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REGULAR ARTICLE

Identification of intestinal M cells in isolated lymphoid follicles and Peyer's patches of the Angora rabbit

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Abstract The presence, distribution, and localization of M cells in isolated lymphoid follicles (ILF) and in follicle-associated epithelia (FAE) covering Peyer's patches (PP) in Angora rabbits were investigated by immunohistochemistry and electron microscopy. Although PP could macroscopically be identified along the length of the mucosal and serosal surfaces of jejunum and ileum, the presence of ILF could only be located microscopically. Typical M cells in FAE were detected within the periphery of the dome regions of the PP, and immature columnar M cells in the FAE resided in the vicinity of the crypts. M cells in the FAE of both ILF and PP showed vimentin-positive reaction. M cells in the FAE of ILF were morphologically similar to the immature M cells found in the FAE of PP. Typical mature M cells were also observed in the FAE of a few ILF. In contrast to FAE of PP, numerous goblet cells were observed in the FAE of many ILF. Moreover, among intestinal villi, we noticed villi-like solitary lymphoid structures that showed abundant lymphocytes in their lamina propria and that were surrounded with vimentin-positive cells and goblet cells. Thus, the occurrence of copious immature M cells and goblet cells, in addition to the detection of villi-like solitary lymphoid structures full of lymphocytes in the FAE of many ILF, indicate that ILF do not complete their immunological maturation in contrast to PP. Various antigenic stimulations conceivably induce the formation and maturation of ILF along the length of the small intestine. The morphological resemblance between ILF M cells and PP M cells suggests that these two types of cells perform similar or the same immunological functions.

Keywords Intestine · Isolated lymphoid follicles · M cells · Peyer's patches · Vimentin · Rabbit (Angora)

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Introduction

The main function of the mucosa-associated lymphoid tissue (MALT) is to generate specific immune responses or tolerance against antigens invading mucosal surfaces (Liebler-Tenorio and Pabst 2006; Mowat 2003). Such tissue is more precisely named in relation to the sites in which it is found, e.g., gut-associated lymphoid tissue (GALT), bronchus-associated lymphoid tissue (BALT), nasal-associated lymphoid tissue, and conjunctiva-associated lymphoid tissue (Brandtzaeg et al. 2008; Cesta 2006).

The intestines are exposed to numerous potential pathogens, food-borne antigens, and commensal micro-organisms throughout the life of an animal (Cesta 2006). GALT, which develops immune response or tolerance against pathogens invading mucosa, is functionally divided

into inductive and effecter sites. The inductive sites consist of Peyer's patches (PP) and isolated lymphoid follicles (ILF), which ensure the secretion of IgA and the production of clonal B cells, whereas the effecter sites comprise intestinal epithelia and lamina propria containing plentiful lymphocytes (Mowat 2003; Newberry 2008). In addition to PP and ILF, the gastrointestinal tract of the rabbit also possesses the sacculus rotundus composed of aggregated lymph follicles, appendix, and cecal patches (Haley 2003). Furthermore, some mammals have been shown to have novel lymphoid entities, e.g., lymphocyte-filled villi (LFV; Hitotsumatsu et al. 2005; Mayrhofer and Brooks 1995; Mayrhofer et al. 1999; Moghaddami et al. 1998) and cryptopatches (CP; Kanamori et al. 1996).

PP are collections of many lymphoid follicles that are closely packed together. They reside in the lamina propria layer, extend into the submucosa of the small intestine and form oblong elevations on the mucous membrane and are also called aggregate glands or agminate glands (Cesta 2006; Newberry and Lorenz 2005). They are easily observable macroscopically through the mucosal and serous surfaces on the antimesenteric wall of the small intestine (Newberry 2008). PP are covered with follicle-associated epithelia (FAE) and specialized dome epithelia (Magalhaes et al. 2007; Neutra 1998). Because of the presence of the membranous epithelial cell (M cell or microfold cell) in FAE, they are strikingly different from the epithelia of the villi (Brayden et al. 2005; Corr et al. 2008). M-cells play an important role in the transport of antigens from the lumen of the small intestine to mucosal lymphoid tissues in which the processing and initiation of immune responses occur (Gebert et al. 2004; Hathaway and Kraehenbuhl 2000; Kucharzik et al. 2000). Even though no universal marker is available for the identification of M cells, *Ulex europaeus* agglutinin-1 (UEA-1) lectin in mice (Clark et al. 1993), cytokeratin 8 in rats (Rautenberg et al. 1996), and cytokeratin 18 (Gebert et al. 1994) and vimentin intermediate filament in rabbits (Gebert et al. 1992; Jepson et al. 1992, 1993) are used for studying these specific cells.

ILF consist of a solitary follicle (Hamada et al. 2002) and resemble PP by having M cells containing FAE (Pabst et al. 2005). However, in contrast to PP, ILF do not possess T cell areas (Glaysher and Mabbott 2006; Wang et al. 2006) and have been described only in the intestines of mice (Hamada et al. 2002), guinea pig (Rosner and Keren 1984), and human (Moghaddami et al. 1998). Few studies exist concerning the presence and structure of ILF in the intestine of the rabbit (Iwatsuki et al. 2002; Keren et al. 1978). In addition, ILF are thought function as the inductive sites for mounting the immune response (Lorenz and Newberry 2004; Pabst et al. 2005).

The structure of GALT is more highly developed in rabbits than in other mammalian species (Cesta 2006). Furthermore, the number of M cells in PP in the rabbit is greater than that in other mammals (Haley 2003). The information that is available on ILF in rabbits is however limited. To the authors' knowledge, no previous study exists on the structure of M cells in the FAE of PP and ILF in the Angora rabbit, a breed endemic to Turkey. In this study, we have determined the presence, distribution, localization, and differences of M cells in ILF and in FAE covering PP in Angora rabbits by immunohistochemistry and electron microscopy.

Materials and methods

Ten healthy adult Angora rabbits, obtained from the Research Farm of the Faculty of Veterinary Medicine at Erciyes University, were provided with standard laboratory feed and fresh water and housed at a temperature of 23±2°C. They were killed by an intravenous injection of 0.1 ml/kg sodium pentobarbital. After the abdominal cavities of the animals had been opened, samples of the jejunal and ileal parts of the gut, with PP and without PP, were removed, and their intestinal contents were washed out with physiological saline. The jejunal and ileal tissues were fixed in Bouin's solution for 2 h, treated with a series of graded alcohol, methyl benzoate, and benzene, and then embedded in paraffin. Sections (5 μm) were prepared from the blocks and were stained with Mallory's triple staining in order to investigate the general composition of PP and ILF (Crossmon 1937).

