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Heterogeneity of M-cell-associated B and T cells in human Peyer's patches

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SUMMARY

The specialized M cells in the follicle-associated epithelium (FAE) of Peyer's patches (PP) represent an intimate interphase between luminal antigens and gut-associated lymphoid tissue (GALT). M cells form pockets that contain clusters of leucocytes probably involved in the first encounter with antigens from the gut lumen. Three-colour immunofluorescence *in situ* phenotyping of these leucocytes in humans revealed about equal numbers of B ($CD19/20^+$) and T ($CD3^+$) lymphocytes, the latter mainly $CD4^+$ (median 73%, range 40–90%), but relatively few macrophages ($CD68^+$). Most B cells (90%) were positive for surface IgM (sIgM) and often co-expressed sIgD (median 34%, range 6–60%). Occasional B cells (median 2%) did not express CD45RA (range 0–15%) and 13% virtually lacked HLA-DR (range 0–40%). Some B and T lymphocytes expressed the nuclear proliferation marker Ki-67 (range 1–10%). The M-cell pockets also contained occasional cells with cytoplasmic IgA or IgM. These sites thus contained a heterogeneous B-cell population with features of both follicular mantle ($sIgD^+ sIgM^+$) and marginal zone ($sIgD^- sIgM^+$) B lymphocytes. Adjacent T lymphocytes were generally of the memory phenotype ($CD45RO^+$). Our findings suggest that the M-cell-associated B lymphocytes represent local extensions of B-cell follicles towards the gut lumen, developed topically to facilitate antigen presentation and diversification of mucosal immune responses.

INTRODUCTION

Peyer's patches (PP) are assumed to be important for antigen priming of lymphocytes that subsequently migrate to distant intestinal lamina propria and other secretory tissue sites.[1–3] The follicle-associated epithelium (FAE) covering the domes of these organized lymphoid structures contains specialized M ('membrane') cells that constitute very thin ($\approx 0{\cdot}3\ \mu m$) parts of the epithelial barrier.[4,5] These cells have characteristic ultrastructural features with short, irregular microvilli and basal invaginations ('pockets') containing lymphocytes and macrophages.[5–7] M cells are probably the main entrance for poliovirus type 1 in humans.[8] In animal experiments, they have been shown to take up and transport both soluble and especially particulate antigens, such as bacteria and viruses (including human immunodeficiency virus type-1), as well as non-organic particles, to the subepithelial dome area (reviewed in ref. 9). However, the subsequent handling of these antigens is incompletely understood, particularly with regard to leucocytes involved in further antigen processing and presentation.

FAE of rats, mice and rabbits[6,10,11] contains more B and T lymphocytes than villous epithelium; even a few plasma cells have been reported to occur in the rat M-cell pockets. Previous studies in humans have revealed increased proportions of $CD4^+$ T lymphocytes related to M cells in FAE,[12] as well as aggregates of B lymphocytes positive for surface IgM (sIgM).[13] Such $sIgM^+ sIgD^-$ B cells were also localized around the follicular mantle zones and towards the serosal aspect;[13,14] they were described by Spencer *et al.*[13] as the PP counterpart of splenic marginal zone cells.

The aim of this study was to phenotype leucocytes in human M-cell pockets with regard to immunological markers of functional interest. The well-recognized nuclear proliferation marker detected by monoclonal antibody (mAb) Ki-67 was applied to detect putative proliferating lymphocytes, and mAb to CD25 and CD69 to detect activated cells. The various CD45 isoforms were examined because these molecules distinguish naive and memory T cells,[15] and CD45RO may be expressed by B cells during differentiation to plasma cells.[16] HLA-DR was also investigated because this molecule is lost during B-cell differentiation to plasma cells,[17] which were identified together with plasma blasts by their cytoplasmic immunoglobulin. In addition, the presence of L-selectin on M-cell-associated B and T cells was examined as it is known to be expressed by a proportion of human appendix[18] as well as PP[19] lymphocytes, and because of its assumed role in PP homing, at least in mice.[20] However, integrin α_4, when coupled to the β_7-chain, is regarded as the main PP lymphocyte homing receptor[21,22] and was

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Abbreviations: FAE, follicle-associated epithelium; GALT, gut-associated lymphoid tissue; PP, Peyer's patches.

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therefore also evaluated with regard to M-cell-associated expression.

