoxicity is augmented by gamma-interferon (Tagliabue et al. 1981; Landazuri et al. 1981). In human small intestine approximately 90% epithelial lymphocytes express the CD3 phenotype as well as the surface differentiation antigen, CD8 (Selby et al. 1981a, b, 1983; Cerf-Bensussan et al. 1983). Despite the presence of granules, few EL express putative NK-cell sur-

face receptors (Leu-11, Leu-7) (Cerf-Bensussan et al. 1983; Greenwood et al. 1983).

Because of the difficulty in obtaining sufficient numbers of EL for in vitro studies, virtually little in known about gEL in the normal human intestine, what changes in their proportion might occur in diseased intestine and what factors, if any, influence such changes. In order to gain some insight into these questions, we determined the size of gEL populations in a wide variety of mucosal biopsies obtained from defined groups of patients, disease-controls and volunteers.

We were also interested in studying the dynamics of gEL within the intestinal epithelium. In order to permit such studies, we evaluated the effect of gluten challenge on gEL during a five-day period of observation. To this end groups of volunteers, and coeliac patients in remission, received varying doses orally of either Frazer's fraction III (FF3) (Frazer et al. 1959) or the control protein $\beta$-lactoglobulin ($\beta$-LG).

## Patients and methods

Patients studied: there were 60 subjects comprising the following groups:

(a) *Human volunteers (HV)*: 10 young and healthy paramedical volunteers (age range: 20–26).

(b) *Family member controls (FMC)*: these comprised 9 first-degree relatives of known coeliac patients, and who were asymptomatic with normal mucosal morphology (Rubin et al. 1960).

(c) *Normal disease controls (NDC)*: the biopsies in this group, which also fulfilled established criteria of normality (Rubin et al. 1960) were obtained from 9 subjects referred with intestinal symptoms, and whose ages ranged from 18 through 65.

(d) *Flat disease controls (FDC)*: this comprised a heterogeneous group of 9 subjects with flat mucosae considered not to be gluten-related, and in which the mitotic index of EL was less than 0.2% (Marsh and Haeney 1983). The patients, whose age range was 17–70, comprised severe Crohn's disease of jejunum (3); small intestinal lymphoma (3); $\alpha$-chain disease (1), untreated tropical sprue (1) and common variable immunodeficiency (1).

(e) *Untreated coeliac disease (UCD)*: These mucosae were obtaiend from 12 patients presenting with a combination of anaemia, malnutrition, diarrhoea or other deficiency together with a flat mucosa in which the mitotic index of EL > 0.2% (Marsh 1982). The age range was 18–64 years.

(f) *Treated coeliac disease ($R_xCD$)*: this comprised eleven well-treated patients (GFD for > 6 months) who, by accepted criteria, all responded to dietary treatment. At the time of biopsy they were all well and required no additional nutritional supplements: none complained of diarrhoea. Their age range was 22–72 years.

These six groups yielded a total of 60 mucosal specimens for measurement.

*Challenge protocol*

Peroral challenges were performed according to a protocol approved by Salford District Health Authority's Ethical Committee. Each challenge was performed on small groups of volunteers, or treated coeliac disease patients: each group of subjects received either 500, 1000, 1500 or 3000 mg of Frazer's peptic-tryptic digest of gluten (FF3) (Frazer et al. 1959), or 500 mg of the control milk protein beta-lactoglobulin ($\beta$-LG).

Challenge groups comprised approximately six individuals. For each separate challenge, a control biopsy was first undertaken. The particular dose of FF3, or $\beta$-LG, was then taken orally and further jejunal biopsies were obtained 12, 36, 60 and 84 h later. From these various series of antigen challenges, a total of 210 specimens was obtained, coded and analyzed.

