# Changes in intraepithelial lymphocyte subpopulations in coeliac disease and enteropathy associated T cell lymphoma (malignant histiocytosis of the intestine)

JO SPENCER, T T MacDONALD*, T C DISS, J A WALKER-SMITH*, P J CICLITIRA†, AND P G ISAACSON

From the Dept of Histopathology, University College and Middlesex School of Medicine, London, *Dept of Paediatric Gastroenterology, St Bartholomew's Hospital, London, and †The Rayne Institute, St Thomas's Hospital, London

SUMMARY    Studies of the morphologic and phenotypic diversity of intraepithelial T cells in human small intestine have shown them to be heterogeneous, yet distinct from most extra intestinal T cells. In this study sequential immunoenzymatic staining was used to define new intraepithelial lymphocyte subpopulations in man. In normal human jejunum approximately 6% of the intraepithelial T cells expressing CD3 (an antigen associated with the T cell receptor) do not express the T cell subset antigens CD4 or CD8. Approximately 20% of CD7+ cells (T cells and null cells) do not express CD4 or CD8 and 14% of the CD7+ cells do not express CD3 and are therefore not T cells. The CD7+, CD3+/−, CD4−, CD8− population is concentrated in the tips of the villi. In coeliac disease, the ratios of the subsets change significantly. The percentage of CD3+, 4−, 8− cells increases to 28%, the proportion of CD7+, 4−, 8− cells remains unchanged and the CD7+, CD3− (non-T cell) population is reduced to 1·4% of the CD7+ cells. In contrast, in patients with villous atrophy of uncertain aetiology, all CD4−, CD8− lymphocyte subsets are decreased compared with normal biopsies. Finally, in enteropathy associated T cell lymphoma (malignant histiocytosis of the intestine) in which the 'uninvolved mucosa' is histologically similar to untreated coeliac disease, the changes in the intraepithelial T cell sub-sets are indistinguishable from those in coeliac disease, suggesting that the lymphoma is a complication of coeliac disease.

The intraepithelial lymphoid population, a compartment of gut associated lymphoid tissue, is distinct phenotypically and functionally from lymphoid cells in other organs. Studies of human intraepithelial lymphocytes in tissue sections and in preparations of isolated cells have shown them to be predominantly CD3 positive T cells, most of which also express CD8. A large proportion of intraepithelial T cells do not express CD5, which is a pan T cell marker in other tissues.[1,2] Approximately 20-35% of human intraepithelial lymphocytes contain cytoplasmic

lysosomal granules.[3,4] The distinctive nature of these lymphocytes is further substantiated by the recent development of a monoclonal antibody (HML-1) which recognises the entire heterogeneous intraepithelial lymphocyte population, including the lymphocytes with cytoplasmic granules, but which recognises very few cells outside the mucosae.[2]

A T cell lymphoma which arises in the small intestine has been described in detail using immunocytochemistry and DNA analysis.[5,6] The tumour cells, which are often present within the epithelium and which contain lysosomal granules, express the antigens CD7 and sometimes CD3, but not CD4 or CD8. They also express the antigen recognised by HML-1, suggesting that they are derived from intraepithelial lymphoid cells.[7] Patients with this

lymphoma also have small intestinal pathology, indistinguishable in many cases from that in coeliac disease.[8] It is not known whether the enteropathy is true coeliac disease, or whether it is secondary to the lymphoma, resulting from the action of the malignant T cells themselves. The tumour, previously thought to be a histiocytic malignancy and therefore termed malignant histiocytosis of the intestine, is now more accurately also referred to as enteropathy associated T cell lymphoma.[9]

Evidence to date suggests that the tumour cells in enteropathy associated T cell lymphoma are derived from an intraepithelial lymphoid population.[10] In a recent study it has been shown that intraepithelial lymphocytes in coeliac disease include a population which is CD4−, CD8−.[11] We were interested therefore to determine whether a similar population was also present in 'uninvolved' mucosa from patients with enteropathy associated T cell lymphoma, and also to determine the phenotypes of the CD7+ cells in 'uninvolved' mucosa in patients with enteropathy associated T cell lymphoma and in coeliac disease. We also aimed to determine whether the CD7+, CD3+/−, CD4−, CD8− cells had a counterpart in normal mucosa.