MATERIALS AND METHODS

Tissue specimens

Two to four biopsy specimens were obtained from the terminal ileum during ileocolonoscopy with an Olympus CF 1T 100I colonscope and a large biopsy forceps (FB-13Q) (both from Olympus, Japan) in patients with suspected colitis ($n = 4$), abdominal pain ($n = 4$) or suspected malignancy ($n = 2$) (median age 28·5 years, range 7–72 years). Colonic disease was found in four patients (Crohn's colitis, tubulovillous adenoma of the rectum, solitary ulcer of the rectum, and unspecific low-grade inflammation of the colon, respectively). The patient with Crohn's colitis and one with selective IgA deficiency both had prominent lymphoid aggregates. The terminal ileum and colon were otherwise macroscopically and histologically normal in all patients.

The specimens were immediately orientated on a slice of carrot, embedded in OCT compound (Tissue-Tek, Mile Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°. Cryosections were serially cut at 4 μm, acetone-fixed (10 min, room temperature), and stored at −20° until use. Staining with haematoxylin and eosin revealed between one and six activated lymphoid follicles (median three) with corresponding FAE in each biopsy sample. One specimen from each patient was selected for immunohistochemistry.

Sections (4 μm) from parallel paraffin-embedded specimens fixed in 10% formalin for 20 hr at room temperature, were stained with haematoxylin and eosin for morphological evaluation (Fig. 1a,b). Such sections were also used to study cytoplasmic immunoglobulin by direct immunofluorescence (Fig. 1c, see below). Electron microscopy was performed on two glutaraldehyde-fixed specimens from the IgA-deficient patient and an M cell is identified in Fig. 1d.

Immunofluorescence staining

Cryosections were subjected to three-colour indirect immunofluorescence staining as detailed elsewhere.[23] Mixtures of unlabelled primary antibodies (Table 1) were applied for 1 hr at room temperature. B lymphocytes were identified by mAb to either CD19 (murine IgG1) or CD20 (murine IgG2a), depending on the subclass of the complementary mAb. Secondary antibody reagents (1·5 hr) were subclass-specific biotinylated (0·02 g/l) or fluorescein isothiocyanate (FITC)-conjugated (0·05 g/l) goat anti-mouse IgG (Southern Biotechnology, Birmingham, AL) mixed with rabbit antiserum to human cytokeratin (Table 1). The final incubations (30 min) included Streptavidin–Texas Red (0·0025 g/l; British Research Laboratory, Gaithersburg, MD) combined with aminocoumarin (AMCA)-conjugated goat anti-rabbit IgG (0·075 g/l; Vector Laboratories, Burlingame, CA).

Two sections from every tissue sample were fixed in 96% ethanol at 4° for 10 min and stained with mAb to CD3 or CD20 in combination with antiserum to cytokeratin. M cells were identified by their lack of a typical brush border with alkaline phosphatase. The latter marker was developed by a fourth incubation (5 min) with naphthol phosphoric acid and fast red violet, whose colour product emits red fluorescence.[12] Sections subjected to trypsin (1 mg/ml, 1:250, 30 min; Difco Laboratories Inc., Detroit, MI) were prepared from all paraffin-embedded tissue samples and incubated (room temperature,

Table 1. Primary unlabelled antibodies and primary fluorochrome conjugates used for immunohistochemistry

Antibody reagents	Antibody specificity (human)	Isotype	Working dilution	Source
RIV 9	CD3	Murine IgG3	Purified immunoglobulin, 1:10	Sanbio, Am Uden, the Netherlands
Anti-Leu-3	CD4	Murine IgG1	Purified immunoglobulin, 1:20	Becton Dickinson, Mountain View, CA
Anti-Leu-4	CD8	Murine IgG1	Purified immunoglobulin, 1:20	Becton Dickinson
Anti-HLA-DR	HLA-DR	Murine IgG2a	Purified immunoglobulin, 1:50	Becton Dickinson
Anti-Leu-18	CD45RA	Murine IgG1	Purified immunoglobulin, 1:10	Becton Dickinson
FN61	CD69	Murine IgG1	Purified immunoglobulin, 1:40	S. Funderud, Oslo, Norway
KP1	CD68	Murine IgG1	Supernatant, 1:50	K. Pulford, Oxford, UK
HD37	CD19	Murine IgG1	Supernatant, 1:10	Dakopatts, Glostrup, Denmark
UCHL-1	CD45RO	Murine IgG2a	Supernatant, 1:10	P.C.L. Beverly, London, UK
L26	CD20	Murine IgG2a	Supernatant, 1:20	Dakopatts
ACT-1	CD25	Murine IgG1	Supernatant, 1:10	Dakopatts
Anti-Leu-8	L-selectin	Murine IgG2a	Purified immunoglobulin, 1:10	Becton Dickinson
B5G10	VLA-4 (α_4)	Murine IgG1	Ascites fluid, 1:1000	M. Hemler, Dana-Farber Inst., Boston, MA
Ki-67	Proliferating cells	Murine IgG1	Supernatant, 1:10	Dakopatts
Anti-IgD	δ-chain (IgD)	Murine IgG1	Supernatant, 1:10	D.Y. Mason, Oxford, UK
Anti-IgM	μ-chain (IgM)	Murine IgG1	Supernatant, 1:10	D.Y. Mason
F204	α-chain (IgA)	Rabbit IgG–TRITC	Purified, 1:80	Dakopatts
R152	μ-chain (IgM)	Rabbit IgG–TRITC	Purified, 1:160	Dakopatts
R151	γ-chain (IgG)	Rabbit IgG–FITC	Purified, 1:160	Dakopatts
R-157	δ-chain (IgD)	Rabbit immunoglobulin–FITC	Antiserum, 1:50	Authors' laboratory
R-505	Cytokeratin	Rabbit immunoglobulin	Antiserum, 1:100	Authors' laboratory

