Rabbit M cells and dome enterocytes are distinct cell lineages

Hugues Lelouard, Alain Sahuquet, Hubert Reggio and Philippe Montcourrier*

Laboratoire de Dynamique Moléculaire des Interactions Membranaires, CNRS UMR 5539, cc 107, Université Montpellier II, 34095 Montpellier Cedex 5, France

*Author for correspondence (e-mail: montcour@univ-montp2.fr)

Accepted 7 March 2001
Journal of Cell Science 114, 2077-2083 (2001) © The Company of Biologists Ltd

SUMMARY

We have studied the M cell origin and differentiation pathway in rabbit gut-associated lymphoid tissues. Micro-dissected domes and epithelium isolated by ethylene diamine tetra acetic acid detachment allowed us to view the whole epithelial surface from the bottom of crypts to the top of domes. We used monoclonal antibodies specific to the apex of either M cells or dome enterocytes, lectins, and antibodies to vimentin in appendix, distal Peyer's patches and caecal patches.

The earliest vimentin-labeled M cells were observed in the BrdU-positive proliferative zone of dome-associated crypts. Gradual differentiation of the M cell vimentin cytoskeleton started at this site to progressively give rise to the first pocket-forming M cells in the upper dome. Therefore, these mitotic cells of the crypts appear as the direct precursors of M cells. In addition to an early appearance of M cell markers, a regular mosaic-like relative distribution of M cells and dome enterocytes was already detected in the vicinity of crypts, similar to that observed on the lateral surface of domes where functional M cells lie. This constant distribution implies that there is no *trans*-differentiation of enterocytes to M cells along the crypt-dome axis. Together, these observations provide very strong evidence in favor of an early commitment in crypts of M cell and enterocyte distinct lineages.

Key words: M Cells, Follicle-associated epithelium, Mucosal immunity, Rabbit intestinal epithelium, Differentiation

INTRODUCTION

Epithelial cells are frequently exposed to pathogens that can proliferate in the mucosal environment. Secreted mucus is believed to be the main way of trapping and eliminating most of these pathogens, but mucosal immunity is necessary to prevent their action on epithelial surfaces completely. This is achieved by specific lymphoid tissues that underlie the different mucosae. In gut-associated lymphoid tissues (GALTs), sampling of luminal antigens is achieved by specialized epithelial cells, i.e. M cells, that bind and rapidly transport pathogens from the apical plasma membrane to the basolateral invagination, which contains enough lymphoid cells to trigger an immune response (for reviews, see Neutra et al., 1996; Niedergang and Kraehenbuhl, 2000).

In the small intestine, pluripotent epithelial stem cells reside deep within the crypts and, through commitment and proliferation of lineage precursors, give rise to progressively differentiating absorptive enterocytes and mucous cells, which migrate up towards the crypt mouth and along the villi to be shed from the tips a few days later (for reviews, see Bjerknes and Cheng, 1999; Cheng and Leblond, 1974; Potten and Loeffler, 1990). In GALT, M cell origin and differentiation steps are still a matter of controversy. It has been postulated that, like epithelium cells from the small intestine, they arise and segregate directly from stem cells of crypts. Bye et al. (Bye et al., 1984) have found that transitional forms of M cells at the neck of crypts and upward share some morphological and functional properties (i.e. binding and transport of inert or living particles) with fully differentiated M cells. These findings are supported in mouse and rabbit studies by other authors using different markers or morphological parameters (Clark et al., 1993; Gebert et al., 1999; Gebert et al., 1992; Gebert and Posselt, 1997; Giannasca et al., 1994; Pappo, 1989; Sicinski et al., 1986). Conversely, several authors have challenged the lineage hypothesis. They propose that mature enterocytes could switch to the M cell phenotype under the influence of lymphoid cells from the underlying follicles (Kerneis et al., 1997; Savidge, 1996; Smith and Peacock, 1980) or of pathogens from the lumen (Borghesi et al., 1996; Meynell et al., 1999; Savidge et al., 1991), followed by de-differentiation to the enterocyte phenotype at the apex of domes (Sierro et al., 2000). This has been strengthened by the nature of the role of follicular lymphoid cells both in vivo, in the induction of new Peyer's patches in mouse duodenal mucosa, and in vitro in the acquisition of the M cell phenotype by polarized Caco-2 cells (Kerneis et al., 1997).

We have decided to revisit the M cell differentiation pathway and focus on two important questions that remain unsolved. First, do pre-M cells, previously detected by several authors, originate directly from proliferative cells of the crypts, or could they derive from still poorly differentiated enterocytes of the upper part of the crypts or higher in the dome? Second, are pre-M cells sufficiently numerous to represent the total pool of mature M cells of the median part of the dome or is there a need for conversion of enterocytes into M cells?

Several markers can now be considered to be reliable for selective labeling of M cells in rabbit GALT (Gebert and Hach,

1993; Gebert et al., 1992; Jepson et al., 1993a; Jepson et al., 1992). More recently, we have found new mucin-like markers that are selective for rabbit M cells or dome enterocyte apical surfaces (Lelouard et al., 1999; Lelouard et al., 2001; Maury et al., 1995), and allow simultaneous visualization of M cells and enterocytes in entire domes. We have used multi-labeling to detect M cell and enterocyte precursors in three different GALTs and have designed a rabbit GALT epithelium dissociation method with ethylene diamine tetra acetic acid (EDTA) (Lelouard et al., 1999) that allows visualization of labeled cells continuously from the flank of the dome to the bottom of crypts. We have also assessed micro-dissected domes to obtain large surface views of the follicle associated epithelium (FAE) and cryostat sections.

We identified M cells in the mid-crypt area, among rapidly dividing epithelial cells, and showed that the spatial organization of M cells and dome enterocytes was already established at the neck of crypts. These observations strongly favor the hypothesis that both cell types constitute distinct cell lineages derived from common stem cells.