We have therefore used a very sensitive sequential staining technique to study intraepithelial lymphocytes in well orientated villus/crypt units in normal jejunum, in coeliac disease, 'uninvolved' mucosa from enteropathy associated T cell lymphoma and mucosa from a group of adults with villous atrophy of uncertain aetiology.

## Methods

### TISSUE

Thirty jejunal biopsies taken using the Crosby capsule were cut into two. Half was fixed in formalin for routine histology in paraffin section. The other half was orientated mucosa side up on the side of a freezing vial and immediately snap frozen in liquid nitrogen. Frozen biopsies were stored at −70°C. Eleven well orientated frozen normal biopsies, each including longitudinal sections through villus crypt units, were selected for study. Normality was defined histologically in paraffin sections as a villus:crypt ratio of 3:1 or more, no abnormality of the brush border at the villus tips, and an intraepithelial lymphocyte count within normal limits (5–20 intraepithelial lymphocytes/100 epithelial cells). Of the 11 normal biopsies studied, seven were from patients with diarrhoea of unknown cause, one patient had a jejunal diverticulum, two patients had general physical weakness and inability to gain weight, and one patient was anaemic.

Biopsies taken by Crosby capsule from seven

adults (patients 1–7) showing histological changes characteristic of those in coeliac disease, that is increased number of intraepithelial lymphocytes, plasmacytosis of the lamina propria, flattened mucosa and crypt hyperplasia were studied. They were either clinically refractory to a gluten free diet or their biopsies showed only partial improvement on a gluten free diet. We termed this group villous atrophy of unknown aetiology. Details of each of these patients is shown in Table 1. Also studied were biopsies showing total villous atrophy from three children (patients 15–17) and four adults (patients 11–14) with coeliac disease on normal diet who subsequently responded to a gluten free diet. Biopsies from five adults with established coeliac disease on a gluten free diet were also studied (patients 18–22). Normal villous architecture had been restored in the adults on gluten free diets,

Table 1   *Details of age at which biopsy studied was taken, history of coeliac disease at time biopsy studied was taken and subsequent response to gluten free diet of patients studied*

| Patient | Age (yr) | Previous diagnosis of CD at biopsy | Diet at biopsy | Subsequent response to GFD |
|---|---|---|---|---|
| 1 | 50 | No | Normal | Partial biopsy response, but also taking steroids for asthma/oral ulceration |
| 2 | 43 | No | Normal | Poor biopsy response, but patient well on GFD |
| 3 | 73 | No | Normal | No follow up notes |
| 4 | 26 | No | Normal | No |
| 5 | 52 | No | Normal | No |
| 6 | 58 | No | Normal | Failed to adhere to GFD |
| 7 | 42 | No | Normal | No |
| 8 | 70 | Yes from age 56 | GFD | Yes until the onset of lymphoma |
| 9 | 41 | No | Normal | No |
| 10 | 56 | No | Normal | No |
| 11 | 36 | No | Normal | Biopsy and clinical response |
| 12 | 43 | No | Normal | Biopsy and clinical response |
| 13 | 28 | No | Normal | Biopsy and clinical response |
| 14 | 38 | No | Normal | Biopsy and clinical response |
| 15 | 18 mo | No | Normal | Biopsy and clinical response |
| 16 | 22 mo | No | Normal | Biopsy and clinical response |
| 17 | 13 mo | No | Normal | Biopsy and clinical response |
| 18 | 41 | Yes from age 35 | GFD | Yes |
| 19 | 49 | Yes from age 38 | GFD | Yes |
| 20 | 46 | Yes from age 33 | GFD | Yes |
| 21 | 72 | Yes from age 70 | GFD | Yes |
| 22 | 66 | Yes from age 44 | GFD | Yes |

Patients 1–7=Villous atrophy of uncertain aetiology, 8–10= enteropathy associated T cell lymphoma, 11–22=coeliac disease. GFD=gluten free diet. Intraepithelial lymphocytes/100 epithelial cells was above normal in all of these patients.

Table 2 *Antibodies used in this study*

| Antibody | Specificity | Reference |
|---|---|---|
| UCHT1 | T cells (CD3) | 12 |
| Leu3a | Helper/Inducer T cells (CD4) | 13 |
| UCHT2 | T cell subset (CD5) | 14 |
| 3A1 | T cells and null cells (CD7) | 15 |
| UCHT4 | Suppressor/cytotoxic T cells (CD8) | 16 |

although the number of intraepithelial lymphocytes/ 100 epithelial cells in each case was higher than normal. The dietary status' of the patients at the time of biopsy, their age, and their subsequent response to a gluten free diet is shown in Table 1.