20 hr) with mixtures of FITC- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated rabbit anti-IgA, anti-IgM and anti-IgG (Table 1).

Immunofluorescence microscopy, photography and phenotype recording

Microscopy was performed at ≈ × 300 with a Leitz Orthoplan (Wetzlar, Germany) equipped with a Ploem-type vertical illuminator for selective observation of green (FITC), red (rhodamine or fast red) and blue (AMCA) emissions. A Leitz DMRXE microscope equipped with a filter for simultaneous observation of FITC and Texas Red emissions was also used. Triple-exposures were made on Ektachrome 800/1600 daylight film.

Identification of putative M-cell areas was based on three criteria: (1) lack of apical alkaline phosphatase; (2) lack of detectable cytokeratin in FAE invaginations; and (3) presence of clustered B lymphocytes in the corresponding areas (Fig. 2a). Phenotyping of co-localized leucocytes was performed in such areas easily identified in serial sections. In every biopsy specimen, the total numbers of B and T lymphocytes present within putative M-cell pockets were enumerated by paired staining (Fig. 2b) and the proportion of each phenotype was calculated. A median of 17 (range 9–254) B lymphocytes ($CD19^+$ or $CD20^+$) was evaluated in M-cell pockets of each section for expression of sIgD (Fig. 2d), sIgM, HLA-DR (Fig. 2e), α_4 integrin, L-selectin, CD45RA and CD45RO. A median of 20 (range 8–228) T lymphocytes ($CD3^+$) was similarly evaluated for expression of CD4, integrin α_4, L-selectin, CD45RA and CD45RO. Macrophages ($CD68^+$) were likewise enumerated in the putative M-cell pockets.

All specimens were additionally stained with mAb Ki-67 (against nuclear proliferation marker) in combination with mAb to CD3 or CD20. T and B cells were also examined for the activation markers CD25 and CD69 in five specimens which were of sufficient size to provide M-cell areas. Finally, cells with cytoplasmic IgG, IgA or IgM were recorded in trypsinized sections of representative formalin-fixed, paraffin-embedded tissue specimens.

Methodological considerations

Lack of a reliable positive marker for M cells made it necessary to localize putative M-cell areas by indirect methods because electron microscopy could not be applied for these studies. As clusters of B lymphocytes in FAE were always juxtaposed to brush-border interruptions, these intraepithelial lymphoid aggregates were taken to be hallmarks of M-cell pockets; lack of staining for cytokeratin further outlined these specific sites in FAE (Fig. 2a, c).

Most biopsy specimens contained only two or three putative M-cell areas, which were often small and provided relatively few adequate sections. Moreover, the M-cell areas were susceptible to damage and often contained artefacts with disrupted cells. All immunological markers could therefore not be studied in every specimen. Nevertheless, the reproducibility of both cell enumerations and the complete phenotyping could be tested in serial sections of two specimens stained and evaluated 6 weeks apart; comparable phenotype proportions for all immunological markers were obtained at the two time-points (χ^2 test, $P > 0{\cdot}05$). The KP1 antibody to CD68 might also recognize neutrophils,[24] but no granulocytes were observed in the M-cell areas by ordinary histology. Overestimation of M-cell-associated macrophages was therefore unlikely.

