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T cell distribution is different in follicle-associated epithelium of human Peyer's patches and villous epithelium

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SUMMARY

Immunohistochemical analyses performed on specimens of normal human ileum showed a significantly raised number of T cells in the follicle-associated epithelium (FAE) of Peyer's patches compared with the epithelium of distant villi. The T cells tended to be clustered in all layers of the FAE and were significantly more numerous adjacent to interruptions of the brush border (revealed by lack of staining for alkaline phosphatase). Such interruptions were taken to indicate 'membrane' (M) cells. Our findings therefore suggested a spatial relationship between M cells and aggregation of T cells. The ratio of $CD4^+$ to $CD8^+$ T cells ($\sim$4:10) was significantly higher in the FAE than in the villous epithelium ($\sim$0·6:10). This suggested that the FAE may be involved to a greater extent in induction of 'helper' T cell functions, perhaps depending on luminal antigens transported by M cells, whereas the villous epithelium may be more involved in stimulation of 'suppressor' T cell functions as indicated by recent studies *in vitro*.

Keywords Peyer's patches M cells T cells CD4 CD8 mucosal immunity intestine

INTRODUCTION

Peyer's patches (PP) are assumed to play a major role in antigen uptake from the gut lumen and in induction of intestinal immune responses (Carlson & Owen, 1987). The follicle-associated epithelium (FAE) of PP and solitary lymphoid follicles throughout the gut contains specialized epithelial cells, so-called 'membrane' or M cells, which are thought to mediate most of the inward translocation of antigens to the lymphoid cells (Owen, 1977; Rosen *et al.*, 1981; Shakhlamov, Gaidar & Baranov, 1981; Wolf *et al.*, 1981). As most B-cell responses are T cell-dependent, a particular spatial relationship between T cells and M cells may be of importance for the induction of intestinal humoral immunity.

Studies of solitary lymphoid follicles in the human appendix have indicated that the fetal development of FAE takes place in parallel with subepithelial accumulation of lymphoid cells (Bockman & Cooper, 1975). There is apparently no similar information available for human PP and it is unknown whether epithelial immigration of lymphoid cells explains the development of FAE, or whether the subepithelial accumulation of lymphoid cells is induced by micro-environmental factors released from FAE. In the fetal rat, accumulation of lymphocytes in the subepithelial region of PP takes place after an initial occurrence of dendritic cells, and the FAE already contains M cells at birth (Wilders, Sminia & Janse, 1983).

Intra-epithelial lymphocytes occur both in human intestinal villi (Marsh, 1980; Selby *et al.*, 1981) and FAE of PP (Owen & Jones, 1974). They are especially numerous in the latter epithelium, as demonstrated in animals (Faulk *et al.*, 1970; Ferguson & Parrott, 1972; Abe & Ito, 1977; Chu, Glock & Foss, 1979; Smith, Jarvis & King, 1980). Owen & Jones (1974) showed in humans and Bye, Allan & Trier (1984) in the mouse that there is an intimate contact between mature M cells and lymphocytes. It has therefore been assumed that lymphocytes migrating into the FAE induce differentiation of absorbing epithelial cells into M cells (Bhalla & Owen, 1982; Smith & Peacock, 1982).

In this study we quantified intra-epithelial T cells and mapped their distribution in villous and PP-associated surface epithelium in the normal human ileum. There were significantly more T cells in the latter epithelium and the ratio between the $CD4^+$ and $CD8^+$ subsets was significantly raised in the FAE compared with the epithelium of the distant villi. A quantitative investigation in the mouse had previously shown a non-random distribution of lymphoid cells in the FAE, but the M cells were not considered (Rowinski, Lamprecht & Sicinski, 1984). We likewise observed that T cells were unevenly distributed in human FAE and, moreover, that they were apparently clustered in relation to the M cells.