*Histological technique*

Mucosal specimens were quickly retrieved from a Watson capsule located previously under fluoroscopic control to the first loop of jejunum, spread flat on card, fixed in cacodylate-buffered 2.5% ultrapure glutaraldehyde and embedded in epoxy resin (Araldite). 1 µm sections cut with a Reichert OMU-3 ultramicrotome were stained with aqueous toluidine blue. 5–6 sections were mounted per slide: a 10 µm gap was discarded between successive sections. Selected sections (one per slide) that were free of technical artefact and sectioned perpendicular to the mucosal surface were observed through a ×100 oil-immersion objective with an Olympus BHS-2 research microscope. Photographs were taken on Ilford Pan F 35 mm film and suitably enlarged.

*Enumeration of gEL*

With the staining technique as described above, cytoplasmic granules of gEL appear as dense blue inclusions. Slides were coded, and the number of gEL containing one or more granules was counted and expressed (i) in absolute terms (per mucosal unit overlying $10^4$ µm$^2$ muscularis mucosae) and (ii) as a percentage of total EL per specimen.

In the challenged patients, the absolute number of gEL was determined for surface epithelium only.

*Statistical analysis*

Because values for EL are not uniformly distributed, data were analysed by the Mann-Whitney U-test (two-tailed). Values of $p < 0.05$ were taken as statistically significant. The analyses were performed with the SPSS package by University of Salford Computing Services Section.

## Results

### *Cytologic aspects of gEL*

Granular EL are easily recognizable in toluidine blue-stained 1 µm plastic sections (Fig. 1). They are heterogeneous in size, often with expanded cytoplasm in which densely-staining granules are usually clustered around one nuclear pole. The nuclei of these lymphocytes frequently demonstrate increased euchromatin, and contain prominent nucleoli. Such cells are easily distinguished from mast cells and eosinophils which also occasionally enter