The number of evaluated lymphocytes in individual sections was relatively small (median 18, range 7–52), except in two samples that contained several M-cell areas with a median of 76 (range 40–254) B and T lymphocytes in every section. The phenotypic profiles obtained in these two cases were fairly similar to the results for the remainder, except for a somewhat smaller proportion of T cells expressing CD45RO (88% compared with 100%) and a few T cells (7–9%) with L-selectin (Fig. 3).

RESULTS

Morphology

Lymphoid follicles often occupied parts of the domes and approached the lateral aspects of FAE (Fig. 1a); in these cases clustered leucocytes, probably reflecting M-cell pockets,[12] were seen within the adjoining epithelium (Fig. 1b) as well as in putative pockets apparently unrelated to the follicles (see later). One to three such contact areas between follicles and FAE occurred in every biopsy specimen, most frequently in samples from young persons (< 35 years old). They were especially numerous in one of the normal subjects (23 years old) and in the patient with unspecific low-grade colitis (7 years old). In all specimens some leucocyte clusters apparently penetrated to the gut lumen (Fig. 1b). In addition to lymphoid hyperplasia, the IgA-deficient patient had increased numbers of intraepithelial lymphocytes in villi, while the Crohn's colitis patient had scattered clusters of intraepithelial granulocytes outside M-cell areas, as well as subepithelial plasma cell accumulations.

Immunohistochemistry

General. The number of sites within FAE deemed to represent M-cell pockets varied among the samples, from 1 to 15 (median 3), including the obvious contact areas with lymphoid follicles (see above); only specimens from two subjects contained more than 10 putative pockets. At some such sites clusters containing up to 30–40 B lymphocytes occurred, indicating that several M cells were juxtaposed. T lymphocytes were usually accumulated along with B lymphocytes in the putative M-cell pockets (Fig. 2b), on average in equal proportions (median 50% $CD19^+/CD20^+$, range 39–66%; median 50% $CD3^+$, range 33–61%). $CD68^+$ macrophages were relatively rare at these sites, two $CD68^+$ cells being found per putative M-cell pocket at the most (median 2, range 1–20/specimen). These cells were always $HLA\text{-}DR^+$ and 50% (range 50–100%) were weakly $CD4^+$.

B cells. The calculated lymphoid phenotype proportions associated with putative M cells are shown in Fig. 3. The B lymphocytes ($CD20^+$) were mainly (median 90%) $sIgM^+$ but co-expressed on average 34% sIgD. The latter subset usually expressed L-selectin although $sIgD^-$ L-$selectin^+$ cells were also identified; a median of 55% of the B lymphocytes ($CD19^+$) in putative M-cell pockets were L-$selectin^+$. Most of them also expressed HLA-DR but a fraction (median 13%, range 0–40%) showed only weak or no staining for this marker. Occasional M-cell-associated $CD19^+$ lymphocytes (< 10%) expressed CD45RO weakly. A median of 65% of the M-cell-