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MATERIALS AND METHODS

Tissue specimens

Intestinal mucosal tissue was obtained from 10 kidney donors with maintained peripheral circulation. Small tissue blocks from PP, including surrounding villi at some distance, were excised, fixed in cold ethanol and processed for paraffin embedding (Sainte-Marie, 1962). Sections from appropriately oriented samples were cut at 1–2 μm perpendicular to the surface of the mucosa.

In addition we obtained biopsy specimens from normal ileum including Peyer's patches from eight patients undergoing endoscopy for diagnostic purposes. The specimens were covered by Tissue-Tek O.C.T. Compound (Miles Scientific, Naperville, IL, USA), quick-frozen in liquid nitrogen, and thereafter stored at $-20°C$ until cryostat sections were cut at 8 μm perpendicular to the mucosal surface.

Immunofluorescence and histochemical staining

Attempts to demonstrate M cells were performed by the method of Owen & Bhalla (1983), based on the absence of a brush border with alkaline phosphatase on these cells. Co-staining for T cells and alkaline phosphatase was carried out as follows. De-waxed paraffin sections were first incubated with a murine monoclonal antibody to CD3$^+$ T cells (dilution 1:20 of anti-Leu-4; Becton Dickinson, Mountain View, CA, USA) for 20 h at room temperature, followed by biotinylated horse anti-mouse-IgG (Vector Laboratories, Burlingame, CA, USA) at 0·05 g/l for 3 h and then fluorescein isothiocyanate (FITC)-conjugated avidin (Vector) at 0·025 g/l for 30 min (Brandtzaeg & Rognum, 1983). Alkaline phosphatase was thereafter stained with naphthol phosphoric acid and fast red violet for 3 min (1 mg naphthol AS-TR phosphate (N-6125; Sigma Chemical Co., St. Louis, MO) dissolved in 100 μl dimethyl formamide was mixed with 5 ml 0·1 M Tris HCl buffer, pH 9·0, containing 4 mg fast red violet LB salt (F-1625; Sigma)). Goblet cells could be excluded because of their empty cytoplasm, but as a control the sections were finally stained with alcian blue and Periodic Acid-Schiff (PAS) which should demonstrate most or all mucin in this part of the gut (Mowry, 1956; Bancroft & Cook, 1984).

The cryostat sections were dried overnight at room temperature and fixed for 10 min in acetone before being co-stained with rabbit antiserum (1:100) to keratin (Huitfeldt & Brandtzaeg, 1985) to demonstrate the epithelium, along with monoclonal antibody (supernatant, neat) to CD4 or CD8 (Dakopatts, Glostrup, Denmark) for 30 min at room temperature. Additional sections were stained with monoclonal antibody to HLA-DQ (except DQw3) (anti-Leu-10, 1:20; Becton Dickinson) to detect other intra-epithelial leucocytes including B cells. After a preliminary wash, all sections were fixed for 10 min at 4°C in 96% ethanol, and were further incubated for 3 h with biotinylated anti-mouse IgG followed by 30 min with FITC-conjugated avidin (as described above) mixed with tetramethyl rhodamine (TRITC)-conjugated swine anti-rabbit IgG (Brandtzaeg & Rognum, 1983).

Microscopy

The fluorescence microscope was a Leitz Orthoplan equipped with an Osram HBO 200 W lamp for fluorescence emitted by the fast red colour product or TRITC and an XBO 150 W lamp for green (FITC) fluorescence. A Ploem-type epi-illuminator provided narrow-band excitation light and selective filtration of the contrasting emission colours. Paired colours could be observed simultaneously by means of a less selective filter system (Leitz filter block K2 for excitation (450–490 nm) and barrier filter BP 515), which was used during cell counting. Paraphenylenediamine (1 g/l) was added to the mounting medium (polyvinyl alcohol) to prevent fading of FITC fluorescence and double-exposed colour slides were obtained with Agfa colour 1000 RS daylight film and the selective filter systems.