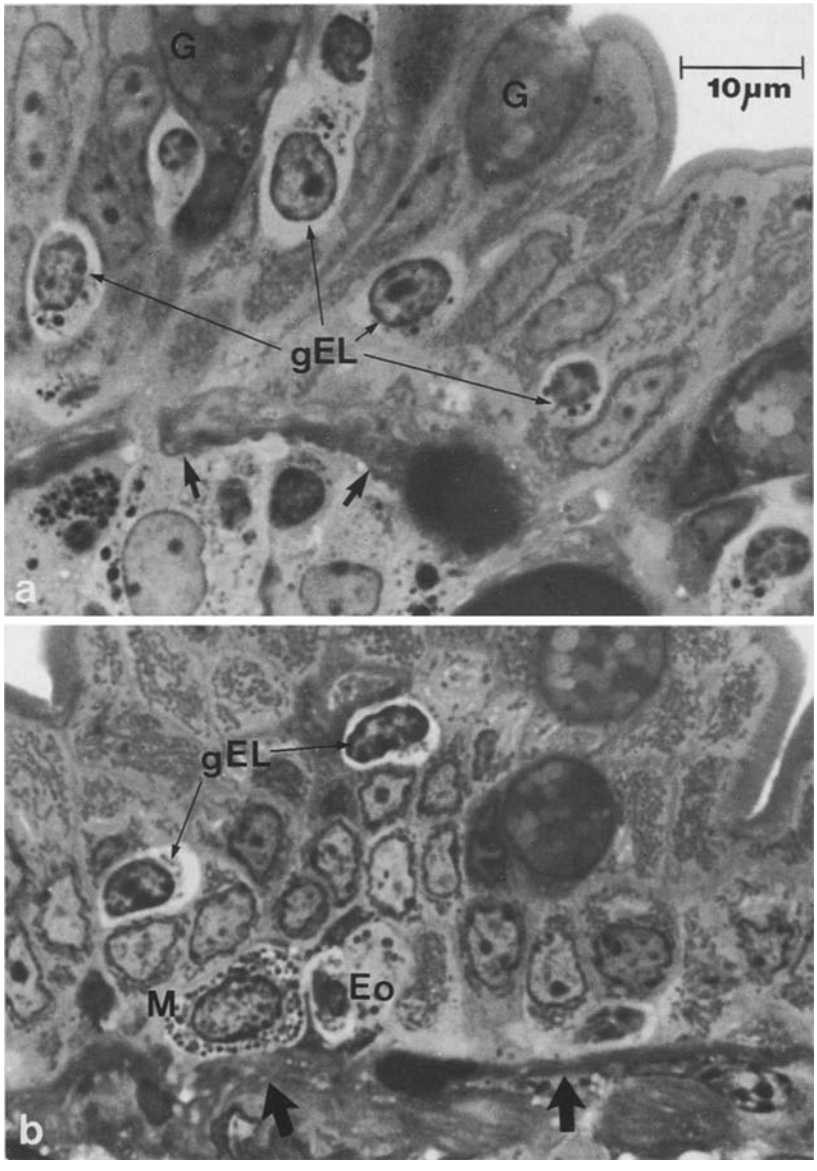


**Fig. 1. a** This shows a group of epithelial lymphocytes containing cytoplasmic granules (*gEL*). Some of these cells are large with expanded cytoplasm and nuclei exhibiting euchromatin and multiple nucleoli. Goblet cells, G. Basement membrane denoted by heavy arrows. **b** This 1 μm toluidine blue-stained section illustrates comparison between granulated epithelial lymphocytes (*gEL*) and adjacent mast cell (*M*) and eosinophil (*Eo*). Heavy arrows locate basement membrane. (Magnifications ×1350)

the epithelium and contain large and more numerous, cytoplasmic granules (Fig. 1). gEL are also present within crypt epithelium and display identical cytologic features.

*Quantitation of gEL within patient groups*

The percentage distribution of gEL was similar between surface and crypt epithelium in each of the six groups of mucosae studied (Table 1). Thus, the absolute number of total, and of gEL, per unit of mucosa overlying $10^4$ $\mu m^2$ muscularis mucosae, were employed for the purposes of comparison.

There was no significant difference in either the total number of epithelial lymphocytes, or in the gEL compartment, between the volunteers, family relatives, or the two groups of disease-controls with either a villous, or a flat, mucosa (Fig 2).

While there was no significant difference between these four control groups or the eleven untreated coeliac patients in terms of total EL, there was a highly significant increase in gEL in the latter patients, compared with volunteers ($p=0.001$), coeliac relatives ($p=0.001$) and normal disease-controls ($p<0.001$). There was no difference, however, in gEL content between untreated coeliac mucosae and the 'flat' disease-control group, reflecting the larger proportion of gEL in the latter mucosae (Table 1).

During treatment with a gluten-free diet, there

**Table 1.** The percentage gEL in surface and crypt epithelium is similar in each of the six groups examined. Note the increased proportion of gEL in flat disease-controls[d] as well as in untreated coeliac patients[e], and the reduction following treatment with a gluten-free diet[f]

| Group: | HV[a] | FMC[b] | NDC[c] | FDC[d] | UCD[e] | $R_xCD$[f] |
|---|---|---|---|---|---|---|
| gEL (%) | | | | | | |
| surface epithelium | 34 | 41 | 32 | 56 | 64 | 41 |
| crypt epithelium | 35 | 38 | 28 | 58 | 65 | 42 |

[[a] vs [b] vs [c] NS; [a, b, c,] vs [d] $0.05<p>0.02$; [a, b, c,] vs [e] $p<0.001$; [d] vs [e] NS; [e] vs [f] $p<0.02$]
*Abbreviations:* HV healthy volunteers: FMC family members of coeliacs: NDC disease-controls with normal mucosae: FDC disease-controls with flat mucosae. UCD, $R_x$ CD untreated and treated coeliac disease