Apparently uninvolved mucosa from three cases of enteropathy associated T cell lymphoma (patients 8–10) was snap frozen in liquid nitrogen and stored at −70°C before study. The mucosa was flat in all three cases. The details of these cases have been reported previously[5] and are shown in Table 1.

IMMUNOHISTOCHEMISTRY
The source and specificity of the antibodies used are shown in Table 2.

Sequential staining of all material studied was achieved using a two stage technique as shown in Figure 1.

STAGE ONE: INDIRECT IMMUNOPEROXIDASE
Cryostat sections were cut at 8 μm, air dried and fixed in acetone for 30 minutes. Staining was carried out using the indirect immunoperoxidase technique, with rabbit antimouse immunoglobulin conjugated to peroxidase as the secondary antibody.[17] Reactivity was visualised using the diamino benzidene reagent. This stage of the technique was used to identify a population of cells recognised by the first 'primary' antibody (or cocktail of mouse monoclonal antibodies) designated antibody A (Fig. 1, and Table 3).

STAGE TWO: IMMUNOALKALINE PHOSPHATASE
A second mouse 'primary' antibody designated antibody B was then applied which was followed by three repeat applications of goat antimouse immunoglobulin and mouse alkaline phosphatase antialkaline phosphatase complexes (APAAP) as shown in Figure 1. Akaline phosphatase activity was visualised using a fast blue reagent.[18] Cells stained with the fast blue reaction product only were assumed to express antigen recognised by the antibody B but not antibody A. The combinations of antibodies used as antibody A and antibody B and their meaning are summarised in Table 3.

In these preparations, despite the lack of counterstain, it was not difficult to determine intraepithelial location of cells (Fig. 2). The sequential staining was clear and highly reproducible. Any staining in which the colour intensities of the peroxidase and alkaline phosphatase reaction products were not of comparable intensity was not used.

QUANTITATION OF INTRAEPITHELIAL LYMPHOCYTE SUBPOPULATIONS
The relative numbers of intraepithelial lymphocytes with different phenotypes were determined from the
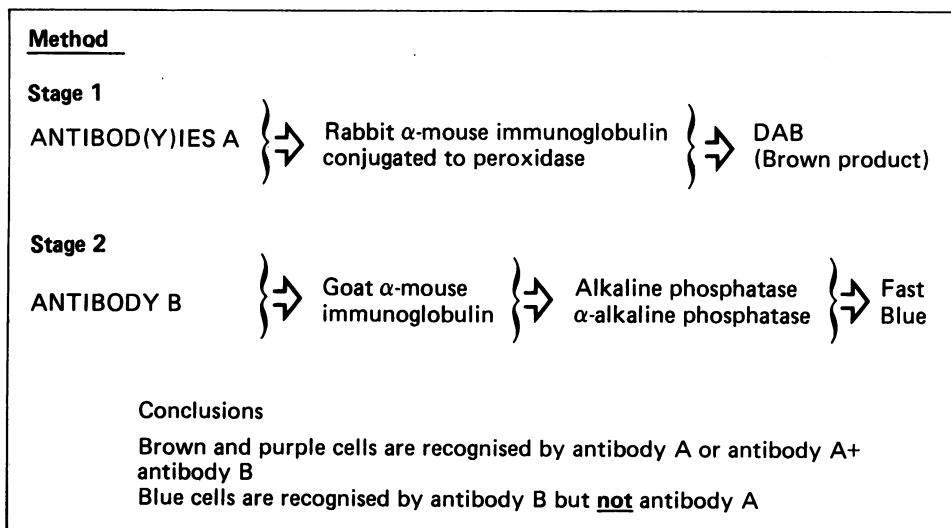


**Method**

**Stage 1**

ANTIBOD(Y)IES A ⟶ Rabbit α-mouse immunoglobulin conjugated to peroxidase ⟶ DAB (Brown product)

**Stage 2**

ANTIBODY B ⟶ Goat α-mouse immunoglobulin ⟶ Alkaline phosphatase α-alkaline phosphatase ⟶ Fast Blue

Conclusions

Brown and purple cells are recognised by antibody A or antibody A+ antibody B
Blue cells are recognised by antibody B but **not** antibody A