MATERIALS AND METHODS

Animals

New-Zealand albino rabbits weighing 2 to 3 kg were obtained from the Institut National de la Recherche Agronomique (Montpellier, France). Animals were housed and cared for according to French regulation 87-848 and to EEC-L358.

Antibodies and reagents

Monoclonal antibodies (MAbs) to rabbit intestinal glycoproteins, namely MAb 58 and MAb 214, have previously been described and characterized (Lelouard et al., 1999; Lelouard et al., 2001), and are detailed in Table 1. Goat anti-mouse IgG coupled to FITC or TRITC were from Biosys (Compiègne, France); mouse anti-vimentin (clone V9) conjugated to Cy3, lectins VVA-FITC from *Vicia villosa* and WGA-RITC from *Triticum vulgaris* were obtained from Sigma (St Louis, MO). The BU-1 MAb was kindly provided by the Mayo Foundation (Rochester, MN).

Preparation of epithelium sheets, single domes and cryostat sections for immunofluorescence microscopy

Epithelium sheets from rabbit appendix, distal Peyer's and caecal patches were detached with EDTA as previously described (Lelouard et al., 1999). Briefly, tissues were incubated in dissociation buffer containing 200 mM sucrose, 40 mM EDTA and protease inhibitors. After 1 to 2 hours, epithelium sheets were released and fixed for 1 hour with 2% formaldehyde in 100 mM potassium phosphate buffer, pH 7.4. Epithelial sheets were further micro-dissected under a stereo-microscope (see Fig. 1). Sheets were permeabilized with 0.2% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.4, and labeled in a droplet of MAb followed by FITC- or RITC-coupled secondary antibodies. Double-labeling with two MAbs was performed by indirect labeling with the first MAb and FITC- or RITC-coupled secondary goat anti-mouse antibody, followed by incubations with an unrelated MAb, then 4,6-dichlorotriazinyl-aminofluorescein (DTAF)-coupled MAb 58 or Cy3-coupled anti-vimentin MAb. Lectins were used at 5 μg/ml concentration in the same buffer.

Whole domes were micro-dissected from fixed tissues, permeabilized and labeled as above. Sections (8 μm) were cut from fixed tissues in a Reichert 2700 cryostat permeabilized and labeled as above. Labeled epithelial sheets, domes and sections were mounted with Mowiol containing 2.5% 1.4-diazabicyclo[2.2.2]octane (DABCO). Samples were observed under a Leica TCS 4D confocal microscope (Wetzlar, FRG) equipped with an argon-krypton laser. Epithelial sheets, pictures of whole domes and cryostat sections were maximal projections of 5 to 60 focal planes, depending on the thickness of the sample. All experiments with Peyer's patches were performed on the two distal ones, which gave the most reproducible labeling with several markers (Lelouard et al., 2001).

Proliferative area detection by nucleus pulse labeling

20 ml of 5 mg/ml bromodeoxyuridine (BrdU) in sterile phosphate-buffered saline was administered intraperitoneally to rabbits. The animals were killed 1 hour later and the appendix, distal Peyer's and caecal patch epithelium were detached with EDTA as described above. The first epithelial sheets were obtained approximately 1 hour after the beginning of dissociation and were immediately fixed at room temperature with 2% formaldehyde in 100 mM phosphate buffer, pH 7.4, for 1 hour. Sheets were rinsed and permeabilized for 5 minutes in Tris-saline containing 0.05% Tween 20. Labeling was then performed with MAb BU-1, which is specific to BrdU (Gonchoroff et al., 1985; Gonchoroff et al., 1986).

RESULTS

We recently described a method to dissociate FAE from rabbit appendix using EDTA (Lelouard et al., 1999). The released epithelial sheets frequently displayed a ring shape, which was formed by one dome with its adjacent surrounding colonic epithelium (Fig. 1A). The top of the dome was often torn off upon shaking, thus producing the ring shape. Crypts associated with the dome and adjacent colonic epithelia were generally intact. It was, thus, possible to unfold the epithelial sheets by gently pulling with tweezers (Fig. 1B), and to further dissect them and isolate the dome from the colonic epithelium by tearing them apart (Fig. 1C). Most crypts remained connected to the dome. A single dome was usually surrounded by 40 to 50 crypts (Fig. 1C). These crypts were short, ranging from 45

Table 1. Summary of M cell and enterocyte markers used in this study

Lectin/MAb	Specificity	Appendix	Caecal patch	Peyer's patch	Refs
MAb 58	Mucin carbohydrate epitope	M	(–)	M	Lelouard et al., 1999; Lelouard et al., 2001
MAb 214	Mucin peptidic epitope	E	E	E	Lelouard et al., 1999; Lelouard et al., 2001
MAb V9	Vimentin	M	M	M	Gebert et al., 1992; Jepson et al., 1992
VVA	D-GalNac	(–)	M	(–)	Gebert and Hach, 1993
WGA	$(\beta(1\text{-}4)$D-GlcNac$)_2$	(–)	M	(–)	Gebert and Hach, 1993; Jepson et al., 1993a

M, M cell labeling; E, Enterocyte labeling.
(–), no labeling or unspecificity.
GalNac, N-acetylgalactosamine; GlcNac, N-acetylglucosamine; MAb, monoclonal antibody.