For vimentin immunoreactivity, sections were placed on poly-L-lysine-coated slides and immunohistochemically stained with the avidin-biotin-peroxidase complex (ABC) method. The sections were incubated in 3% hydrogen peroxide in methanol for 20 min to eliminate endogenous peroxide and then heated at 96°C in citrate buffer (pH 6.0) for 20 min to restore antigenic properties. The sections, which were left to cool at room temperature for 20 min, were washed in phosphate-buffered saline (PBS, pH 7.4) and treated with 10% goat serum (Thermo Scientific, USA) for 5 min to prevent non-specific binding. Subsequently, the sections were incubated with a 1:200 dilution of monoclonal mouse anti-vimentin (Clone; V9; DAKO) primary antibodies at room temperature for 1 h. Following incubation, the sections were treated with anti-mouse secondary antibodies conjugated with biotin (Thermo Scientific) for 20 min and with avidin-peroxidase solution (Thermo Scientific) for 20 min. In order to visualize the antigen-antibody reaction, the chromogen 3-amino 9-ethyl carbazole (AEC; Thermo Scientific) was applied for 5–10 min, followed by Gill's hematoxylin for background staining. All washing steps were performed with PBS, and all staining procedures were performed in a humid chamber (Shandon, UK). The assessment of immunohistochemical

staining was performed by using positive and negative controls. Rabbit kidney sections were used as positive controls for anti-vimentin primary antibodies. Goat serum replaced primary antibodies as the negative control, and staining procedures were carried out as usual.

Sections for electron-microscopic studies were fixed in glutaraldehyde-paraformaldehyde fixative at pH 7.4 (Karnovsky 1965). After the first fixation, the sections were fixed in 1% osmic acid for 2 h and were left in uranyl acetate for an additional 2 h. Subsequently, the sections were treated with graded alcohols and propylene oxide and blocked in Araldite M. Semithin sections (1 μm) were cut from the blocks and were stained with toluidine blue. After the desired regions on the semithin sections has been marked, ultrathin sections were cut at a thickness of 300–400 Å and contrasted according to Veneable and Coggeshall (1965). Finally, the sections were examined under a Carl Zeiss EM 9S2 electron microscope (Carl Zeiss, Oberkochen, Germany).

The animals used in this study were killed with the approval of the Ethics Board of Erciyes University Faculty of Veterinary Medicine (date of meeting: 09.04.2003; decision no. 25).

Results

We macroscopically observed 5 to 6 PP on both mucosal and serosal surfaces of the jejunum and ileum; however, we microscopically noticed a number of ILF in these organs. We examined PP and ILF by light and electron microscopy.

Light-microscopic findings

Peyer's patches

We noted that PP were localized on the antimesenteric borders of the jejunum and ileum and were composed of numerous aggregated lymph follicles. The luminal surfaces of the aggregated lymph follicles were lined with a FAE, and each follicle contained a subepithelial dome region with a centrally located, lightly stained, germinal center, a coronal area separating these two sites, and a wide interfollicular area between neighboring follicles (Fig. 1a). Abundant intraepithelial lymphocytes (IEL) lay among the epithelial cell of the FAE, but strikingly, no goblet cells were noted. The M cells located in the FAE at the periphery of dome region were characterized as having a thin membrane-like apical cytoplasm and basolateral invaginations encircling IEL beneath them. These cells were basally located, possessed a euchromatic nucleus, and were easily distinguishable from the adjacent enterocytes because of their lightly staining aspect (Fig. 1b).

Moreover, we observed abundant vimentin-immunoreactive cells in the FAE surrounding the dome regions of jejunal and ileal PP. In some dome regions, vimentin-positive cells were present throughout the FAE or in the FAE covering the apex of the dome region and lined up only with the enterocytes (Fig. 1c). We determined the presence of two different vimentin-positive cells in FAE. One type was located in an intraepithelial pocket and enclosed IEL in FAE around the dome region (Fig. 1d–f), whereas the other was columnar in appearance and neighbored one or two IEL found in FAE of nearby crypts and was not located in an intraepithelial pocket (Fig. 1g, h). Vimentin immunoreactivity in the FAE around the dome region was localized to perinuclear areas and to the cytoplasm encircling IEL (Fig. 1e, f). Furthermore, we did not detect marked vimentin immunoreactivity within the apical and basal cytoplasm of these cells. The cells with a thin apical cytoplasm and basolateral invaginations encircling IEL were typical M cells (Fig. 1e, f). By contrast, immature M cells were columnar and located in FAE near the crypts of dome regions, showed immunoreactivity only within the perinuclear cytoplasm, and did not surround IEL with an intraepithelial invagination (Fig. 1g, h). Moreover, the staining properties of vimentin-positive cells did not differ between the FAE of the jejunal PP and ileal PP.

Isolated lymphoid follicles

When we examined the sections obtained from the parts of the jejunum and ileum containing no PP, we observed many solitary ILF located between intestinal villi on the antimesenteric border. The dome regions of ILF were surrounded with FAE. However, no separation was seen between the germinal center, corona, and interfollicular areas. In contrast to PP, we noticed numerous goblet cells in the FAE of many ILF (Fig. 2a). However, a small number of ILF lacked goblet cells. In semi-thin sections prepared from ILF, which lacked goblet cells, we found typical M cells with thin membrane-like apical cytoplasm, surrounding the IEL beneath them with their basolateral invaginations; they were also located in FAE lining the periphery and apex of the dome region. M cells in FAE of ILF were readily distinguishable from neighboring enterocytes because of their light staining and basally located euchromatic nucleus (Fig. 2b).

Moreover, FAE in the ILF also contained vimentin-positive cells exhibiting differences in their localization. Whereas some vimentin-positive cells were localized together, particularly at the apex of the dome regions (Fig. 2c), some were found throughout the FAE (Fig. 2d), and only a few were detected in some of the FAE (Fig. 2e). When compared with the M cells in PP, fewer IEL were generally present in the pockets provided by the M cells in most of the FAE of ILF (Fig. 2c–e). The appearance of the

M cells in many ILF more closely resembled the immature M cells in PP. These cells were columnar, showed immunoreactivity only in the perinuclear cytoplasm, and did not surround IEL with an intraepithelial invagination (Fig. 2c–e). Vimentin immunoreactivity in mature M cells in ILF was localized to the perinuclear area, the basal cytoplasm, and the cytoplasm surrounding IEL (Fig. 2f, g).

Furthermore, apart from ILF, villi-like solitary lymphoid structures that enclosed abundant lymphocytes in their lamina propria and surrounded them with a FAE containing vimentin-positive cells were also present between long intestinal villi. The vimentin-positive cells of the FAE of these structures were notably similar to immature M cells found in the FAE of PP and ILF. In addition, akin to ILF, we observed numerous goblet cells in these villi-like solitary lymphoid structures. The villi-like solitary lymphoid structures were rarely seen and were morphologically suggestive of LFV (Fig. 2h).

Electron-microscopic findings

Electron-microscopic studies showed more irregular and shorter microvilli on the apical surfaces of typical mature M cells in the FAE of jejunal and ileal PP with regard to those of neighboring enterocytes. These cells also surrounded the IEL beneath them with their prominent invaginations (Fig. 3a) and included several vesicles, vacuoles, and mitochondria in their apical cytoplasm (Fig. 3b, c). In addition, the nuclei of these cells resided under IEL invaginations next to the basal membrane. Cell connections between M cells and enterocytes were observed in the form of lateral extensions.