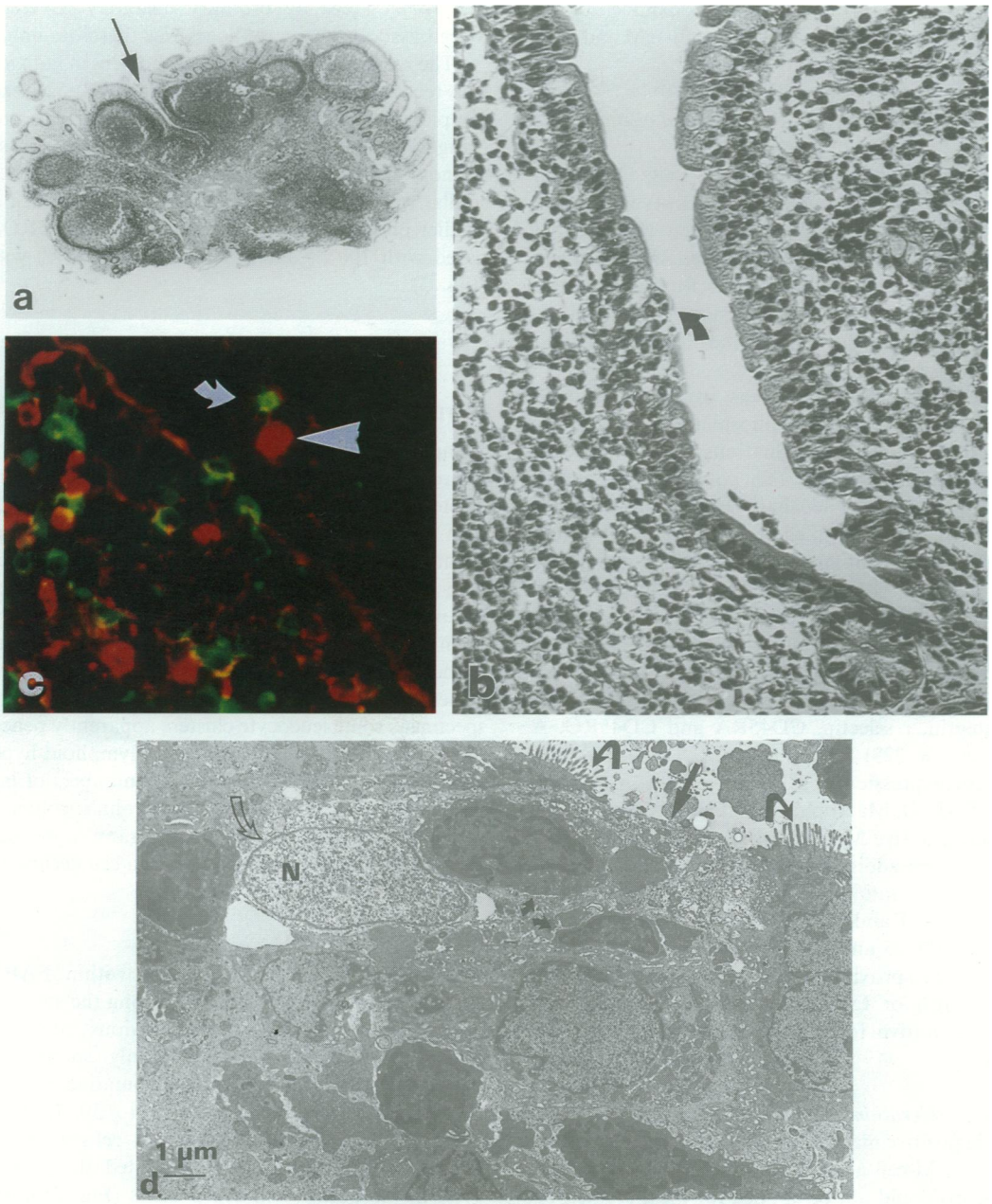

Figure 1. (a) Photomicrograph of human PP. Three follicles approach the epithelium of a crypt (arrow) in the middle (original magnification × 25). (b) Detail of the crypt indicated in (a). Leucocyte cluster (arrow) in putative M-cell area approaches the gut lumen (original magnification × 250). (c) Paired immunofluorescence staining for IgA (green) and IgM (red) in putative M-cell area of parallel formalin-fixed specimen. One cell with cytoplasmic IgM (arrow head) and part of a cell containing IgA (small arrow) are seen in a putative M-cell pocket; two IgA-positive cells are seen traversing the discontinuous basement membrane below (original magnification × 312·5). (d) Electron micrograph of an M cell with distorted microvilli (large arrow) and pale nucleus (open arrow, N) encircles two lymphocytes (small arrows). Two adjacent enterocytes are also seen (curved arrows) (original magnification × 4000).

associated B lymphocytes expressed integrin α_4 weakly. In the formalin-fixed, paraffin-embedded material four specimens contained up to two cells with cytoplasmic immunoglobulin in putative M-cell pockets, usually either IgA or IgM (Fig. 1c) except for one IgG cell in the IgA-deficient case.

T cells. Most aggregated T lymphocytes (median 73%, range 40–90%) in M-cell areas were $CD4^+$, whereas those found scattered throughout the FAE were mainly $CD8^+$. The IgA-deficient case and the Crohn's patient both had M-cell-associated $CD4^+$ T lymphocytes in the lower range (40% and 52%, respectively). Virtually all (median 100%) T lymphocytes in the putative M-cell pockets expressed CD45RO, a fraction was positive for integrin α_4 (median 38%), and some (median 5%) were $CD45RA^+$. T lymphocytes in M-cell pockets generally lacked HLA-DR and usually also L-selectin (Fig. 3). In the two M-cell-rich specimens, however, the figures were