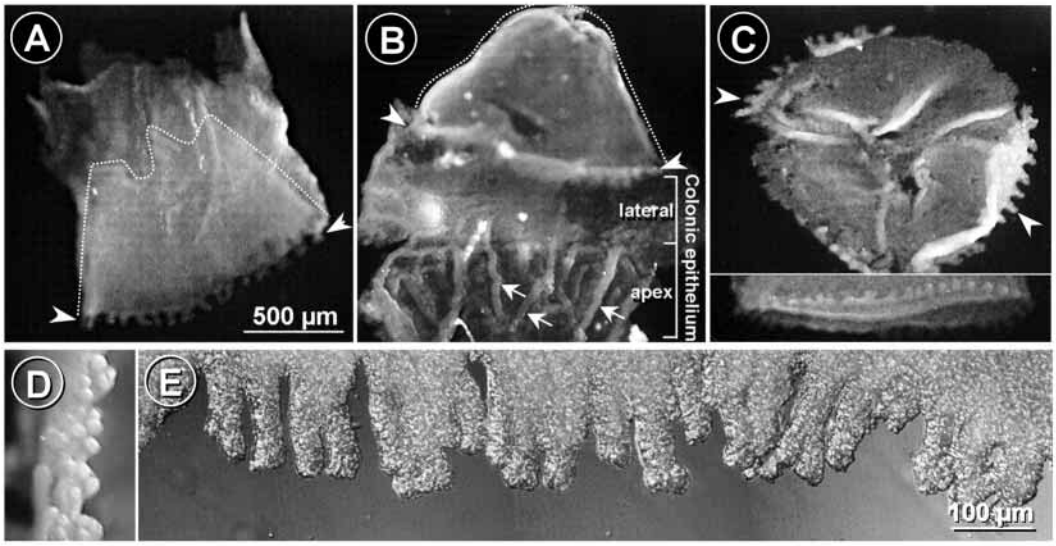

Fig. 1. EDTA treatment allows the visualization of whole rabbit dome epithelium and its associated crypts. Rabbit epithelium from appendix (A-C) and caecal patch (D,E) were isolated from the underlying tissue by EDTA before fixation according to the Materials and Methods. (A) Side view of an apex-less dome (broken line) seen through its associated surrounding colonic epithelium by trans-illumination; intact crypts are distributed regularly around the dome base (arrowheads). (B) Dome epithelium revealed by pulling down the surrounding colonic epithelium. Arrowheads mark each end of the crevice from which crypts supply, first, M cells and enterocytes to the dome above (broken line), and, second, goblet cells and enterocytes to the colonic epithelium (lower part of the figure, bracket). The colonic epithelium, normally lying in apposition to the dome, is clearly visible under the crypt row (arrowheads) down to the beginning of its apical surface, characterized by long tubular structures opening on the apex (arrows). (C) Tearing away the colonic epithelium left only the FAE with its associated crypts (arrowheads and inset). Note the central apex torn off during isolation treatment. (D) Bottom view (in bright field) of dissociated caecal patch crypts lined up in several rows. (E) DIC side view of caecal patch crypts, with some of them branched.

to 70 µm long, and often branched (see DIC pictures in Fig. 2). In caecal patch epithelium detached with EDTA, crypts were organized in several rows (Fig. 1D). They were grouped into three or four, sometimes branched (Fig. 1E), and much longer than in the appendix (170 to 230 µm). EDTA treatment of Peyer's patches released dome epithelial sheets whose crypts were very often torn off. This was possibly due to the fact that villi surrounding every dome were separated from each other, thus weakening the epithelium that broke during dissociation, contrary to other GALTs. However, a few rare domes with crypts were sometimes observed.

To enhance the reliability of recognition and selective discrimination of M cells and dome enterocytes, especially in crypts, we used a combination of several markers on the three different GALTs described above. These markers, labeled by MAbs or lectins, are summarized in Table 1. They gave complementary labeling of M cells and dome enterocytes (see below).

Precursors of M cells arose directly from proliferative cells in rabbit GALT crypts

The different epithelial lineages began their differentiation in the proliferative area of the villus crypts. It was therefore important to localize the first pre-M cells relative to this area in dome crypts. Proliferative cells were detected by in vivo 1 hour pulse labeling with BrdU, and M cells were identified by vimentin labeling (vim^+ cells), which is characteristic of M cells in rabbit intestinal epithelium (Gebert et al., 1992; Jepson et al., 1992). Labeling was performed in isolated permeabilized FAE. In the appendix, pulse labeling with BrdU indicated that the proliferative areas were located in crypts, but also at the crypt neck (Fig. 2A-D). The first vim^+ cells were also located in these areas (Fig. 2C,D), and some of them were co-labeled with BrdU (Fig. 2D, inset). Many epithelial cells weakly expressed cytoplasmic vimentin at the crypt neck. Vimentin-labeled cells rapidly increased in number to finally reflect the proportion of mature M cells present in the mid-part of the dome. Along the crypt-dome axis, vimentin expression in each cell increased and the intermediate filament structural arrangement was progressively organized, from the apico-basal perinuclear bundles in the crypt to the typical cage structure surrounding the lymphoid cell-containing pocket (Fig. 2E-G). In Peyer's patches, the first vim^+ cells were also found in the proliferative area of crypts, as illustrated in Fig. 2H, and co-labeling with BrdU was evident in several cells (Fig. 2H inset). In the caecal patch, the first vim^+ cells were also found at mid-crypt in spots labeled by BrdU (Fig. 2I). From mid-crypt upwards, vim^+ cells represented an important subpopulation of cells. At the neck of crypts, apical VVA co-labeling of vim^+ cells confirmed that they were M cells (not shown).

The relative distribution of M cells and dome enterocytes was already established in the crypts of rabbit GALTs

As pre-M cells arise directly from mitotic cells of the crypts, it appeared important to investigate whether the proportion of pre-M cells was similar to that of mature M cells in the dome flanks. The first approach involved serial cryostat cross-sectioning of intestinal tissue. Epithelium in crypts and villi from normal ileum was devoid of vimentin labeling (Fig. 3A). On the dome side of crypts from Peyer's patches, starting from the proliferative BrdU-labeled area (Fig. 3B), numerous regularly spaced vim^+ cells were detected in a proportion similar to that observed for M cells on the dome (compare Fig. 3C with 3D). MAb 58, the only known apical marker of M cells in rabbit appendix and Peyer's patch (Table 1), confirmed that these cells were M cells (vim^+58^+ cells) (Fig. 3C,D). A few vim^+58^+ cells were also detected on the villus side of crypts (Fig. 3C) and on the adjacent villus (Fig. 3D), as in the appendix and caecal patches (not shown). This indicated that