Typical mature M cells in the FAE of the ILF were similar to those of PP when viewed by electron microscopy. Like the M cells in PP, the M cells in the FAE of the ILF also had several vesicles and mitochondria in their apical cytoplasm, and the basolateral invaginations of these cells surrounded the IEL (Fig. 3d, e).

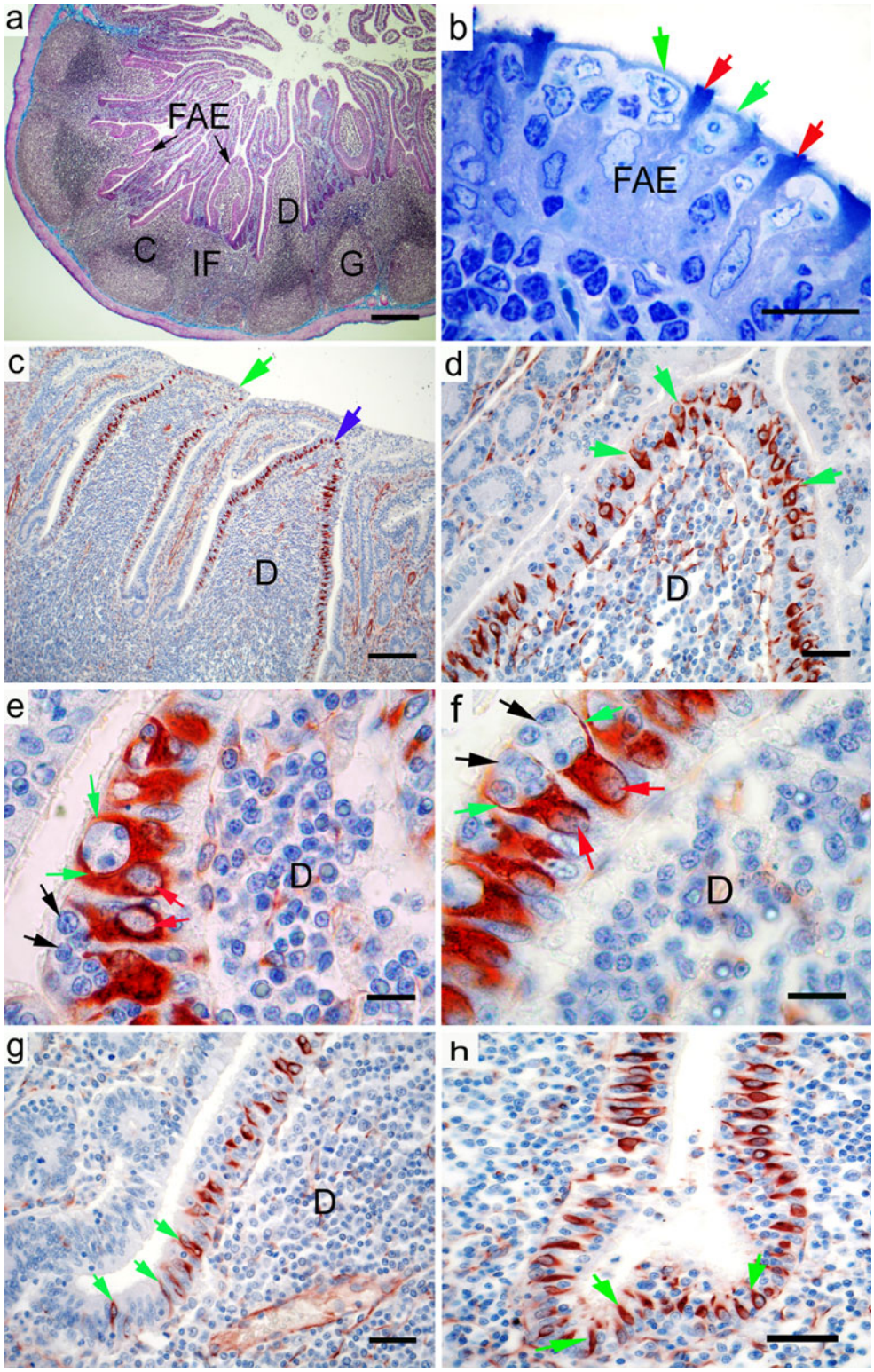
Discussion

GALT is present throughout the gastrointestinal tract in mammals and consists of aggregated and solitary lymph nodes (Macdonald 2003; Newberry 2008). The rabbit contains better developed GALT in comparison with other mammalian species (Cesta 2006). The GALT in the rabbit is commonly considered to constitute PP, sacculus rotundus, appendix, and cecal patches (Haley 2003); therefore, most of the studies regarding the lymphoid structure in the rabbit focus on these structures (Gebert and Bartels 1991; Gebert et al. 1992; Jepson et al. 1992, 1993; Regoli et al. 1995). Few studies consequently exist concerning the presence of ILF in the rabbit (Iwatsuki et al. 2002; Keren et al. 1978).

Two locally, developmentally, and functionally different types of PP are found in ruminants, pigs, and dogs (Haley 2003; Yasuda et al. 2006). In these species, uninterrupted ileal PP serve as primer lymphoid organs, whereas solitary jejunal PP function as secondary lymphoid organs (Beyaz and Asti 2004; Yasuda et al. 2006). In the present study, we have macroscopically detected five to six PP on both mucosal and serosal surfaces of the jejunum and ileum. The appearance and organization of these PP are consistent with previous reports regarding classical PP (Cesta 2006; Haley 2003). In the current study, PP have been found to be composed of numerous aggregated lymph follicles, each of which contain four different sites: a dome region, a germinal center, a coronal area, and a wide interfollicular area between neighboring follicles. The dome regions are also surrounded by a specialized FAE.

In contrast to PP, we have been able to view ILF only microscopically in the jejunum and ileum. ILF are similar to PP in terms of having a follicle with a germinal center and FAE containing M cells and might represent induction sites for the mucosal immune system in a similar manner as PP (Pabst et al. 2005). The presence of ILF has been