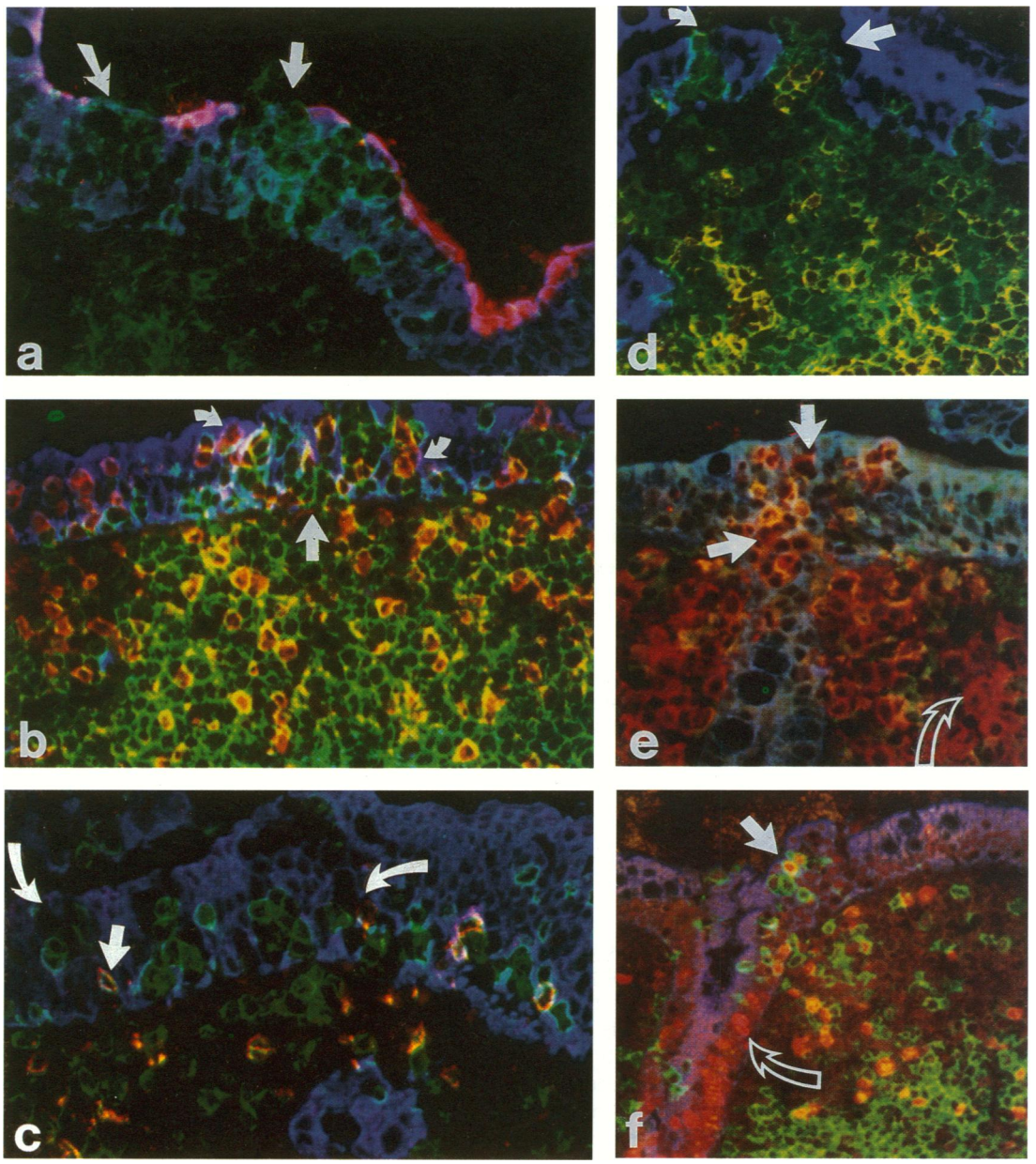

Figure 2. Three-colour immunofluorescence staining in cryosections of normal human PP. (a) Identification of M-cell areas. Interruptions of apical alkaline phosphatase (red) correspond to putative M cells whose pockets contain clustered $CD20^+$ B cells (green). FAE is visualized by staining for cytokeratin (blue). Oblique arrow (left) points at M-cell area with few $CD20^+$ cells. (b) Triple-staining for CD3 (red), CD20 (green) and cytokeratin (blue). Clustered B lymphocytes dominate in these M-cell areas (green cells, vertical arrow), but some T-cell clusters are also seen (red cells, small curved arrows). Yellow staining is caused by juxtaposed green and red cells, and light blue by juxtaposed green and blue. Note the large fraction of green B cells immediately below the blue epithelium. (c) Co-staining for CD3 (green), CD8 (red) and cytokeratin (blue). A few $CD8^+$ cells (yellow, vertical arrow) are seen in some of the cytokeratin-deficient, putative M-cell pockets (curved arrows). (d) Triple-staining for CD20 (green), IgD (red), and cytokeratin (blue). $sIgD^+$ B lymphocytes (mantle zone phenotype, yellow) are found in one M-cell area (large arrow), but there are only $sIgD^-$ cells (marginal zone phenotype, purely green) in another (left, small curved arrow). (e) Triple-staining for CD19 (red), HLA-DR (green), and cytokeratin (blue). Occasional $CD19^+$ B cells lack HLA-DR (red cells, solid arrows) in M-cell area, while most are yellow ($CD19^+$ $HLA\text{-}DR^+$). Note several larger $CD19^+$ $HLA\text{-}DR^-$ cells in dome area below (open arrow). (f) Triple staining for CD20 (green), Ki-67 (red) and cytokeratin (blue). Some B cells in M-cell area express Ki-67 (arrow; red nucleus with yellow periphery). Several $Ki\text{-}67^+$ epithelial cells are seen in crypt (open arrow) (original magnification a–d, ×312·5; e–f, ×250).