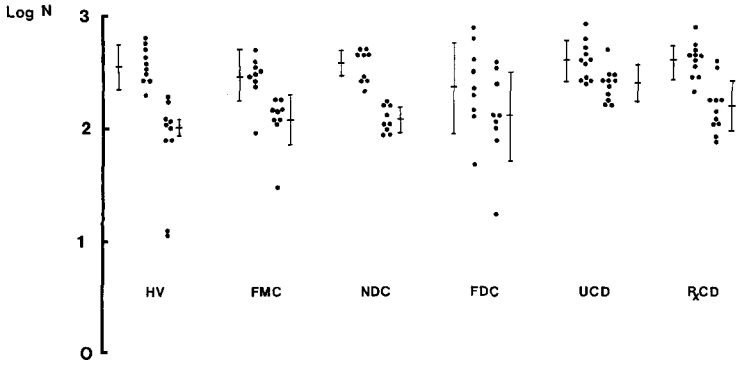


**Fig. 2.** This diagram illustrates total, and granular EL (mean SD) for each of the six groups studied. For each group, left hand data refer to total EL, and right hand data to gEL. For convenience of display, data are expressed logarithmically. Total EL for all groups differed insignificantly, whereas gEL in untreated coeliac patients were significantly increased compared with HV, FMC and NDC but not FDC. During treatment with a gluten free diet, gEL fell significantly in comparison with the untreated group. *Abbreviations:* HV healthy volunteers: FMC family members of coeliacs: NDC disease-controls with normal mucosae: FDC disease-controls with flat mucosae. UCD, $R_xCD$ untreated and treated coeliac disease

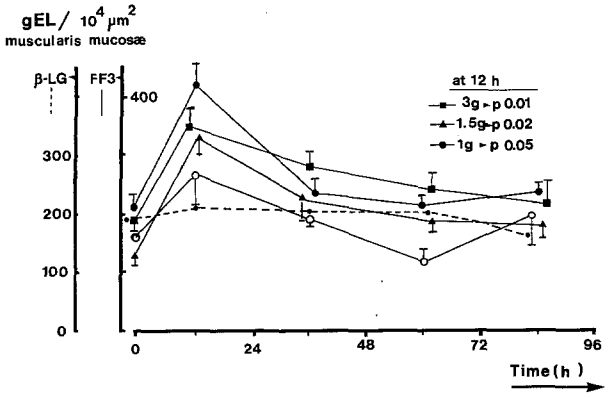


**Fig. 3.** This diagram illustrates the effect on treated coeliacs of oral challenge with varying doses of FF3 [3 g (■); 1.5 g (▲); 1 g (●); 0.5 g (○)] or 500 mg β-LG. A significant absolute increase in villous gEL occurred 12 h post-challenge. Coeliac patients did not respond to β-LG (*dotted line*). Controls did not respond to FF3 or β-LG (data not shown)

was a significant fall in gEL, in comparison with the untreated coeliac group ($0.05<p>0.02$), although there was no difference in the total numbers of EL between these groups (Fig. 2).

*Effect of antigen challenge on gEL populations*

Oral challenges were performed either with graded doses (500–3000 mg) of FF3, or with a standard (500 mg) dose of β-LG on groups of (i) treated coeliac patients and (ii) young healthy volunteers.

An absolute rise in villous gEL over basal (pre-challenge) levels occurred at 12 h after gluten ingestion (Fig. 3) which was significant statistically for doses of 1 gram FF3 and above. This response was no longer evident beyound 36 hours. There was no response in surface gEL populations to oral challenge with 500 mg β-LG, and control subjects did not respond to either immunogen.

## Discussion

These studies indicate that in toluidine blue-stained 1 µm epon sections of human small intestinal biopsies, the mean percentage gEL from three control groups consisting of young healthy volunteers, miscellaneous disease-controls with normal mucosal architecture and a group of first degree relatives

of known coeliac disease patients, varies between 30–40%. In less detailed studies, other investigators appear to have underestimated these cells, for example, 21% by Greenwood et al. (1983) employing in vitro suspensions, and 20–30% by Cerf-Bensussan et al. (1983, 1985) using immunohistologic techniques.