Fig. 1 Peyer's patches (PP) and the M cells in the follicle-associated epithelia (*FAE*) surrounding the dome regions of the PP. **a** Note the overall appearance of a jejunal PP that consists of aggregated lymph follicles (*D* dome region, *C* corona, *G* germinal center, *IF* interfollicular area). Mallory's triple staining. *Bar* 100 μm. **b** Note the presence of M cells (*green arrows*) having a thin membrane-like apical cytoplasm and basolateral invaginations encircling intraepithelial lymphocytes (IEL) beneath them and the occurrence of neighboring enterocytes (*red arrows*) in a ileal PP. Toluidine blue staining. *Bar* 8 μm. **c** Vimentin-positive cells in the FAE cover the dome region of a jejunal PP. Numerous vimentin-positive cells (*blue arrow*) are present at the apex of a dome region, and enterocytes (*green arrow*) are found in the FAE at the apex of a neighboring dome region. Immunohistochemical staining, 3-amino 9-ethyl carbazole (AEC). *Bar* 50 μm. **d** Vimentin-positive cells (*green arrows*) lie in the FAE at the periphery and apex of the dome region of a jejunal PP. Immunohistochemical staining, AEC. *Bar* 25 μm. **e** Note the vimentin-positive M cells (*green arrows*) surrounding IEL (*black arrows*) with their basolateral invaginations in the FAE at the periphery of the dome region of an ileal PP. Vimentin immunoreactivity in the M cells is localized in the perinuclear area and in the cytoplasm surrounding the IEL (*red arrows* nuclei of M cells). Immunohistochemical staining, AEC. *Bar* 10 μm. **f** Note the vimentin-positive M cells (*green arrows*) surrounding IEL (*black arrows*) with their basolateral invaginations in the FAE at the periphery of the dome region of a jejunal PP. Vimentin immunoreactivity in the M cells is localized in the perinuclear area and in the cytoplasm surrounding the IEL (*red arrows* nuclei of M cells). Immunohistochemical staining, AEC. *Bar* 10 μm. **g** Vimentin-positive columnar immature M cells (*green arrows*) lie in the FAE next to crypts and crypt epithelia of a jejunal PP. Immunohistochemical staining, AEC. *Bar* 25 μm. **h** Vimentin-positive columnar immature M cells (*green arrows*) lie in the FAE next to crypts and crypt epithelia of an ileal PP. Immunohistochemical staining, AEC. *Bar* 25 μm



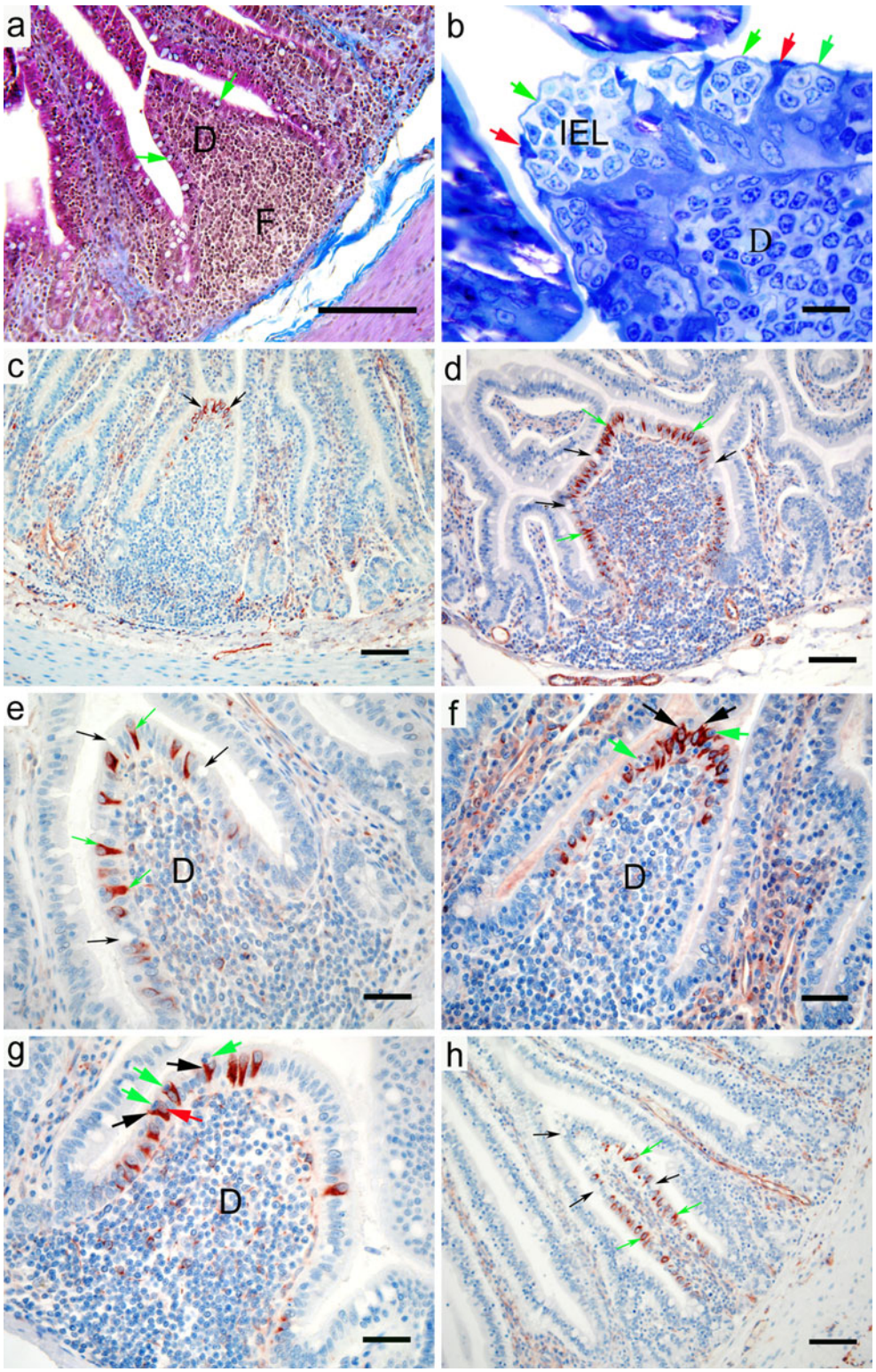
established in the intestines of mice (Hamada et al. 2002), guinea pig (Rosner and Keren 1984), human (Moghaddami et al. 1998), and rabbit (Iwatsuki et al. 2002; Keren et al. 1978). In addition, depending on the extent of antigenic stimulation, variable numbers of mature and immature ILF have been recorded in the intestine of mice (Lorenz et al. 2003; McDonald et al. 2005) suggesting that ILF serve as reservoir lymphoid structures for the intestinal immune system. Our inspection of the ILF in the intestine of the Angora rabbit has shown that several solitary ILF are

located between the intestinal villi on the antimesenteric border of the jejunum and ileum. Each ILF is reminiscent of a follicle with a dome region. The dome regions of ILF are surrounded with FAE akin to that of mice and guinea pig; however, no separation occurs between the germinal center, corona, and interfollicular areas. Moreover, in contrast to the FAE of PP, abundant goblet cells are present in the FAE of many ILF. However, a few ILF, which lack goblet cells, have also been observed. Typical M cells have also been seen in semithin sections prepared from these ILF . Overall, all the features of the ILF of the Angora rabbit are similar to those of mice (Hamada et al. 2002) and guinea pig (Rosner and Keren 1984), other than containing follicles with a well-developed germinal center.