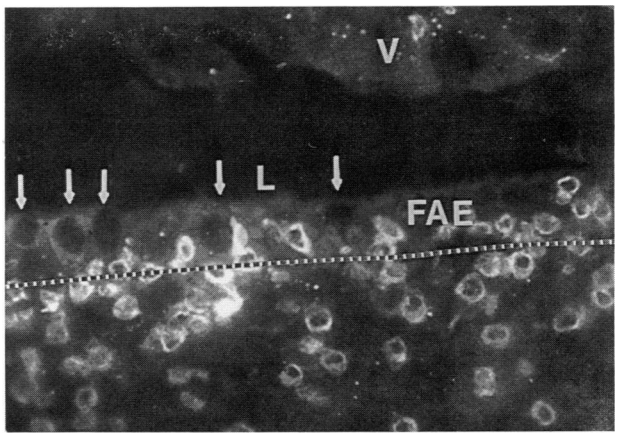

Fig. 1. Section of ethanol-fixed ileum mucosa stained for CD3. Note distribution of intra- and sub-epithelial CD3$^+$ T lymphocytes in the follicle-associated epithelium (FAE). The follicle center is to the right of this field. There are few intra-epithelial T cells on the left where goblet cells (arrowed) are numerous in contrast to the situation in the typical FAE on the right. Broken line indicates basement membrane. L lumen; V villus. (original magnification × 450).

Histomorphometry

Enumeration of intra-epithelial CD3$^+$ T cells was performed in paraffin sections with an ×40 Leitz immersion objective and a grid (Leitz code No. 519902) that was moved systematically along the FAE with one side of the frame touching the epithelial surface. The epithelium was thereby divided into segments 0·055 mm long. T cells in the corresponding subepithelial part of the lamina propria included within the grid, were additionally counted. Intra-epithelial T cells within a distance of 0·028 mm from the centre of interruptions in alkaline phosphatase staining (indicative of M cells) were separately recorded. Usually we evaluated only one dome region per subject, but in one case five and in two cases two such regions were included. Villi at some distance (at least two crypts away from PP) were subjected to T cell enumeration as described for PP.

Intra-epithelial CD4$^+$ and CD8$^+$ T cell subsets and HLA-DQ$^+$ leucocytes (including B cells) were enumerated in serial cryostat sections in a manner similar to that described above. Identification of the epithelium was facilitated by co-staining for keratin, and the same epithelial areas were evaluated in serial sections. One PP follicle and villous epithelium at some distance were included in each specimen and sufficient sections were counted to obtain an average of about 200 intra-epithelial T cells per subject.

Statistical analysis

Differences in T cell numbers were evaluated by the Wilcoxon's test for unpaired samples (two-tailed).

Table 1. T cells/grid (median and 95% confidence interval) enumerated in normal ileum of 10 subjects*

FAE	Villous epithelium	Subepithelial area	
		PP	Villi
3·0 (2·1–5·3)	1·2 (1·0–1·6)	3·5 (3·2–6·1)	1·2 (0·8–1·7)

* The median number of examined grid segments per subject in PP and villi was 14 (range, 5–30) and 15·5 (range 14–19), respectively. FAE follicle-associated epithelium of Peyer's patch.

Table 2. Intra-epithelial T cells/grid (median and 95% confidence interval) enumerated in Peyer's patches of 10 subjects

Adjacent to putative M cells*	Remaining FAE
3·6 (3·3–4·9)	2·6 (1·7–4·0)

* The median number of examined grid segments was 4·5 per subject (range 3–14).

FAE follicle-associated epithelium.