In addition to studying a wide range of villus-bearing control mucosae, we also extended our observations to include two other groups of patients with 'flat' mucosae, comprising gluten-induced (untreated coeliac) and non-gluten-associated patients. In these two latter groups, the percentage gEL was significantly (55–65%) higher than in the 3 control groups but there was no difference between the untreated coeliac patients and those with other conditions such as Crohn's disease of jejunum, $\alpha$-chain disease, lymphoma or immunodeficiency. Thus an increased gEL compartment cannot be used as a histological marker for untreated coeliac disease: neither is this phenomenon exclusive to untreated coeliac disease. There was, however, a significant fall in gEL in the treated coeliac group, which is presumably consistent with the ongoing regeneration of the mucosa in these patients.

There has been considerable interest in the nature and function of gEL. They are not mast cells, since the granule glycoproteins are different (Bland et al. 1986), but are related (Tagliabue et al. 1981) to the circulating large granular lymphocytes (LGL) that subserve NK activity (Timonen et al. 1981). NK cells comprise a markedly heterogeneous population of cells effecting lysis of unrelated targets i.e. tumours, parasites or virus-infected cells in a non-antigen-specific, non-MHC-restricted, manner (Mowat 1987). In most species (Tagliabue et al. 1982; Ritchie et al. 1983; Mowat et al. 1983) including man, intestinal gEL differ from circulating LGL in (i) carrying the T cell marker (CD8) associated with presumptive cytolytic-suppressor lymphocytes (ii) lacking typical NK markers (Selby et al. 1983; Greenwood et al. 1983; Mowat et al. 1983) as confirmed with in situ immunohistological techniques and (iii) requiring "activation" before their cytotoxic potential is realised (Tagliabue et al. 1982; Parrott et al. 1982).

The question arises what role gEL play in protection of the intestinal mucosa. Although few gEL are detectable within the lamina propria (Tagliabue et al. 1981; Cerf-Bensussan et al. 1983; Parrott et al. 1982; Borland et al. 1983; Gibson et al. 1984), the majority of gEL appear to be specifically concentrated in the epithelial frontier separating host from environment. Despite this differential localization, there are no in vivo data that verify the presumptive lytic functions of gEL demonstrated in vitro against chosen target cells.

It was shown recently that gEL accumulate within villous and crypt epithelium of rodents mounting acute graft-versus-host reactions (Guy-Grand and Vassalli 1986). In time-course studies involving autoradiographic analysis of labelled cells within the mucosa, lymphocytes appeared to migrate directly across the lamina and into the epithelium from the blood stream as non-granulated precursors. Once inside the epithelium, the cells rapidly developed granules. These data help to explain why so few gEL are present within the lamina propria.

These data are similar to our own observations concerning the effects of gluten challenge on gEL populations in treated coeliac patients. In addition to the absolute rise in gEL demonstrated approximately 12 h post-challenge, it was noted that the cells at this time contained far more granules than those in pre-challenge mucosae, or the disease-control biopsies. Indeed, it was often possible to identify the post-challenge biopsies from this feature alone, although the specimens were coded before their evaluation was undertaken.

There are few data to explain what in vivo factors induce gEL hyperplasia within the intestinal epithelium, although inflammatory factors may play a role. However, despite increases of similar magnitude in coeliac mucosae and other flat-lesions, including Crohn's disease in which epithelial damage is not a factor in pathogenesis, it seems certain that gEL are unlikely to cause damage to coeliac enterocytes, contrary to other claims (Flores et al. 1982). During a GVH reaction, NK cell activity is considerably boosted, and within the intestinal epithelium such activity resides among the gEL component (Borland et al. 1983; Guy-Grand and Vassalli 1986). However, the GVH lesion, which is essentially driven by MHC Class II incompatibilities, is dependent absolutely on CD4 (helper-inducer) cells and lymphokine production, and is therefore a pure T cell-mediated phenomenon (Guy-Grand and Vassalli 1986; Mowat et al. 1986; Mowat 1987; Ferguson 1987). In this model, intestinal damage still occurs even when NK cell activity is abrogated by depleting animals of gEL. Again, such data indicate that a rise in gEL does not necessarily imply obligatory cell or tissue destruction.