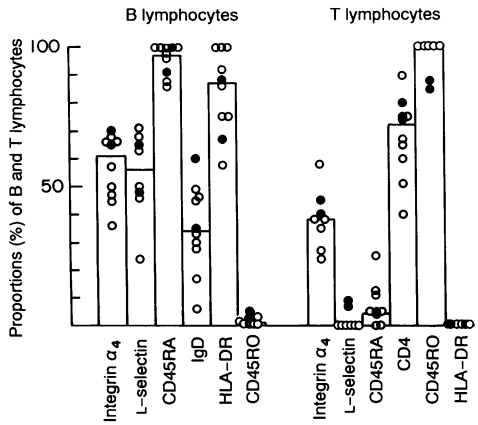

Figure 3. Phenotype distribution (%) of B and T lymphocytes in M-cell pockets of human PP ($n = 10$). Filled circles represent data obtained in two M-cell-rich specimens. Columns indicate medians.

80% and 75% for CD4, 85% and 88% for CD45RO, 7% and 9% for L-selectin, and 4% and 12% for CD45RA, respectively.

Lymphocyte activation and proliferation. Most M-cell-associated T lymphocytes (84%, range 25–100%), but relatively few B lymphocytes (34%, range 0–50%), expressed CD69. Only few T cells (three altogether in the sections examined) and no B cells expressed detectable levels of CD25.

Cells reactive with Ki-67 were identified in putative M-cell pockets of all specimens, ranging from 1 to 14 cells/section; the highest numbers were found in the two M-cell-rich specimens (12–14 positive cells). They constituted only 1–10% of all M-cell-associated lymphocytes, and were more often B cells than T cells (38% and 29% of all Ki-67-reactive cells, respectively). The Ki-67 reactive B lymphocytes were never $sIgD^+$ but often expressed sIgM.

DISCUSSION

This study is apparently the first attempt to phenotype in detail leucocytes present in M-cell areas of human PP. These parts of FAE are considered crucial in the induction of mucosal immune responses and are usually located laterally on the domes. We have previously shown that human M-cell pockets lack T-cell receptor γ/δ^+ lymphocytes.[25] Here we report that the M-cell-associated leucocytes differ markedly from intraepithelial lymphocytes in other respects as well; they consist on average of equal numbers of B and T cells, the latter mainly of memory phenotype ($CD45RO^+$) and belonging predominantly to the $CD4^+$ subset. The B cells are strikingly heterogeneous, with characteristics both of mantle ($sIgD^+$ $sIgM^+$) and marginal ($sIgD^-sIgM^+$) zone lymphocytes,[13,14] in addition to occasional plasma blasts or plasma cells with cytoplasmic immunoglobulin. Notably, the M-cell pockets contain only few macrophages.

The functional role of sIgD is controversial, but its cross-linking has been shown to augment humoral immune responses in animal studies.[26] Most authors consider sIgD as a marker of 'naive' B lymphocytes.[17] This is supported by the recent finding that $sIgD^+$ $sIgM^+$ peripheral blood lymphocytes express $V\kappa$ genes with no or little somatic mutation.[27] The marginal zones of the spleen white pulp contain $sIgD^-sIgM^+$ B lymphocytes;[13] a proportion of this population in the rat apparently represents memory cells.[28] Although the precise functions of the $sIgD^-$ and $sIgD^+$ subsets revealed in M-cell pockets of human PP remain unknown, their presence at these sites might reflect that B cells with both naive and memory properties participate in immune responses within FAE.