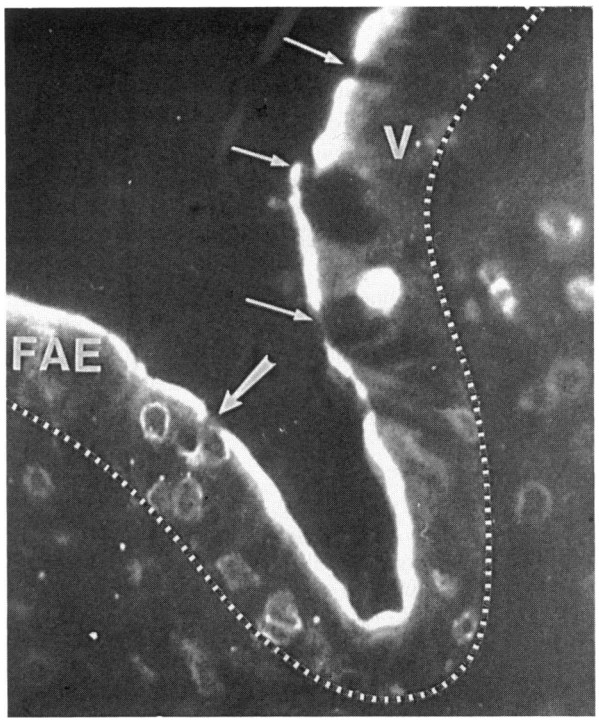

Fig. 2. Section of ethanol-fixed ileum mucosa co-stained for CD3 and alkaline phosphatase. Note difference of intra-epithelial CD3$^+$ T-lymphocyte distribution in follicle-associated epithelium (FAE) and villous epithelium (V). The brushborder is positive for alkaline phosphatase. T cells are clustered near an interruption (large arrow) in the FAE, whereas they appear as scattered single lymphocytes adjacent to the basement membrane in the villous epithelium. Broken line indicates basement membrane. Small arrows indicate goblet cells (original magnification × 610).

RESULTS

T cells were distributed in all layers of the FAE (Fig. 1) and they tended to be clustered. Conversely, the intra-epithelial T cells of the villi were more evenly dispersed and appeared mainly along the basement membrane. The number of both intra- and subepithelial T cells per grid was significantly higher ($P < 0{\cdot}01$) in the FAE than in the distant villi (Table 1). About five putative M cells per subject were identified by brush border interruption (median, 4·5; range, 3–14). T cells were significantly more numerous ($P = 0{\cdot}02$) adjacent to such interruptions than elsewhere in the FAE (Table 2, Fig. 2).

In the cryostat sections used to enumerate CD4$^+$ and CD8$^+$ subsets, we evaluated per subject a median of 68 (range, 39–107) intra-epithelial T cells in the FAE and 113 (range, 30–183) in the epithelium of distant villi. The proportion of CD4$^+$ T cells in the FAE was 40% compared with 6% in the villous epithelium (Table 3 and Fig. 3). In both PP and villi there was always a marked subepithelial predominance of CD4$^+$ T cells. In the FAE the median percentage of intra-epithelial HLA-DQ$^+$ leucocytes (including B cells) was about 3% of the total number of CD4$^+$ and CD8$^+$ T cells. HLA-DQ$^+$ cells were virtually lacking in the villous epithelium.

Table 3. Median proportion (and range) of CD4$^+$ T cells in relation to the total number of CD4$^+$ and CD8$^+$ intra-epithelial T cells in normal ileum of eight subjects

FAE	Villous epithelium
40·0% (30·8–48·8)	6·3% (2·0–11·0)

FAE follicle-associated epithelium of peyer's patches.

DISCUSSION

Due to limited resolution of the light microscope, we tried to identify M cells of human PP indirectly by their reduced expression of brush border alkaline phosphatase as described by Owen & Bhalla (1983). Adjacent epithelial cells with bright enzyme staining could possibly have masked interruptions in our 1–2 μm sections. In addition, the three dimensional distribution of M cells in the FAE obviously rendered a clear-cut mapping of T cells in relation to M cells difficult. Nevertheless, we were able to show a significant aggregation of T cells adjacent to putative M cells.