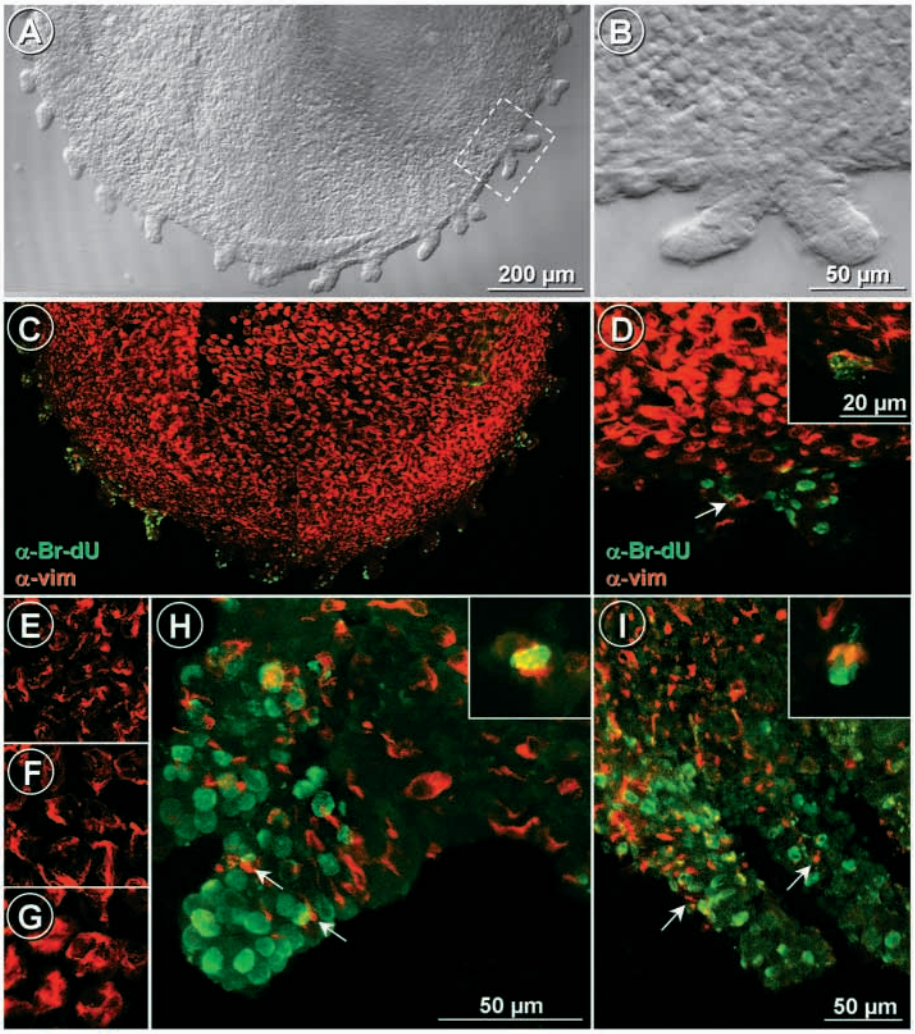

Fig. 2. Vimentin-positive immature M cells appear in the area of rapidly proliferative cells of crypts in three rabbit GALTs. After 1 hour in vivo incorporation of BrdU, FAE was dissociated, fixed, permeabilized and labeled with anti-vimentin (red) and anti BrdU (green) antibodies. (A) Half dome epithelium from rabbit appendix revealed with DIC; (B) fluorescent labeling of the corresponding field. BrdU labeling is confined to crypts and the neck of crypts. (C,D) Enlarged fields from the delimited area in A, showing vimentin labeling starting around BrdU-labeled cells in crypts (arrow). (E-G) Vimentin-labeled cytoskeleton in the three GALTs studied is progressively organized along the crypt-dome axis starting from fine apico-basal filaments in the crypts (E), here seen from the top of cells, to pockets in dome flanks (G). Isolated FAE from Peyer's patch (H) and caecal patch (I) shows the first vimentin-expressing cells around BrdU-incorporating cells of crypts (arrows). Insets in D,H,I show vimentin-positive cells co-labeled with BrdU.

even M cells recently reported on villi facing the dome (Borghesi et al., 1999) arise, already pre-differentiated, from dome crypts.

We looked at the relative distribution of M cells and enterocytes to determine whether it was the same at the base of the dome and at mid-height, where lymphoid cells penetrate the epithelium and M cells are known to be functional. We performed double-labeling on micro-dissected domes with MAb 58 and MAb 214, the only markers specific to the apex of dome enterocytes in all rabbit FAE (see Table 1). At the crypt neck of Peyer's patches, round 214^+ dome enterocytes and 58^+ M cells were already labeled and never co-localized (Fig. 4A). The spatial organization of M cells and enterocytes at the base and on the lateral side of the dome was similar, three to four M cells surrounding each enterocyte (Fig. 4A,B). M cells of Peyer's patches are known to have varying apical surfaces (Jepson et al., 1993b). We looked for correlations between the apical surface of M cells and the size of vimentin-delimited pockets. Large vimentin-outlined pockets corresponded to large apical M cell surfaces, whereas small pockets corresponded to small apical M cell surfaces (Fig. 4C). Staining of M cells by MAb 58 even revealed the existence of such small apical surfaces (Fig. 4B), which were undistinguishable by scanning electron microscopy (not shown).

In appendix, M cells and enterocytes were distributed in a very regular mosaic pattern from the base to a short distance from the top of the dome, with every M cell surrounded regularly by 5 to 7 dome enterocytes (Fig. 4D). In caecal patches, double-labeling of enterocytes with MAb 214 and M cells with WGA revealed that the spatial arrangement between M cells and enterocytes was already established at the crypt neck (not shown) as in the appendix and Peyer's patches.