In some species, novel lymphoid components, e.g., LFV (Hitotsumatsu et al. 2005; Mayrhofer and Brooks 1995; Mayrhofer et al. 1999; Moghaddami et al. 1998) and CP (Kanamori et al. 1996) are defined as new GALT regions. CP is reported to consist of lymphoid clusters containing about a thousand cells and is located at the bottom of the intestinal crypts (Kanamori et al. 1996). In addition, CP are found only in the small intestine of mice and do not contain T and B lymphocytes (Lügering and Kucharzik 2006). LFV have been detected in the small intestines of rat, mouse, and human to date (Mayrhofer et al. 1999). Unlike CP, LFV are encircled with FAE containing M cells, and the LFV present in the small intestines of rat and human have been proposed as the counterpart of CP in mice (Hitotsumatsu et al. 2005). Although we have not detected CP in the small intestine of the rabbit, we have noted a few solitary lymphoid structures resembling the LFV in rat, mouse, and human. Lymphoid cells occur in the lamina propria of the LFV-like structures, and their surfaces are covered with an FAE. In addition, we have observed vimentin-positive cells, resembling more closely the immature M cells described in the FAE of the PP and ILF, in the FAE of these structures. We consider that LFV-like structures are the primitive forms of the ILF, and we also assume that the formation of the ILF is sustained by luminal stimuli and normal bacterial flora. By contrast to PP, the existence of goblet cells and vimentin-positive cells, generally reminiscent of immature M cells, suggests that the diverse lymphoid structures that we have noted here are at discrete stages of development, reflecting the strength of various antigenic stimulations.

M cells, which are specialized epithelial cells, play an important role in the transport of intraluminal macromolecules and pathogens from the lumen of the small intestine to underlying mucosal lymphoid tissues in which the processing of the antigen and initiation of immune responses occur (Kyd and Cripps 2008; Man et al. 2004; Neutra 1998). During the immune response, B cells differ from plasma cells secreting specific immunoglobulin A (IgA). M cells are characterized by the possession of thin membrane-like apical cytoplasm and surround the IEL beneath them via their intraepithelial invaginations (Corr et al. 2008; Jepson et al. 1996; Miller et al. 2007; Neutra et al. 2001; Nicoletti 2000). Furthermore, M cells have been shown to be shorter than adjoining enterocytes, having irregular microvilli and containing numerous vesicles, vacuoles, and mitochondria in their apical cytoplasm (Brayden et al. 2005; Neutra et al. 2001; Niedergang and Kraehenbuhl 2000). In agreement with others (Hathaway and Kraehenbuhl 2000; Kucharzik et al. 2000; Magalhaes et al. 2007; Neutra 1998), we have discerned typical M cells, containing thin membrane-like apical cytoplasm and surrounding IEL beneath them with their basolateral invaginations in the FAE of the ILF and PP. M cells in the present study are easily discernable from the neighboring enterocytes because of their light staining and basally located euchromatic nuclei. Our electron-microscopic examinations have also shown that M cells contain numerous vesicles and mitochondria in their apical cytoplasm. The present findings thus indicate that the M cells exhibit representative features of the mature M cells found in the peripherally located FAE of PP and apically located FAE of ILF.

Fig. 2 Isolated lymphoid follicles (ILF) and the presence of M cells in the FAE surrounding the dome region of a ILF. **a** Note the overall exterior of a jejunal ILF composed of solitary lymph follicle (*green arrows* goblet cells, *F* follicle, *D* dome region). Mallory's triple staining. *Bar* 20 μm. **b** Note the presence of typical mature M cells (*green arrows*), which possess thin membrane-like apical cytoplasm and basolateral invaginations encircling intraepithelial lymphocytes (*IEL*) beneath them, in the FAE surrounding the apex of the dome region of a mature ILF and the neighboring enterocytes (*red arrows*). Toluidine blue staining. *Bar* 8 μm. **c** Vimentin-positive cells (*black arrows*) lie in the FAE encircling the dome region of an ileal ILF. Immunohistochemical staining, AEC. *Bar* 50 μm. **d** Vimentin-positive cells (green arrows) are plentiful in the FAE encasing the dome region of a jejunal ILF (*black arrows* goblet cells). Immunohistochemical staining, AEC. *Bar* 50 μm. **e** Immature vimentin-positive cells (*green arrows*) lie in the FAE enveloping the dome region of an ileal ILF (*black arrows* goblet cells). Immunohistochemical staining, AEC. *Bar* 50 μm. **f** Note the vimentin-positive mature M cells (*black arrows*) surrounding IEL (*green arrows*) with their basolateral invaginations in the FAE at the periphery of the dome region of a jejunal ILF. Vimentin immunoreactivity in the M cells is also localized in the perinuclear area and in the cytoplasm surrounding the IEL. Immunohistochemical staining, AEC. *Bar* 50 μm. **g** Note the vimentin-positive mature M cells (*black arrows*) surrounding IEL (*green arrows*) with their basolateral invaginations in the FAE at the periphery of the dome region of a jejunal PP. Vimentin immunoreactivity in the M cells is localized in the perinuclear area and in the cytoplasm surrounding the IEL (*red arrow* nucleus of M cell). Immunohistochemical staining, AEC. *Bar* 50 μm. **h** Lymphocyte-filled villi-like solitary lymphoid structures are found between the intestinal villi. In addition, abundant vimentin-positive cells (green arrows) lie in the FAE surrounding the dome region (*black arrows* goblet cells). Immunohistochemical staining, AEC. *Bar* 50 μm



The results of earlier studies of M cells in various mammals correspond to our current findings. The FAE of GALT (Gebert et al. 1992; Jepson et al. 1992, 1993), BALT (Gebert and Hach 1992), and tonsils (Gebert et al. 1995) of the rabbit are reported to have vimentin intermediate-filament-containing M cells. Gebert et al. (1992) have demonstrated two different vimentin-positive cells in the FAE of PP, a result similar to our findings. We have also noted that the cells encasing the IEL in the FAE at the periphery of the dome regions of PP are typical M cells.