Ferguson & Parrott (1972) first reported that luminal antigens are required for infiltration of the FAE by lymphocytes. Our study showed that significantly more T cells appeared

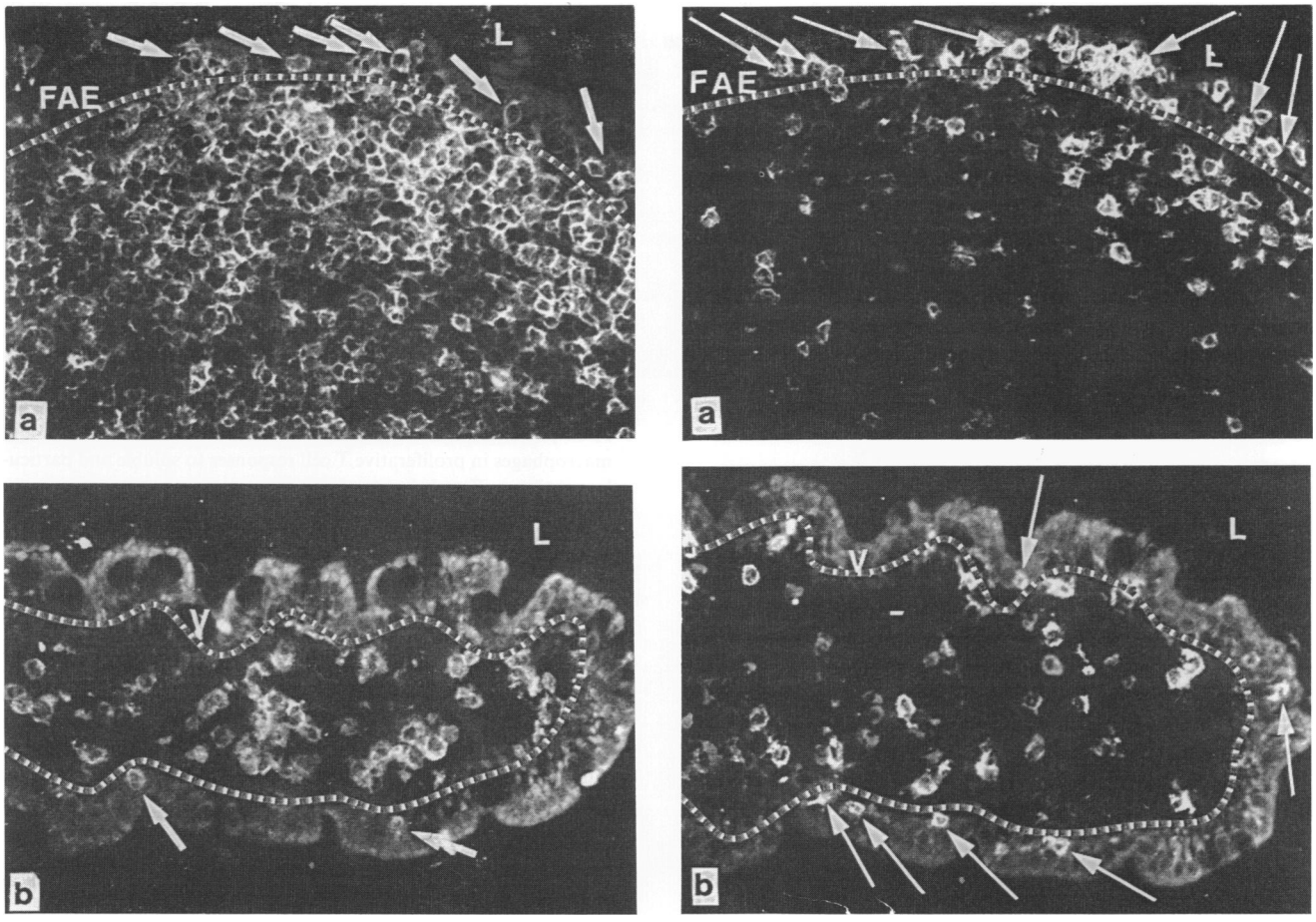

Fig. 3. Cryostat sections of (a) Peyer's patches and (b) villus stained for CD4 (left panels) and CD8 (right panels). Note that there are only two $CD4^+$ T cells (heavy arrows) in the villous epithelium (V) compared with the relatively large number in the follicle-associated epithelium (FAE). $CD8^+$ T cells (thin arrows) predominate in both types of epithelium. Broken line indicates baement membrane. L lumen (original magnification × 265).