It is more difficult to explain the rise in gEL during challenge with doses of FF3 previously shown not to damage mucosal structure (Leight et al. 1985). However, normality of structure does not exclude the possibility of mediator release that

could influence the rapid infiltrate of gEL occurring during the 24 h immediately post-challenge. Clearly this phenomenon, which requires further elucidation, may be explained as knowledge of the functional role of intestinal gEL is increased.

## References


Arnaud-Battandier FA, Bundy BM, O'Neill M, Bienenstock J, Nelson DL (1978) Cytotoxic activities of gut mucosal lymphoid cells in guinea pigs. J Immunol 121:1059–1065

Bland CE, Rosenthal KL, Pluznik DH, Dennert G, Hengartner H, Bienenstock J, Metcalfe DD (1986) Glycosaminoglycan profiles in cloned granulated lymphocytes with natural killer function and in cultured mast cells: their potential use as biochemical markers. J Immunol 132:1937–42

Borland A, Mowat A, Parrott DMV (1983) Augmentation of intestinal and peripheral natural killer cell activity during the graft-versus-host reaction in mice. Transplantation 36:513–9

Cerf-Bensussan N, Guy-Grand D, Griscelli C (1985) Intraepithelial lymphocytes of human gut: isolation, characterisation and study of natural killer activity. Gut 26:81–88

Cerf-Bensussan N, Schneeberger EE, Bhan AK (1983) Immunohistologic and immunoelectron microscopic characterisation of the mucosal lymphocytes of human small intestine by the use of monoclonal antibodies. J Immunol 130:2615–2622

Collan Y (1972) Characteristics of non-epithelial cells in the epithelium of normal rat ileum. Scand J Gastroenterology (Suppl) 18:7

Ernst PB, Befus AD, Bienenstock J (1985) Leukocytes in the intestinal epithelium: an unusual immunological compartment. Immunol Today 6:50–55

Ferguson A (1977) Intraepithelial lymphocytes of the small intestine. Gut 18:921–937

Ferguson A (1987) Models of immunologically-driven small intestinal damage. In: Marsh MN (ed) The Immunopathology of the Small Intestine. Wiley, Chichester, pp 225–52

Flores AF, Winter HS, Bhan AK (1982) In vitro model to assess immunoregulatory T lymphocyte populations in gluten-sensitive enteropathy. Gastroenterology 82:A1058

Frazer AC, Fletcher RF, Ross CAC, Shaw B, Sammons HC (1959) Gluten-induced enteropathy. The effect of partially-digested gluten. Lancet 2:252–55

Gibson PR, Down EL, Selby WS, Strickland RG, Jewell DP (1984) Natural killer cells and spontaneous cell-mediated cytotoxicity in the human intestine. Clin Exp Immunol 56:438–44

Greenwood JH, Austin LL, Dobbins WO (1983) In vitro characterisation of human intestinal intraepithelial lymphocytes. Gastroenterology 85:1023–1035

Guy-Grand D, Vassalli P (1986) Gut injury in mouse graft-versus-host reaction: study of its occurrence and mechanisms. J Clin Invest 77:1584–1595

Landazuri MO, Lopez-Botet M, Timonen T, Ortaldo JR, Herberman RB (1981) Human large granular lymphocytes: spontaneous and interferon-boosted NK activity against adherent and non-adherent cell lines. J Immunol 127:1380–1383