Fig. 1 *Summary of sequential staining method.*

Table 3   *Combinations of antibodies used in stage A and stage B of this study*

| Stage 1 Antibody(y)ies A | Stage 2 Antibody B | Phenotype of blue cells |
|---|---|---|
| 1 Anti-CD4+anti-CD8 | Anti-CD3 | CD3+, CD4−, CD8− |
| 2 Anti-CD4+anti-CD8 | Anti-CD7 | CD7+, CD4−, CD8− |
| 3 Anti-CD3 | Anti-CD7 | CD3−, CD7+ |
| 4 Anti-CD7 | Anti-CD3 | CD3+, CD7− |
| 5 Anti-CD4+anti-CD8 | Anti-CD5 | CD5+, CD4−, CD8− |

sequentially stained sections. Counts were made using the ×400 total magnification, in triplicate, with at least 200 intraepithelial cells counted each time. At least two separate preparations were counted for each case. First of all a ratio of brown and purple cells which were recognised by antibody A or antibody A+antibody B to blue cells recognised by antibody B only was determined and from this figure, the percentage of blue cells (stained by antibody B only) of the total stained cells was calculated.

To examine the distribution of a particular lymphocyte phenotype throughout the length of a villus, villi cut in longitudinal section were divided into three using an eyepiece graticule as described by Marsh.[19] The percentage of cells expressing the antigen recognised by antibody B but not antibody A was determined as described above for each villus third.

STATISTICAL ANALYSIS

Statistical comparisons were carried out using a 'Microstat' statistical program on an Amstrad PC 1512 microcomputer. Populations were tested to see whether they conformed to a normal distribution and if they did comparisons were made using analysis of variance. If they were not normally distributed, they were compared using the non-parametric Kolmogorov-Smirnoff 2 group test.

Table 4   *Percentages of cells of various phenotypes in 11 well orientated normal jejunal biopsies*

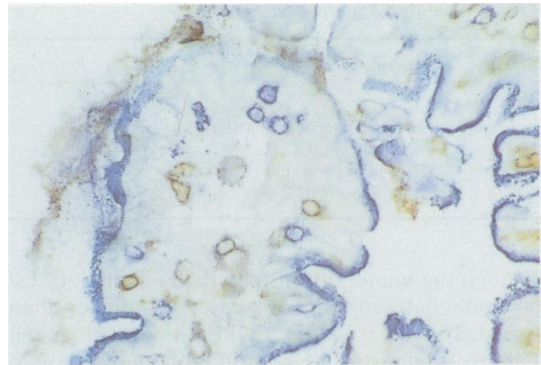|  | % CD3+, 4−, 8− of total CD3+ | % CD7+, 4−, 8− of total CD7+ | % CD7+, CD3− of total CD7+ |
|---|---|---|---|
| 1 | 8 | 29 | 22 |
| 2 | 3 | 31 | 25 |
| 3 | 10 | 14 | 2 |
| 4 | 3 | 9 | 11 |
| 5 | 8 | 12 | 11 |
| 6 | 5 | 18 | 18 |
| 7 | 5 | 18 | 15 |
| 8 | 9 | 23 | 10 |
| 9 | 2 | 19 | 10 |
| 10 | 10 | 34 | 18 |
| 11 | 2 | 8 | 7 |
| Mean | 6·6 | 19·6 | 13·5 |



Fig. 2   *Double immunoenzymatic stain of a normal jejunal biopsy. There is no counterstain. Cells which are CD4+ or CD8+ are stained using immunoperoxidase (brown reaction product) using a cocktail of anti-CD4 and anti-CD8 antibodies. Cells which are CD7+ are stained by the immunoalkaline phosphatase technique (blue reaction product) using anti-CD7. Cells which are stained blue only are CD7+, CD4−, CD8−. They are concentrated in the villus tips. Brush border enzymes give background positivity on the epithelial cells.*

Results

NORMAL JEJUNAL BIOPSIES

Cells with the phenotype CD3+, CD4−, CD8− were present in all 11 normal jejunal biopsies studied (Table 4). They constituted a mean value (SE) of 5·9% (0·9) of the total CD3+ intraepithelial T cells.

Cells with the phenotype CD7+, CD4−, CD8− were also present in the epithelium in all normal jejunal biopsies. They represented on average 19·5% (2·5) of the total intraepithelial CD7+ population and appeared to be concentrated in the tips of the villi (Fig. 2).