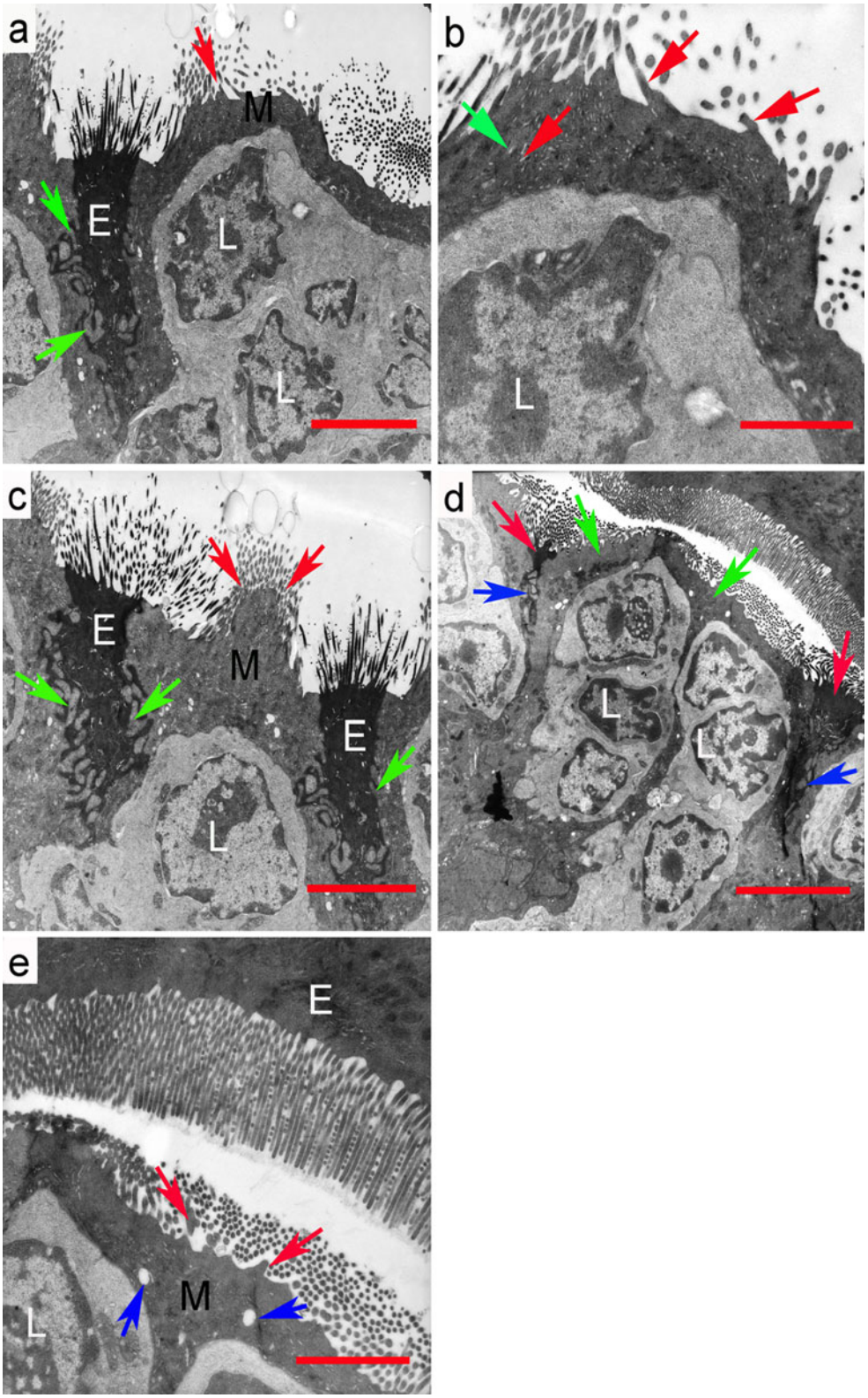


Fig. 3 Electron-microscopic appearance of typical mature M cells in the follicle-associated epithelia (FAE) of Peyer's patches (PP) and isolated lymphoid follicles (ILF). **a** The M cell (*M*) is characterized by a thin membrane-like apical cytoplasm in the FAE of ileal PP; note the adjoining enterocyte (*E*) and the intraepithelial lymphocytes (*L*) surrounded by the M cell (*red arrow* short irregular microvilli on the apical membrane of the M cell, *green arrows* lateral tight apposition between M cells and neighboring enterocytes). *Bar* 2 μm. **b** Appearance of M cell at higher magnification (*red arrows* short irregular microvilli on the apical membrane of the M cell, *green arrow* numerous small vesicles in the apical cytoplasm of the M cell, *L* intraepithelial lymphocyte encircled by the M cell). *Bar* 1 μm. **c** An M cell lying between two neighboring enterocytes in the FAE of a jejunal PP (*green arrows* lateral tight appositions between M cells and bordering enterocytes, *red arrows* short irregular microvilli on the apical membrane of the M cell, *L* intraepithelial lymphocyte encircled by the M cell). *Bar* 2 μm. **d** Note the mature M cell in the FAE of an ILF (*green arrows*) and the neighboring enterocytes (*red arrows*), the lateral tight appositions between M cells and bordering enterocytes (*blue arrows*), and the intraepithelial lymphocytes (*L*) encircled by the M cell. *Bar* 3 μm. **e** Appearance of mature M cell (*M*) at higher magnification in the FAE of an ILF (*red arrows* short irregular microvilli on the apical membrane of the M cell, *blue arrows* abundant minute vacuoles in the apical cytoplasm of the M cell, *E* enterocyte, *L* intraepithelial lymphocyte encircled by the M cell). *Bar* 1.5 μm

Our vimentin-positive cells near the FAE of the crypts are similar to the immature columnar M cells described in mice by Bye et al. (1984). We have detected vimentin-positive cells in the FAE of ILF with different localizations from those of PP. Although a number of vimentin-positive cells are localized collectively and, predominantly, at the apex of the dome regions, some are present throughout the FAE, and only a few of them have been seen in some of the FAE.

The appearance of the M cells in ILF resembles more that of immature M cells in PP.

Vimentin, an intermediate filament protein, is generally found in mesenchymal-derived cells and is part of the essential machinery of the cell's cytoskeleton (Goldman et al. 1996; Helfand et al. 2005; Ivaska et al. 2007). They might stabilize organelles, such as the nucleus, they might be involved in specialized junctions (Goldman et al. 1996), or they might participate in intracellular cell signaling via the formation of structural connections between the membrane and the nucleus of cells (Helfand et al. 2005; Ivaska et al. 2007). Vimentin intermediate filaments of the intestinal M cells in the rabbit are depicted as spreading from the nucleus to the intraepithelial pocket membrane surrounding the lymphocytes (Gebert et al. 1992; Jepson et al. 1992). In this study, we have detected vimentin immunoreactivity in the perinuclear cytoplasm of the mature M cells in addition to their cytoplasmic regions wrapping the IEL, but only in the perinuclear cytoplasm of the immature M cells. However, we have not observed vimentin immunoreactivity in the apical cytoplasm of the cells. We consider that the intense presence of vimentin in the perinuclear cytoplasm of the mature M cells and in the cytoplasmic regions wrapping the IEL maintains cell morphology by acting against the pressure from the IEL, which is surrounded with a basolateral pocket, on the basally located nuclei of the cells and by providing resilience to cells via allowing an in-and-out trafficking of the lymphocytes. On the other hand, the interaction between the immature M cells and B cells appears to be critical for the immature M cells to acquire the morphology of the mature M cells (Golovkina et al. 1999; Shaykhiev and Bals 2007). Moreover, vimentin intermediate filaments might take part in signaling between the membrane of the M cell and IEL.