The higher proportion of L-selectin$^+$ than of $sIgD^+$ B lymphocytes in the M-cell pockets suggests that some marginal zone cells express this homing receptor, perhaps also reflecting recruitment of memory B cells to these sites. Interestingly, some B lymphocytes reacted with Ki-67, probably reflecting topical proliferation. By contrast, only few of the intermingled T cells were positive for L-selectin and relatively few for Ki-67. Notably, L-selectin$^+$ T lymphocytes were found only in M-cell pockets of specimens rich in such areas. The same specimens contained occasional $CD45RO^-$ and $Ki\text{-}67^+$ T cells in the pockets, probably reflecting that recruitment of naive cells to these sites, as well as topical proliferation of T lymphocytes, may take place under certain stimulatory conditions. In germ-free mice exposure to luminal antigens has been shown to induce rapid increase of M cells,[29] and murine M cells were reduced when cyclosporin A was administered during enteric antigen exposure.[30]

Most M-cell-associated T cells but rather few B cells expressed CD69, which is an early activation marker of both phenotypes.[31] CD69 is not present on cells with a chromatin pattern characteristic of resting cells (S. Funderud, personal communication). Furthermore, CD69 is lost from murine thymocytes deprived of interaction with major histocompatibility complex MHC class II molecules.[32] Expression of CD69 on T cells in the M-cell pockets might suggest that these cells were interacting with class II molecules on adjacent B cells, as only rare HLA-DR$^+$ macrophages were present at these sites.

Integrin α_4 was expressed with variable intensity by the M-cell-associated lymphocytes, but the fractions of α_4^+ B and T cells were seemingly lower at this site than in the follicular mantle and interfollicular zones, respectively (I. N. Farstad, T. S. Halstensen, D. Kvale, O. Fausa & P. Brandtzaeg, manuscript in preparation). This fact could reflect that the immunoreactive epitope was concealed by cell–cell or cell–matrix interactions, or that integrins might be susceptible to degradation through cellular damage during tissue processing.

Some M-cell-associated B lymphocytes expressed CD45RO weakly and 13% showed little or no expression of HLA-DR. Furthermore, some B cells were negative for CD45RA in contrast to those in the follicular mantle and marginal zones (I. N. Farstad, T. S. Halstensen, D. Kvale, O. Fausa & P. Brandtzaeg, manuscript in preparation). Taken together, these findings suggested that B-cell differentiation occurs in the M-cell pockets; normal and malignant human B cells have been reported to express CD45RO during differentiation to plasma cells.[16] Although a subset of human T cells expresses low levels of CD20[33] and thus might account for some of the observed $CD20^+CD45RA^-$ cells, this possibility was deemed unlikely because $CD3^+CD20^+$ cells were not directly observed *in situ* and CD20 was strongly decorated in the tissue sections.

Rat M cells were recently suggested to have antigen-presenting capacity because they contain prelysosome- and lysosome-like structures and express MHC class II, at least basolaterally.[34] Furthermore, human PP M cells have been

reported to contain Cathepsin E[35] which is considered to be important in antigen processing.[36] Both rat and human M cells have been shown by immunoelectron microscopy to express MHC class II,[6,37] but human M-cells were found to be negative for HLA-DR by indirect immunofluorescence, in contrast to the villous and remaining epithelial cells[38] as well as the M-cell-associated macrophages described above. The antigen-presenting capacity of human M cells in class II-restricted immune responses is therefore questionable compared with that of traditional antigen-presenting cells, such as macrophages and B cells. Nevertheless, the M cells might still contribute to the processing of antigens, which could then be more easily recognized and internalized by adjacent B cells. Our findings raise the possibility that M cells provide an opportunity for juxtaposed B lymphocytes to present luminal antigens to adjacent memory T cells and thereby promote the diversification of mucosal immune responses according to recent theories.[39] Indeed, there is evidence that secretory IgA antibodies show broader antibody specificity than comparable serum antibodies.[40,41] This property may be of considerable advantage in coping with antigenic drift of microorganisms, which is probably extensive in the gut.

In conclusion, leucocytes present in the human M-cell pockets were found to consist of equal proportions of phenotypically heterogeneous B lymphocytes, T cells mainly of the $CD4^+CD45RO^+$ 'memory' phenotype, occasional plasma blasts or plasma cells, and a few macrophages. These intraepithelial leucocyte aggregates could represent topical extensions of the underlying lymphoid follicles into FAE, thus facilitating direct access of antigens from the gut lumen to gut-associated lymphoid tissue (GALT). This setting should promote the possibility for B lymphocytes to act as antigen-presenting cells, interact with adjacent memory T cells, and thereby amplify as well as diversify mucosal immune responses. The phenotypic heterogeneity and the presence of various functional markers suggests that B lymphocytes can proliferate and differentiate topically in the M-cell pockets.

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