The number of CD7+ cells exceeded the number of CD3+ cells in each biopsy studied: CD7+, CD3− cells were 13·5% (2·0) of CD7+ cells. These cells were characteristically located in the villus tips. To confirm this observation, villi were divided into thirds and the percentage of CD7+, CD3− in the total CD7+ population was determined for each third. These results are summarised in Table 5.

No cells with the phenotype CD5+, CD4−, CD8− were observed in any biopsy studied.

The results of staining coeliac disease, uninvolved mucosa from enteropathy associated T cell lymphoma and biopsies with villous atrophy of uncertain aetiology with the antibodies in the sequence described above are shown in Figure 3a–c.

There was no difference between newly diagnosed coeliac disease in childhood, newly diagnosed coeliac disease in adults and established adult coeliac

Table 5  *Distribution of CD7+, CD3− cells through normal villi in longitudinal section*

| | IEL's/ villus (n) | CD7+, CD3− IEL's/villus (n) | % CD7+, CD3− of total CD7+ cells in each third of epithelial height | | |
|---|---|---|---|---|---|
| | | | Proximal 1/3 | Middle 1/3 | Distal 1/3 |
| 1 | 56 | 9 | 8·1 | 16·7 | 57·2 |
| 2 | 55 | 17 | 16·6 | 36·8 | 50·0 |
| 3 | 68 | 5 | 0 | 0 | 25·0 |
| 4 | 54 | 8 | 5·0 | 0 | 50·0 |
| 5 | 29 | 4 | 8·3 | 8·3 | 40·0 |
| 6 | 63 | 9 | 4·0 | 10·0 | 27·0 |
| 7 | 54 | 13 | 3·5 | 13·0 | 46·0 |
| 8 | 85 | 10 | 6·5 | 12·0 | 20·0 |
| 9 | 47 | 5 | 0 | 8·3 | 44·4 |
| 10 | 15 | 9 | 33·0 | 16·7 | 100·0 |
| 11 | 25 | 4 | 8·3 | 0 | 37·0 |
| Mean | | | 8·5 | 11·1 | 45·1 |

Table 6  *Summary of results of sequential staining*

| | Normal adult n=11 | VAUA n=7 | Coeliac | EATCL n=3 |
|---|---|---|---|---|
| CD3+, CD4−, CD8− as mean percentage of total CD3+ | 5·9 | 0·9 | 27·6 (n=12) | 24·3 |
| CD7+, CD4−, CD8− as mean percentage of total CD7+ | 19·5 | 4·5 | 22·5 (n=8) | 20·4 |
| CD7+, CD3− as mean percentage of total CD7+ | 13·5 | 1·6 | 1·4 (n=8) | 0·3 |
| CD5+, CD4−, CD8− as mean percentage of total CD5+ | 0 | 0 | 0 (n=8) | 0 |

VAUA=villous atrophy of uncertain aetiology.

patients on a gluten free diet in terms of any of the criteria studied (Fig. 3a–c). There was a significant increase in the proportion of CD3+ cells which were CD4−, CD8− in coeliac disease (patients 11–22; 27·6% (4·9), p=0·0005) and 'uninvolved' mucosa from enteropathy associated T cell lymphoma (patients 8–10; 24·3% (5·0), p=0·0002), compared with the levels in normal villi. There was no significant difference between coeliac disease and

'uninvolved' mucosa from enteropathy associated T cell lymphoma in the proportion of CD4−, CD8− cells in the CD3+ population. There were significantly fewer cells with this phenotype in biopsies with villous atrophy of uncertain aetiology than in normal jejunum (patients 1–7; 0·9% (0·4), p=0·005).