The origin, differentiation mechanism, and death of M cells have not been well defined as yet. Nonetheless, two different concepts regarding the formation of the M cells exist (Mach et al. 2005; Miyazawa et al. 2006). The first suggests that the M cells develop from the stem cells dwelling in the intestinal crypts, although these stem cells do give rise to enterocytes (Gebert et al. 1999, 2004; Lelouard et al. 2001). The second proposes that M cells are transformed from enterocytes upon interaction with B cells (Kerneis et al. 1997) and pathogenic bacterial stimulation (Borghesi et al. 1999). In their study of the development of M cells in pigs, Miyazawa et al. (2006) report that, after being in close contact with lymphocytes at the peripheral FAE of the dome regions, immature M cells turn into mature M cells, which then differentiate into enterocytes nearby the apex of the dome region. These workers also state that the M cells in the PP of pigs, as shown in mice (Sierro et al. 2000) and rabbits (Takeuchi and Gonda 2004), are converted into enterocytes and then undergo apoptosis in the apex of the dome and are discharged into the lumen. In the present study, the detection of M cells, containing narrowed cytoplasm and basolateral pockets surrounding the IEL, in the peripheral FAE of the dome regions and the occurrence of columnar immature M cells, as detected with vimentin immunoreactivity in the FAE adjacent to the crypts and containing no IEL pocketing, support the concept that M cells originate from stem cells in the crypts. However, the present findings do not embrace the idea that M cells, after being converted into enterocytes, undergo apoptosis at the apex of the dome and drift into the lumen, because they cluster in the apex of the dome regions.

Furthermore, Gebert et al. (1992) demonstrate that vimentin-positive cells in the FAE of the PP in newborn rabbits are definitely immature M cells, which in subsequent weeks become mature M cells upon antigenic stimulation. In the current study, by contrast to the FAE of the PP, the presence of goblet cells and immature M cells in the FAE of ILF and LFV-like structures indicates that ILF can be found at various stages of their development based on antigenic stimulation.

In conclusion, the present study has demonstrated that the structural features of M cells in the ileal and jejunal PP were alike and in agreement with features previously reported in other rabbit breeds (Gebert et al. 1992; Jepson et al. 1992, 1993). Furthermore, this study has revealed the structure of M cells in the FAE of ILF in rabbits. The presence of abundant immature M cells and goblet cells in the FAE of ILF and the observation of LFV-like structures assumed to be the primitive forms of the ILF suggest that ILF do not complete their immunological development in contrast to PP in the rabbit. In addition, the development, differentiation, and the maturation of ILF in the intestine appear to take place in the presence of antigen stimulation. The morphological resemblance between ILF M cells and PP M cells suggests that these two types of cells perform similar or the same immunological functions.

References

Beyaz F, Asti RN (2004) Development of ileal Peyer's patches and follicle associated epithelium in bovine foetuses. Anat Histol Embryol 33:172–179

Borghesi C, Taussig MJ, Nicoletti C (1999) Rapid appearence 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

Brandtzaeg P, Kiyono V, Pabst R, Russel MW (2008) Terminology: nomenclature of mucosa-associated lymphoid tissue. Mucosal Immunol 1:31–37

Brayden DJ, Jepson MA, Baird AW (2005) Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today 10:1145–1157

Bye WA, Allan CH, Trier JS (1984) Structure, distribution, and origin of M cells in Peyer's patches of mouse ileum. Gastroenterology 86:789–801

Cesta MF (2006) Normal structure, function, and histology of mucosa-associated lymphoid tissue. Toxicol Pathol 34:599–608

Clark MA, Jepson MA, Simmons NL, Booth TA, Hirst BH (1993) Differential expression of lectin-binding sites defines mouse intestinal M-cells. J Histochem Cytochem 41:1679–1687

Corr SC, Gahan CGM, Hill C (2008) M-cells: origin, morphology and role in mucosal immunity and microbial pathogenesis. FEMS Immunol Med Microbiol 52:2–12

Crossmon G (1937) A modification of Mallory's connective tissue stain with a discussion of the principles involved. Anat Rec 69:33–38

Gebert A, Bartels H (1991) Occluding junctions in the epithelia of the gut-associated lymphoid tissue (GALT) of the rabbit ileum and caecum. Cell Tissue Res 266:301–314

Gebert A, Hach G (1992) Vimentin antibodies stain membranous epithelial cells in the rabbit bronchus-associated lymphoid tissue (BALT). Histochemistry 98:271–273

Gebert A, Hach G, 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, Rothkotter HJ, Pabst R (1994) Cytokeratin 18 is an M-cell marker in porcine Peyer's patches. Cell Tissue Res 276:213–221

Gebert A, Willfuhr B, Pabst R (1995) The rabbit M-cell marker vimentin is present in epithelial cells of the tonsil crypt. Acta Otolaryngol 115:697–700

Gebert A, Fassbender S, Werner K, 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

Gebert A, Steinmetz I, Fassbender S, Wendlandt KH (2004) Antigen transport into Peyer's patches: increased uptake by constant numbers of M cells. Am J Pathol 164:65–72

Glaysher BR, Mabbott NA (2006) Isolated lymphoid follicle maturation induces the development of follicular dentritic cells. Immunology 120:336–344

Goldman RD, Khuon S, Chou YH, Opal V, Steinert PM (1996) The function of intermediate filaments in cell shape and cytoskeletal integrity. J Cell Biol 134:971–983

Golovkina TV, Shlomchik M, Hannum L, Chervonsky A (1999) Organogenetic role of B lymphocytes in mucosal immunity. Science 286:1965–1968

Haley PJ (2003) Species differences in the structure and function of the immune system. Toxicology 188:49–71

Hamada H, Hiroi T, Nishiyama Y, Takahashi H, Masunaga Y, Hachimura S, Takahashi-Iwanaga H, Iwanaga T, Kiyono H, Yamamoto H, Ishikawa H (2002) Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. J Immunol 168:57–64

Hathaway LJ, Kraehenbuhl JP (2000) The role of M cells in mucosal immunity. Cell Mol Life Sci 57:323–332

Helfand BT, Chou YH, Shumaker DK, Goldman RD (2005) Intermediate filament proteins participate in signal transduction. Trends Cell Biol 15:568–570

Hitotsumatsu O, Hamada H, Naganuma M, Inoue N, Ishii H, Hibi T, Ishikawa H (2005) Identification and characterization of novel gut-associated lymphoid tissues in rat small intestine. J Gastroenterol 40:956–963

Ivaska J, Pallari HM, Nevo J, Eriksson JE (2007) Novel functions of vimentin in cell adhesion, migration, and signaling. Exp Cell Res 313:2050–2062

Iwatsuki H, Ogawa C, Suda M (2002) Vimentin-positive cells in the villus epithelium of the rabbit small intestine. Histochem Cell Biol 117:363–370

Jepson MA, Mason CM, Bennett MK, Simmons NL, Hirst BH (1992) Co-expression of vimentin and cytokeratins in M-cells of rabbit intestinal lymphoid follicle associated eptihelium. Histochem J 24:33–39

Jepson MA, Simmons NL, Hirst GL, Hirst BH (1993) Identification of M cells and their distribution in rabbit intestinal Peyer patches and appendix. Cell Tissue Res 273:127–136

Jepson MA, Clark MA, Foster N, Mason CM, Bennett MK, Simmons NL, Hirst BH (1996) Targeting to intestinal M cells. J Anat 189:507–516