DISCUSSION

The data presented in this study indicate that, in rabbit FAE, M cells and dome enterocytes represent distinct pre-programmed lineages already separated before the neck of crypts. The two lineages would be derived from common stem cells, as are epithelial cells in small intestine (Cheng and Leblond, 1974). This is substantiated by the following findings:

(1) a vimentin filamentous cytoplasmic network already appeared in the zone of crypts where rapidly dividing cells incorporate BrdU, in the three GALTs studied;

(2) the vimentin expression pattern was approximately one out of two cells in GALT crypts, in continuity with what is observed in the dome flanks, whereas it was absent in crypts of the small intestine;

(3) the mosaic-like relative distribution of M cells and dome enterocytes at the neck of crypts was similar to that observed on the dome side.

These findings were achieved using a newly designed method for labeling whole FAE epithelium dissociated with

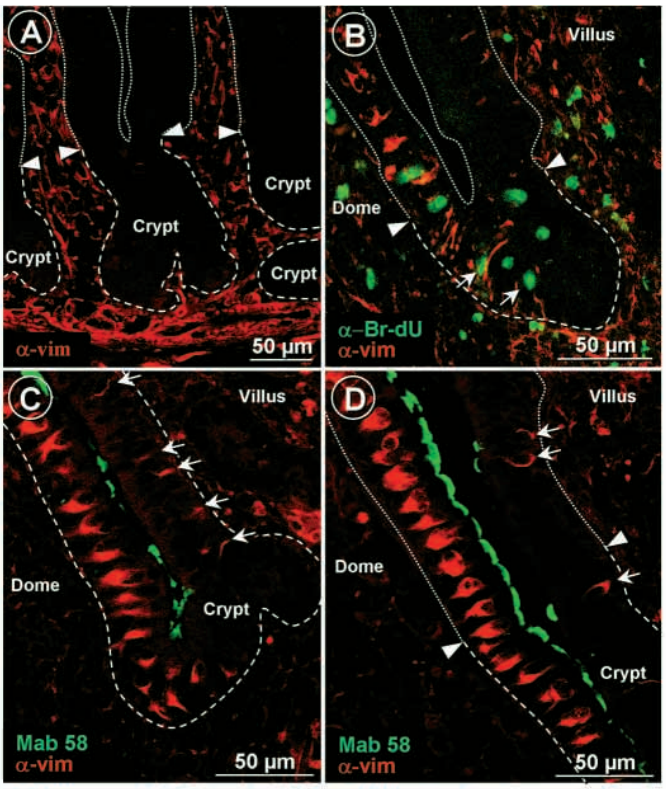

Fig. 3. M cell distribution is already regular in crypts of rabbit Peyer's patches. Cryostat serial cross-sections of crypts in normal ileum villi (A) and the dome region (B-D) were permeabilized and double-labeled for M cells with anti-vimentin (A-D, red) and MAb 58 (C,D, green) or for dividing cells with anti-BrdU (B, green). Arrowheads mark the junction between crypts (delineated by discontinuous lines) and domes or villi (dotted lines). In normal ileum (A), vimentin labels interstitial tissue but is absent from the overall epithelium, including crypts. In FAE (B-D), vimentin labeling surrounds M cell nuclei on the dome side of crypts (B,C) and above the crypt neck (D). In crypts, starting from BrdU-positive cells (arrows in B), vimentin-labeled cells are regularly spaced (B-D). Apical surface of vimentin-positive cells are also labeled with MAb 58 (C), as in the dome flanks (D). A few cells are co-labeled with anti-vimentin and MAb 58 on the villus side (arrows), both in the crypts (C) and above (D).

EDTA (Lelouard et al., 1999) combined with labeling of entire micro-dissected domes and cryostat sections. This allowed us to visualize markers deep in the crypts, down to the zone of rapidly dividing cells pulse labeled with BrdU, and extensively revealed the relative distribution of M cells and enterocytes from the crypts in three different GALTs.

Detection results depend upon the selectivity of the markers used. Vimentin has been widely used as an excellent marker for rabbit M cells on dome flanks (Gebert et al., 1992; Jepson et al., 1992). In the crypts, vim^+ cells were not enterocyte precursors, as vimentin labeling was absent in the crypts of small intestine villi. The other M cell markers used included the glycosidic epitopes recognized by MAb 58 (Lelouard et al., 1999) and lectin receptors (Gebert and Hach, 1993; Jepson et al., 1993a), depending on the GALT under study. In the FAE, they clearly labeled M cells and displayed a pattern complementary to MAb 214-labeled enterocytes (Lelouard et al., 1999; this study). They always co-labeled vim^+ cells as soon as their expression began in the crypt.

Crypts are known to be the region where stem cells divide and segregation of cell lineages takes place in the small intestine (Cheng and Leblond, 1974; Gordon, 1989; Potten and Loeffler, 1990). Previous studies have favored a separate cell lineage for M cells, different from that of enterocytes, originating from crypts and followed by a progressive transition from immature to mature M cells (Bye et al., 1984; Gebert et al., 1999; Gebert et al., 1992; Gebert and Posselt, 1997; Giannasca et al., 1994; Sicinski et al., 1986). The