There was no statistically significant difference between normal jejunum, coeliac disease and 'uninvolved' mucosa from enteropathy associated T cell lymphoma in the percentage of intraepithelial CD7+ cells which were CD4−, CD8− (normal: 19·5% (2·5), coeliac disease patients 15–22: 22·5% (6·3), 'uninvolved' mucosa from enteropathy associ-
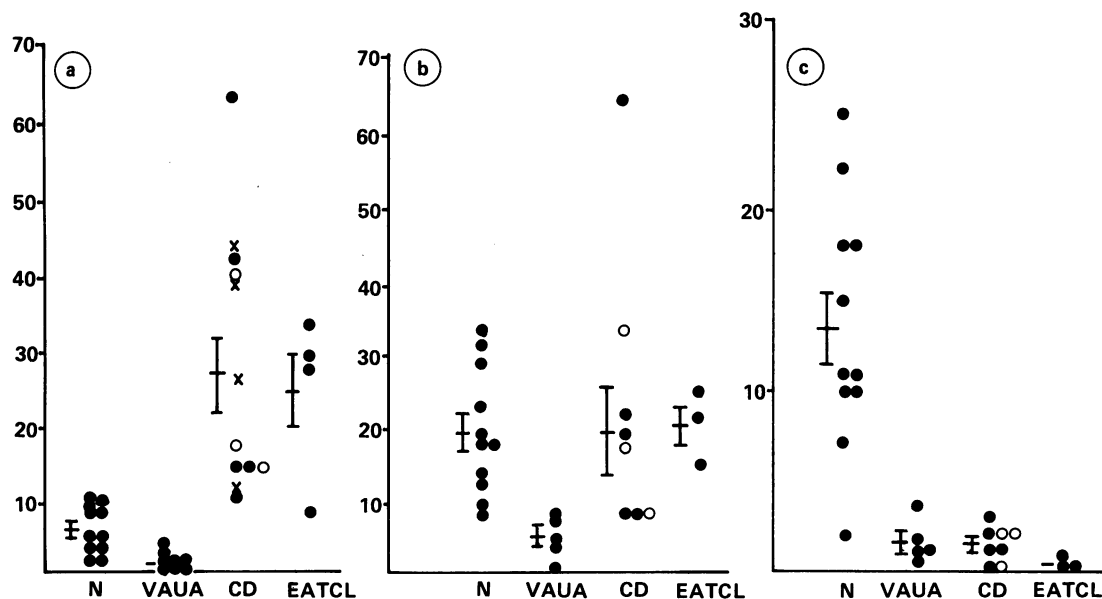


Fig. 3  *In panel (a) is shown the percentages of CD3+, CD4−, CD8− intraepithelial cells; in panel (b) the percentages of CD7+, CD4−, CD8− intraepithelial cells; and in panel (c) the percentages of CD7+, CD3− cells in normal (N) biopsies, in patients with villous atrophy of uncertain aetiology (VAUA), coeliac disease in treated adults (●), untreated adults (X) and untreated children (○), and from patients with enteropathy associated T cell lymphoma (EATCL).*

ated T cell lymphoma patients: 20·4% (2·4)). In biopsies with villous atrophy of uncertain aetiology, however, the percentage was significantly lower than in all other groups (4·5% (1·1), p=0·001).

The proportion of CD7+ cells which were CD3– was statistically significantly reduced compared to the proportion in normal jejunum in all conditions studied (coeliac disease patients 15–22: p=0·0005, enteropathy associated T cell lymphoma: p=0·005, gluten refractory enteropathy: p=0·005). There was no significant difference between the proportion of CD7+ cells which were CD3– in coeliac disease patients 15–22 (1·4% (0·35)), uninvolved mucosa from enteropathy associated T cell lymphoma (0·3% (0·01)) or biopsies with villous atrophy of uncertain aetiology (1·6% (0·5)).

These results are summarised in Table 6.

## Discussion

It is becoming increasingly clear that considerable heterogeneity exists in T cell subpopulations, which can only be revealed by using panels of monoclonal antibodies. In this present study we have attempted to identify subpopulations of CD3+ and CD7+ intraepithelial T cells based on their expression of T cell subset markers (CD4 and CD8). We have described two novel populations of intraepithelial lymphocytes in normal jejunal mucosa: CD7+, CD3+, CD4–, CD8– (6·5% of intraepithelial lymphocytes) and CD7+, CD3–, CD4–, CD8– (13·5% of intraepithelial lymphocytes). We have shown that the latter population is concentrated in the tips of the villi in normal adult jejunum. In coeliac disease and enteropathy associated T cell lymphoma, intraepithelial CD7+, CD3+, CD4–, CD8– cells are present at raised levels whereas the population of CD7+, CD3– cells, is diminished. In contrast, intraepithelial cells from biopsies of jejunum with villous atrophy of uncertain aetiology, were almost exclusively CD7+, CD3+, CD4/CD8+.