Kanamori Y, Ishimaru K, Nanno M, Maki K, Ikuta K, Nariuchi H, Ishikawa H (1996) Identification of novel lymphoid tissues in murine intestinal mucosa where clusters of c-kit$^+$ IL-7R$^+$ Thy1$^+$ lympho-hemopoietic progenitors develop. J Exp Med 184:1449–1459

Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J Cell Biol 27:137–138

Keren DF, Holt PS, Collins HH, Gemski P, Formal SB (1978) The role of Peyer's patches in the local immune response of rabbit ileum to live bacteria. J Immunol 120:1892–1896

Kerneis S, Bogdanova A, Kraehenbuhl JP, Pringault V (1997) Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria. Science 277:949–952

Kucharzik T, Lugering N, Rautenberg K, Lugering A, Schmidt MA, Stoll R, Domschke W (2000) Role of M cells in intestinal barrier function. Ann N Y Acad Sci 915:171–183

Kyd JM, Cripps AW (2008) Functional differences between M cells and enterocytes in sampling luminal antigens. Vaccine 26:6221–6224

Lelouard H, Sahuquet A, Reggio H, Montcourrier P (2001) Rabbit M cells and dome enterocytes are distinct cell lineages. J Cell Sci 114:2077–2083

Liebler-Tenorio EM, Pabst R (2006) MALT structure and function in farm animals. Vet Res 37:257–280

Lorenz RG, Newberry RD (2004) Isolated lymphoid follicles can function as sites for induction of mucosal immune responses. Ann N Y Acad Sci 1029:44–57

Lorenz RG, Chaplin DD, McDonald KG, McDonough JS, Newberry RD (2003) Isolated lymphoid follicle formation is inducible and dependent upon lymphotoxin-sufficient B lymphocytes, lymphotoxin β receptor, and TNF receptor I function. J Immunol 170:5475–5482

Lügering A, Kucharzik T (2006) Induction of intestinal lymphoid tissue: the role of cryptopatches. Ann N Y Acad Sci 1072:210–217

Macdonald TT (2003) The mucosal immune system. Parasite Immunol 25:235–246

Mach J, Hshieh T, Hsieh D, Grubbs N, Chervonsky A (2005) Development of intestinal M cells. Immunol Rev 206:177–189

Magalhaes JG, Tattoli I, Girardin SE (2007) The intestinal epithelial barrier: how to distinguish between the microbial flora and pathogens. Semin Immunol 19:106–115

Man AL, Prieto-Garcia ME, Nicoletti C (2004) Improving M cell mediated transport across mucosal barriers: do certain bacteria hold the keys? Immunology 113:15–22

Mayrhofer G, Brooks A (1995) Lymphopoieses in lymphocyte-filled villi in the small intestine of the rat. Clin Immunol Immunopathol 76:55

Mayrhofer G, Moghaddami M, Murphy C (1999) Lymphocyte-filled villi (LFV): non-classical organized lymphoid tissues in the mucosa of the small intestine. Mucosal Immunol Update 7:9–13

McDonald KG, McDonough JS, Newberry RD (2005) Adaptive immune responses are dispensable for isolated lymphoid follicle formation: antigen-naive, lymphotoxin-sufficient B lymphocytes drive the formation of mature isolated lymphoid follicles. J Immunol 174:5720–5728

Miller H, Zhang J, Kuolee R, Patel GB, Chen W (2007) Intestinal M cells: the fallible sentinels? World J Gastroenterol 13:1477–1486

Miyazawa K, Aso V, Kanaya T, Kido T, Minashima T, Watanabe K, Ohwada V, Kitazawa H, Rose MT, Tahara K, Yamasaki T, Yamaguchi T (2006) Apoptotic process of porcine intestinal M cells. Cell Tissue Res 323:425–432

Moghaddami M, Cummins A, Mayrhofer G (1998) Lymphocyte-filled villi: comparison with other lymphoid aggregations in the mucosa of the human small intestine. Gastroenterology 115:1414–1425

Mowat AM (2003) Anatomical basis of tolerance and immunity to intestinal antigens. Nat Rev Immunol 3:331–341

Neutra MR (1998) Current concepts in mucosal immunity. V. Role of M-cells in transepithelial transport of antigens and pathogens to the mucosal immune system. Am J Physiol 274:785–791

Neutra MR, Mantis NJ, Kraehenbuhl JP (2001) Collaboration of epithelial cells with organized mucosal lymphoid tissues. Immunol Nat 2:1004–1009

Newberry RD (2008) Intestinal lymphoid tissues: is variety an asset or a liability. Curr Opin Gastroenterol 24:121–128

Newberry RD, Lorenz RG (2005) Organizing a mucosal defense. Immunol Rev 206:6–21

Nicoletti C (2000) Unsolved mysteries of intestinal M cells. Gut 47:735–739

Niedergang F, Kraehenbuhl JP (2000) Much ado about M cells. Trends Cell Biol 10:137–141

Pabst O, Herbrand H, Worbs T, Friedrichsen M, Yang S, Hoffmann MW, Körner H, Bernhardt G, Pabst R, Förster R (2005) Cryptopatches and isolated lymphoid follicles: dynamic lymphoid tissues dispensable for the generation of intraepithelial lymphocytes. Eur J Immunol 35:98–107

Rautenberg K, Cichon C, Heyer G, Demel M, Schmidt MA (1996) Immunohistochemical characterization of the follicle-associated epithelium of Peyer's patches: anti-cytokeratin 8 antibody (clone 4.1.18) as a molecular marker for rat M cells. Eur J Cell Biol 71:363–370

Regoli M, Bertelli E, Borghesi C, Nicoletti C (1995) Three-dimensional (3D) reconstruction of M cells in rabbit Peyer's patches: definition of the intraepithelial compartment of the follicle-associated epithelium. Anat Rec 243:19–26

Rosner AJ, Keren DF (1984) Demonstration of M cells in the specialized follicle-associated epithelium overlying isolated lymphoid follicles in gut. J Leukoc Biol 35:397–404

Shaykhiev R, Bals R (2007) Interactions between epithelial cells and leukocytes in immunity and tissue homeostasis. J Leukoc Biol 82:1–15

Sierro F, Pringault E, Assman PS, Kraehenbuhl JP, 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

Takeuchi T, Gonda T (2004) Cellular kinetics of villous epithelial cells and M cells in rabbit small intestine. J Vet Med Sci 66:689–693

Veneable JH, Coggeshall R (1965) A simplified lead citrate stain for use in electron microscopy. J Cell Biol 25:407–408

Wang C, McDonald KG, McDonald JS, Newberry RD (2006) Murine isolated lymphoid follicles contain follicular B lymphocytes with a mucosal phenotype. Am J Physiol Gastrointest Liver Physiol 291:595–604

Yasuda M, Jenne CN, Kennedy LJ, Reynolds JD (2006) The sheep and cattle Peyer's patch as a site of B-cell development. Vet Res 37:401–415