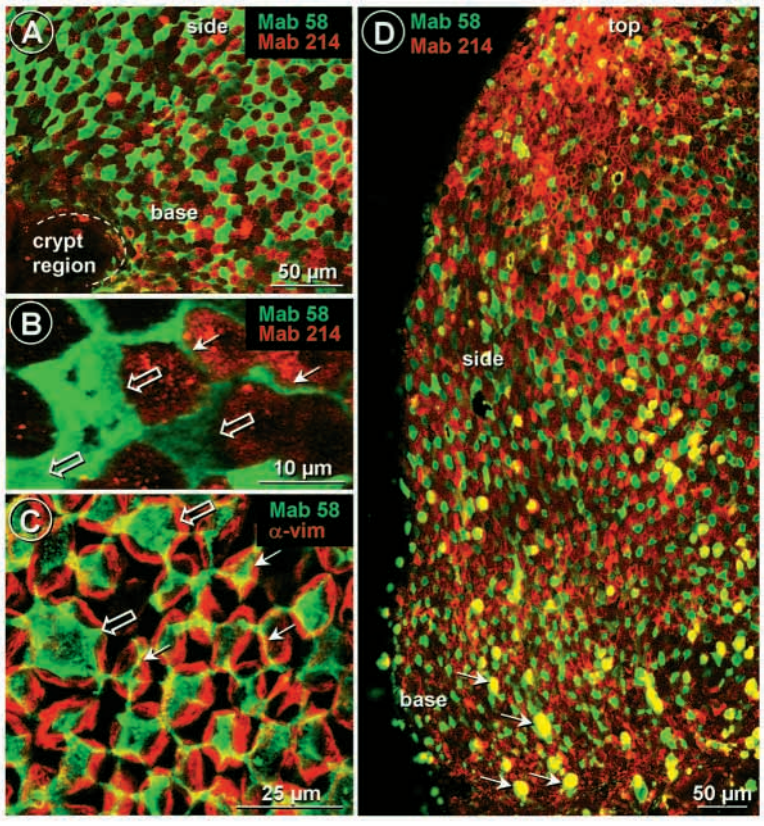

Fig. 4. The spatial distribution of M cells and dome enterocytes is already established at the base of domes from rabbit appendix and Peyer's patch. Micro-dissected domes from Peyer's patch (A-C) and appendix (D) were permeabilized and co-labeled with MAb 58 (green) and MAb 214 (red in A, B, D) or anti-vimentin (red in C). (A) The regular mosaic distribution pattern of M cells and enterocytes, already established at the very base of the dome around a crypt region. (B) A higher magnification reveals marked variations in M cell apical surfaces (thin and open arrows). (C) is the maximal projection of 60 focal planes to localize both the apical surface of M cells and the lateral corresponding pocket: note that M cells with large apical surfaces (open arrows) correspond to large vimentin-delimited pockets, while M cells with compressed apical surfaces (thin arrows) correspond to small pockets. (D) The relative distribution of M cells and enterocytes in appendix reveals a very regular mosaic pattern from the base to the upper part of the dome. A few goblet cells were double labeled in yellow at the lower part of the domes (arrows).

presence of M cell markers that outline radial strips at the base of domes starting from the mouth of crypts suggests a clonal origin for M cells (Clark et al., 1993; Gebert et al., 1999; Gebert and Posselt, 1997). Stronger evidence of early committed M cells is the presence of immature M cells in the upper part of crypts, as recognized by their morphology in electron microscopy and their binding to UEA1 lectin (Gebert et al., 1999). In this paper, we monitored M cell precursors deeper in crypts, down to the zone of rapidly dividing cells, where vimentin-labeled pre-M cells already represent about half the population, as in the flanks of dome where mature M cells lie. The presence of pre-M cells in the dividing zone is crucial and favors the hypothesis of an early commitment of M cell lineage, before terminal differentiation occurs in the upper crypt.

It has been proposed that M cells are derived from differentiated dome enterocytes via an induction step, owing to the influence of lymphocyte penetration into the epithelial monolayer (Kerneis et al., 1997; Savidge, 1996) and/or by an interaction with bacteria (Meynell et al., 1999), and that M cells would revert back to enterocyte phenotype at the top of domes (Sierro et al., 2000). Our results are not in line with such a conversion. The model of conversion of Caco2 cells into M cells (Kerneis et al., 1997) represents the only in vitro model currently available for studying the biology of M cells and interactions between epithelial cells, bacteria (Schulte et al., 2000) and lymphoid cells. The fact that the Caco2 cell line can acquire an M cell phenotype by interaction with lymphoid cells from mouse Peyer's patches does not necessarily mean that fully differentiated enterocytes do the same in vivo. It is known that Caco-2 cells retain crypt cell properties (Grasset et al., 1985; Grasset et al., 1984), and display multipotent phenotypes (Engle et al., 1998), which could indicate that this cell line might rather behave as intestinal crypt cells that could still differentiate, depending on the local environment. The fact that Kerneis et al. (Kerneis et al., 1997) succeeded in inducing Peyer's patch formation in vivo, by injecting lymphoid follicular cells in mouse duodenum, reveals the crucial role of the local lymphoid environment in the early commitment of progenitor epithelial cells and GALT formation.

Our data highlight a striking and unchanged spatial organization of M cells and dome enterocytes along the crypt-dome axis, with a constant ratio between the two populations in three different GALTs. This again is not compatible with a conversion of enterocytes into M cells along the dome flanks, which should lead to a concomitant increase in M cells and a decrease in enterocyte population. Meynell et al. (Meynell et al., 1999) have reported a rapid increase in M cells that are able to transport microspheres across the FAE of rabbit Peyer's patches after exposure to *Streptococcus pneumonia*. They hypothesize that this increase corresponds to enterocyte conversion. However, when sampling microspheres after bacterial stimulation, they observed less than 25 M cells/mm^2, which is very few compared with the total pool of M cells. We alternatively propose that in rabbit Peyer's patches, many M cells may be inaccessible to bacterial-sized antigen or microspheres, owing to their small surface area, which is almost completely covered by adjacent enterocyte microvilli (see Fig. 4B). Consistent with this idea is the fact that M cells that bind microspheres display apical surfaces of more than 100 μm^2 (Meynell et al., 1999). After antigenic stimulation, the surface area of M cells should increase after recruitment of lymphoid cells in their pocket. The recruitment of lymphoid cells has actually been shown by injection of non-invasive *Shigella flexneri* (Sansonetti et al., 1996) or *Streptococcus pneumonia* (Borghesi et al., 1996) in rabbit Peyer's patches, and we observed that large apical M cell surfaces seemed to be associated with large pockets. Enlarged apical surfaces should allow sampling of bacterial-sized antigens or microspheres (Meynell et al., 1999). Our data favor this interpretation rather than the alternative proposed expansion of M cells by conversion from fully differentiated enterocytes upon contact with micro-organisms (Meynell et al., 1999; Savidge et al., 1991). The fact that many small M cell apical surfaces were only visible by immunostaining, shows the difficulty of evaluating M cell numbers in rabbit Peyer's patches by scanning electron microscopic analysis. Moreover, the shape change of M cells and the increase of both their apical surface and their pocket upon lymphocyte recruitment might enhance the chance of detecting M cells on electron microscope sections. This could lead to an apparent increase in the number of M cell profiles in the presence of pathogens (Borghesi et al., 1999). Our data and those of Sansonetti et al. (Sansonetti et al., 1996) indicate that future studies should focus on the size, shape and relative amount of M cells and enterocytes after bacterial challenge.