Leigh RJ, Marsh MN, Crowe P, Kelly C, Garner V, Gordon D (1985) Studies of intestinal lymphoid tissue. IX-Dose-dependent, gluten-induced lymphoid infiltration of coeliac jejunal epithelium. Scand J Gastroenterol 20:715–9

Marsh MN (1975) Studies of intestinal lymphoid tissue. I – Electron microscopic evidence of "blast-transformation" in epithelial lymphocytes of mouse small intestinal mucosa. Gut 16:665–674

Marsh MN (1982) Studies of intestinal lymphoid tissue. IV – The predictive value of raised mitotic indices among jejunal epithelial lymphocytes in the diagnosis of gluten-sensitive enteropathy. J Clin Pathol 35:517–525

Marsh MN (1985) Functional and structural aspects of the epithelial lymphocyte, with implications for coeliac disease and tropical sprue. Scand J Gastroenterology (Suppl) 114:55–75

Marsh MN, Haeney MR (1983) Studies of intestinal lymphoid tissue. VI – Proliferative response of small intestinal epithelial lymphocytes distinguishes gluten- from non-gluten-induced enteropathy. J Clin Pathol 36:149–160

Mowat A McI (1987) The cellular basis of gastrointestinal immunity. In: Marsh MN (ed) The Immunopathology of the Small Intestine. Wiley, Chichester, pp 41–72

Mowat A McI, Borland A, Parrott DMV (1986) Hypersensitivity reactions in the small intestine. VII – Induction of the intestinal phase of murine graft-versus-host reaction by Lyt $2^-$ T cells activated by I-A alloantigens. Transplantation 41:192–8

Mowat A, Tait RC, Mackenzie S, Davies MDJ, Parrott DMV (1983) Analysis of natural killer effector and suppressor activity by intraepithelial lymphocytes from mouse small intestine. Clin Exp Immunol 52:191–8

Parrott DMV, Tait C, MacKenzie S, Mowat A (1982) Analysis of the effector functions of different populations of mucosal lymphocytes. Ann N Y Acad Sci 409:307–20

Ritchie AW, James K, Micklem HS (1983) The distribution and possible significance of cells identified in human lymphoid tissues by the monoclonal antibody HNK-1. Clin Exp Immunol 51:439–47

Rubin CE, Brandborg L, Phelps P (1960) Studies of celiac disease. I. The apparent, identical and specific nature of the duodenal and proximal jejunal lesion in coeliac disease and idiopathic steatorrhoea. Gastroenterology 38:28–49

Rudzik O, Bienenstock J (1979) Isolation and characterstics of gut mucosal lymphocytes. Lab Invest 30:260–266

Selby WS, Janossy G, Bofill M, Jewell DP (1983) Lymphocyte subpopulations in the human small intestine. The findings in normal mucosa and in the mucosa of patients with adult coeliac disease. Clin Exp Immunol 52:219–228

Selby WS, Janossy G, Goldstein G, Jewell DP (1981a) T lymphocyte subsets in human intestinal mucosa: the distribution and relationship to MHC-determined antigens. Clin Exp Immunol 44:453–8

Selby WS, Janossy G, Jewell DP (1981b) Immunohistological characterisation of intraepithelial lymphocytes of the human gastrointestinal tract. Gut 22:169–176

Tagliabue A, Befus AD, Clark DA, Bienentock J (1982) Characteristics of natural killer cells in the murine intestinal epithelium and lamina propria. J Exp Med 155:1785–96

Tagliabue A, Luini W, Soldateschi D, Boraschi D (1981) Natural killer activity of gut mucosal lymphoid cells in mice. Eur J Immunol 11:919–22

Timonen T, Ortaldo JR, Heberman RB (1981) Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. J Exp Med 153:569–582

Toner PG, Ferguson A (1971) Intraepithelial cells in the human intestinal mucosa. J Ultrastr Res 34:329–344


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