Our results are different from those obtained by Jenkins and coworkers.[11] They also described a CD3+, CD4–, CD8– population of intraepithelial cells in coeliac disease, but did not detect these cells in normal jejunum. Similary the CD7+, CD3– and CD7+, CD4–, CD8– cells were not detected by this group. The differences in results are probably due to differences in sensitivity of techniques used. When serial sections are single stained using immunohistochemistry and intraepithelial cells counted as in the study of Jenkins *et al*, small differences in a variable population are not detectable. They are detectable, however, in sequentially stained preparations as used in this study.

It is of interest that newly diagnosed coeliac disease

in childhood and adult life and adult patients with coeliac disease on a gluten free diet all have the same distribution of intraepithelial lymphocyte phenotypes which are distinct from those in normal epithelium. This suggests that expansion of the CD3+, CD4–, CD8– population and depletion of the CD3–, CD7+ population are not transient consequences of an immune response to gliadin, but may be fundamental characteristics of coeliac disease. The differences between the biopsies from patients with confirmed coeliac disease and the flat biopsies from patients with no firm diagnosis are also relevant to this point. Although we cannot exclude the possibility that some of the group of patients with villous atrophy of unknown aetiology are coeliacs not adhering to a gluten free diet, these differences may prove to be significant and useful if follow up studies of biopsies from non-coeliac patients with flat mucosa of known aetiology yield the same results.

It is controversial whether the enteropathy in enteropathy associated T cell lymphoma is indicative of coeliac disease or whether it is caused by some other mechanism such as the action of malignant T cells themselves.[30] Activated T cells have been shown to play a pivotal role in the development of villus atrophy and crypt cell hyperplasia in fetal human small intestine *in vitro*.[31] Although some patients with enteropathy associated T cell lymphoma have an obvious history of gluten sensitive enteropathy, this is not always the case.[32] In addition, patients with enteropathy associated T cell lymphoma do not have high titres of anti α gliadin antibodies in their serum as is usually seen in patients with coeliac disease.[8] Splenic atrophy, however, is seen in both conditions, and they show the same HLA predisposition.[20 23] In our study, there was no statistically significant difference between the various intraepithelial lymphocyte populations in coeliac disease and enteropathy associated T cell lymphoma in any respect, supporting the hypothesis that the enteropathy in enteropathy associated T cell lymphoma is the result of coeliac disease. If this is indeed the case, then the tendency for patients with enteropathy associated T cell lymphoma to become refractory to a gluten free diet might indicate that the T cells activated in the mucosa as a consequence of gluten challenge have undergone malignant transformation.

The function of intraepithelial CD7+, CD3+/–, CD4–, CD8– cells is not clear. Although CD3 is associated with the T cell receptor and is necessary for signal transduction after antigen binding,[34] the role of the CD7 molecule is unknown. It is not likely that they are natural killer cells. While natural killer cells are known to be CD3–, cells expressing natural killer cell phenotype are not present in normal jejunal epithelium[11 26] and Cerf-Bensussan *et al* have

shown that isolated intraepithelial cells do not have natural killer cell activity.[3] The CD7+, CD4−, CD8− cells are not likely therefore to be natural killer cells. They may, however, represent the granular intraepithelial lymphoid population. Studies of isolated cells have shown granular intraepithelial cells to represent 20% of the total intraepithelial lymphocytes,[23] whereas studies of tissue sections suggest around 35%.[4] In this study, CD7+, CD4−, CD8− intraepithelial cells (which account for approximately 20% of intraepithelial cells, and the majority of CD4−, CD8− cells) are concentrated in the villus tips. Moreover their proportion of the intraepithelial population is unchanged in coeliac disease. There are no reports suggesting that granular intraepithelial cells are concentrated in the villus tips, and the percentage of granular intraepithelial lymphocytes is reported to rise to 60% in coeliac disease.[4]

There has recently been interest in the small population of blood CD3+, CD4−, CD8− T cells. These cells when activated show non-specific cytolytic activity for both natural killer sensitive and resistant targets.[26] In addition these cells express the γ/δ dimer rather than the α/β dimer as the antigen receptor, but the functional significance of this is unknown.[27 28] The CD3+, CD4−, CD8−, γ/δ phenotype is that of the immature human thymocyte and T cell clones of this phenotype can be isolated from fetal thymus.[29] Further work is required to investigate the possible link between the blood borne and intraepithelial CD4−, CD8− T cell populations and their role in the small intestinal epithelium in both health and disease.

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