In a recent paper, Sierro et al. (Sierro et al., 2000) observed in mouse that apoptosis was not present in the zone of M cell disappearance, whereas apoptotic enterocytes were numerous at the very top of domes. They suggested a reverse differentiation of M cells to enterocyte phenotype before entering into apoptosis. This was based on the fact that the cell migration rate of BrdU-labeled cells does not slow down in the zone of disappearance of M cells, as expected if release diminished the number of cells. However, M cells represent only 10% of the cell population in mouse (Clark et al., 1993), and extrusion or not of such a low number of M cells would probably not affect the cell migration rate. The situation is different in rabbit FAE: contrary to mouse FAE and rabbit adjacent villi, apoptosis was absent in both the area of the sharp disappearance of M cells and at the top of domes (our unpublished results). Hence, if apoptosis in rabbit could not explain the disappearance of M cells, it could also not explain that of dome enterocytes. A possible explanation is that epithelial cells could be rapidly sloughed off from the dome by anoikis in the early stage of apoptosis (or even before) in the area where the M cell phenotype disappears and also at the tip of domes. This hypothesis is supported by the presence of vimentin-labeled luminal debris and M cell desquamation in this region (Owen and Piazza, 1998).

In conclusion, the presence of M cell precursors has been documented for several years (Bye et al., 1984; Gebert et al., 1999; Giannasca et al., 1994). In addition, we show that M cell precursors can already be detected in the zone of rapidly dividing cells, and that the spatial distribution of M cells and dome enterocytes in crypts reflects that observed in the domes flank. The mosaic organization of M cells and enterocytes, already present in the zone of rapidly dividing cells, suggests that interactions between neighboring cells might involve lateral specification events as in Notch signaling (Artavanis-Tsakonas et al., 1999), which may be important to investigate in the future. All this provides strong evidence in favor of an

early commitment of M cell and enterocyte distinct lineages. This could very well be induced by the influence of lymphoid cells (Kerneis et al., 1997) during the first stem cell division steps.

We thank Dr P. Mangeat, Dr M. Neutra, Dr S. Baghdiguian and Dr A. M. Martinez for helpful discussions. We gratefully acknowledge F. Mérezègue for excellent technical help. This work was supported by the Centre National de la Recherche Scientifique (UMR 5539, and Cell Biology project 96033), the Association pour la Recherche sur le Cancer (contract 9829), and the Ligue Nationale contre le Cancer. H.L. was a fellow of the Délégation à la Recherche et au Développement and the Association pour la Recherche sur le Cancer.

REFERENCES

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-776.

Bjerknes, M. and Cheng, H. (1999). Clonal analysis of mouse intestinal epithelial progenitors. *Gastroenterology* **116**, 7-14.

Borghesi, C., Regoli, M., Bertelli, E. and Nicoletti, C. (1996). Modifications of the follicle-associated epithelium by short-term exposure to a non-intestinal bacterium. *J. Pathol.* **180**, 326-332.

Borghesi, C., Taussig, M. J. and Nicoletti, C. (1999). Rapid appearance of M cells after microbial challenge is restricted at the periphery of the follicle-associated epithelium of Peyer's patch. *Lab. Invest.* **79**, 1393-1401.

Bye, W. A., Allan, C. H. and Trier, J. S. (1984). Structure, distribution, and origin of M cells in Peyer's patches of mouse ileum. *Gastroenterology* **86**, 789-801.

Cheng, H. and Leblond, C. P. (1974). Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am. J. Anat.* **141**, 461-479.

Clark, M. A., Jepson, M. A., Simmons, N. L., Booth, T. A. and Hirst, B. H. (1993). Differential expression of lectin-binding sites defines mouse intestinal M-cells. *J. Histochem. Cytochem.* **41**, 1679-1687.

Engle, M. J., Goetz, G. S. and Alpers, D. H. (1998). Caco-2 cells express a combination of colonocyte and enterocyte phenotypes. *J. Cell. Physiol.* **174**, 362-369.

Gebert, A., Hach, G. and Bartels, H. (1992). Co-localization of vimentin and cytokeratins in M-cells of rabbit gut-associated lymphoid tissue (GALT). *Cell Tissue Res.* **269**, 331-340.

Gebert, A. and Hach, G. (1993). Differential binding of lectins to M cells and enterocytes in the rabbit cecum. *Gastroenterology* **105**, 1350-1361.

Gebert, A. and Posselt, W. (1997). Glycoconjugate expression defines the origin and differentiation pathway of intestinal M cells. *J. Histochem. Cytochem.* **45**, 1341-1350.

Gebert, A., Fassbender, S., Werner, K. and Weissferdt, A. (1999). The development of M cells in Peyer's patches is restricted to specialized dome-associated crypts. *Am. J. Pathol.* **154**, 1573-1582.

Giannasca, P. J., Giannasca, K. T., Falk, P., Gordon, J. I. and Neutra, M. R. (1994). Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. *Am. J. Physiol.* **267**, G1108-G1121.

Gonchoroff, N. J., Greipp, P. R., Kyle, R. A. and Katzmann, J. A. (1985). A monoclonal antibody reactive with 5-bromo-2-deoxyuridine that does not require DNA denaturation. *Cytometry* **6**, 506-512.