in the FAE than in the villous epithelium. This observation accords with the notion that the FAE is an important intestinal entrance for foreign antigens (Carlson & Owen, 1987). There was likewise a more marked subepithelial accumulation of T cells in the PP than in the villi. The porosity of the epithelial basement membrane in PP (McClugage, Low & Zimny, 1986) probably facilitates antigen penetration in addition to the active transport taking place through the M cells (Owen & Nemanic, 1978).

As previously suggested by Owen & Jones (1974), our study likewise indicated that there is a preferential spatial relationship between T cells and M cells in human FAE. However, it is still uncertain whether M cells differentiate from absorbing cells under the influence of T lymphocytes (Bhalla & Owen, 1982; Smith & Peacock, 1982), or whether they originate directly from undifferentiated crypt cells (Bye *et al.*, 1984).

Although the function of intra-epithelial T cells remains obscure, it is tempting to speculate that they respond to luminal antigens. Expression of MHC determinants is crucial for an antigen-presenting cell function, and Hirata *et al.* (1986a) recently reported that occasional human M cells may be MHC class II-positive. Some reports have shown that proteolytic degradation of antigens is a prerequisite for their efficient presentation to T cells (Ziegler & Unanue, 1982; Kapsenberg *et al.*, 1986), but M cells seem to be deficient in lysosomes (Owen, Apple & Bhalla, 1981). Nevertheless, dendritic cells with virtually no endocytic activity are known to be superior antigen-presenting cells (Inaba *et al.*, 1983; Kaye, Chain & Feldmann, 1985). According to our observations, however, M cells are usually not class II (HLA-DR)-expressing in contrast to the remaining FAE of human PP which is always strongly positive (Bjerke & Brandtzaeg, 1988). Local antigen presentation, therefore, may rather be performed by those epithelial cells regularly expressing HLA-DR and by the numerous subepithelial DR-positive dendritic cells (Brandtzaeg, 1985). Luminal antigens may be delivered to both these putative antigen-presenting cell types by the M cells, and the adjacent clustering of T cells may be conducive to the immune responsiveness in PP.

$CD4^+$ T lymphocytes are generally considered to be the subset of immunoregulatory ('helper/inducer') cells responding to MHC class II-positive antigen-presenting cells. Nevertheless, studies *in vitro* have indicated that class II-positive gut epithelial cells from the rat (Bland & Warren, 1986a; 1986b) and human (Mayer & Shlien, 1987) preferentially stimulate $CD8^+$ suppressor cells, which are the dominating intra-epithelial lymphocytes in the villi (Selby *et al.*, 1981). We likewise noted a striking preponderance (89–98%) of $CD8^+$ cells in the ileal villous epithelium, in good agreement with the finding (median, 87%)

of Hirata *et al.* (1986b). Conversely, in the FAE of PP we found about 40% $CD4^+$ lymphocytes. This might suggest that the FAE to a much greater extent than the villous epithelium is involved in stimulation of T cell 'helper/inducer' functions. It is of interest in this context that the so-called 'switch' helper T cells identified in murine PP are autoreactive and can be directly stimulated by class II determinants (Kawanishi, Ozato & Strober, 1985). Such cells are thought to be important for the differentiation of IgM-to IgA-expressing B cells in PP, but it remains to be learned at which level (epithelium, dome or intrafollicular area) this immune regulation mainly takes place. Our study suggested that $CD4^+$ putative helper T cells have the spatial possibility of being stimulated intra-epithelially in human PP.

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