Gonchoroff, N. J., Katzmann, J. A., Currie, R. M., Evans, E. L., Houck, D. W., Kline, B. C., Greipp, P. R. and Loken, M. R. (1986). S-phase detection with an antibody to bromodeoxyuridine. Role of DNase pretreatment. *J. Immunol. Methods* **93**, 97-101.

Gordon, J. I. (1989). Intestinal epithelial differentiation: new insights from chimeric and transgenic mice. *J. Cell. Biol.* **108**, 1187-1194.

Grasset, E., Pinto, M., Dussaulx, E., Zweibaum, A. and Desjeux, J. F. (1984). Epithelial properties of human colonic carcinoma cell line Caco-2: electrical parameters. *Am. J. Physiol.* **247**, C260-C267.

Grasset, E., Bernabeu, J. and Pinto, M. (1985). Epithelial properties of human colonic carcinoma cell line Caco-2: effect of secretagogues. *Am. J. Physiol.* **248**, C410-C418.

Jepson, M. A., Mason, C. M., Bennett, M. K., Simmons, N. L. and Hirst, B. H. (1992). Co-expression of vimentin and cytokeratins in M cells of rabbit intestinal lymphoid follicle-associated epithelium. *Histochem. J.* **24**, 33-39.

Jepson, M. A., Clark, M., Simmons, N. L. and Hirst, B. H. (1993a). Epithelial M cells in the rabbit caecal lymphoid patch display distinctive surface characteristics. *Histochemistry* **100**, 441-447.

Jepson, M. A., Simmons, N. L., Hirst, G. L. and Hirst, B. H. (1993b). Identification of M cells and their distribution in rabbit intestinal Peyer's patches and appendix. *Cell Tissue Res.* **273**, 127-136.

Kerneis, S., Bogdanova, A., Kraehenbuhl, J. P. and Pringault, E. (1997). Conversion by Peyer's patches lymphocytes of human enterocytes into M cells that transport bacteria. *Science* **277**, 949-952.

Lelouard, H., Reggio, H., Mangeat, P., Neutra, M. and Montcourrier, P. (1999). Mucin-related epitopes distinguish M cells and enterocytes in rabbit appendix and Peyer's patches. *Infect. Immun.* **67**, 357-367.

Lelouard, H., Reggio, H., Roy, C., Sahuquet, A., Mangeat, P. and Montcourrier, P. (2001). Glycocalyx on rabbit intestinal M cells displays carbohydrate epitopes from MUC2. *Infect. Immun.* **69**, 1061-1071.

Maury, J., Nicoletti, C., Guzzo-Chambraud, L. and Maroux, S. (1995). The filamentous brush border glycocalyx, a mucin-like marker of enterocyte hyper-polarization. *Eur. J. Biochem.* **228**, 323-331.

Meynell, H. M., Thomas, N. W., James, P. S., Holland, J., Taussig, M. J. and Nicoletti, C. (1999). Up-regulation of microsphere transport across the follicle-associated epithelium of Peyer's patch by exposure to Streptococcus pneumoniae R36a. *FASEB J.* **13**, 611-619.

Neutra, M. R., Pringault, E. and Kraehenbuhl, J. P. (1996). Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* **14**, 275-300.

Niedergang, F. and Kraehenbuhl, J. P. (2000). Much ado about M cells. *Trends Cell Biol.* **10**, 137-141.

Owen, R. L. and Piazza, A. J. (1998). Where do M cells go ? Identification of their desquamation sites in rabbit Peyer's patches and appendix by immunohistochemical labeling of M cell cytoplasmic vimentin. *Gastroenterology* **114**, A1057.

Pappo, J. (1989). Generation and characterization of monoclonal antibodies recognizing follicle epithelial M cells in rabbit gut-associated lymphoid tissues. *Cell. Immunol.* **120**, 31-41.

Pappo, J., Steger, H. J. and Owen, R. L. (1988). Differential adherence of epithelium overlying gut-associated lymphoid tissue. An ultrastructural study. *Lab. Invest.* **58**, 692-697.

Potten, C. S. and Loeffler, M. (1990). Stem cells: attributes, cycles, spirals, pitfalls and uncertainties. Lessons for and from the crypt. *Development* **110**, 1001-1020.

Sansonetti, P. J., Arondel, J., Cantey, J. R., Prevost, M. C. and Huerre, M. (1996). Infection of rabbit Peyer's patches by *Shigella flexneri*: effect of adhesive or invasive bacterial phenotypes on follicle- associated epithelium. *Infect. Immun.* **64**, 2752-2764.

Savidge, T. C. (1996). The life and times of an intestinal M cell. *Trends Microbiol.* **4**, 301-306.

Savidge, T. C., Smith, M. W., James, P. S. and Aldred, P. (1991). Salmonella-induced M-cell formation in germ-free mouse Peyer's patch tissue. *Am. J. Pathol.* **139**, 177-184.

Schulte, R., Kerneis, S., Klinke, S., Bartels, H., Preger, S., Kraehenbuhl, J. P., Pringault, E. and Autenrieth, I. B. (2000). Translocation of *Yersinia enterocolitica* across reconstituted intestinal epithelial monolayers is triggered by *Yersinia* invasin binding to β1 integrins apically expressed on M-like cells. *Cell. Microbiol.* **2**, 173-185.

Sicinski, P., Rowinski, J., Warchol, J. B. and Bem, W. (1986). Morphometric evidence against lymphocyte-induced differentiation of M cells from absorptive cells in mouse Peyer's patches. *Gastroenterology* **90**, 609-616.

Sierro, F., Pringault, E., Simon-Assman, P., Kraehenbuhl, J. P. and Debard, N. (2000). Transient expression of M-cell phenotype by enterocyte-like cells of the follicle-associated epithelium of mouse Peyer's patches. *Gastroenterology* **119**, 734-743.

Smith, M. W. and Peacock, M. A. (1980). "M" cell distribution in follicle-associated epithelium of mouse Peyer's patch. *Am. J. Anat.* **159**, 167-175.