

The Cellular Differentiation of M Cells from Crypt Undifferentiated Epithelial Cells into Microvillous Epithelial Cells in Follicle-Associated Epithelia of Chicken Cecal Tonsils

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ABSTRACT. To clarify the cellular origin and the fate of M cells, detailed distributions of the epithelial cells were investigated scanning electron microscopically on the follicle-associated epithelia (FAE) of chicken cecal tonsils. The distribution of M cells was closely related with the situation of the crypt orifices in chicken cecal tonsils. In undeveloped cecal tonsils, the intestinal crypts were localized at the periphery of the FAE. In these tonsils, M cells without microvilli (M₀) were predominantly populated in the basal region of the FAE, whereas goblet cells and microvillous epithelial cells (MV) were more distributed in the middle to the apical region of the FAE. A few M cells with short microvilli were dispersed throughout the FAE. Significantly shrunk MV (MV_s) clustered together in transitional portions from the lateral face to the roof of the FAE. In well-developed cecal tonsils, the crypts also opened at the lateral surface in addition to the periphery of the FAE. In these tonsils, the M₀ accumulated densely in the small areas around the crypt orifices exclusively. No sign of exfoliation of apoptotic epithelial cells was found in the M₀-accumulated areas and at their peripheral boundaries. The MV_s were often clustered in the central regions among the crypt orifices in addition to the roof of the FAE. These findings suggest that M cells are directly derived from the undifferentiated crypt epithelial cells, not fall into apoptotic cell death and further differentiate into MV in the FAE of chicken cecal tonsils.

KEY WORDS: cecal tonsil, chicken, differentiation, gut-associated lymphoid tissue, M cell.

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M cells are the specialized luminal-antigen sampling cells in the follicle-associated epithelium (FAE). Research has been actively performed regarding possible function of M cells in oral vaccinations and oral drug delivery (for Review, see Gebert *et al.* [7]). Detailed fundamental knowledge of M cells is essential for these applications; however, no more than a small amount of basic information has been hitherto clarified.

The cellular origin of M cells has not been elucidated in the vertebrates. Two hypotheses have been proposed regarding the cellular origin of mammalian M cells. The first is that the M cells differentiate from the mature enterocytes in the FAE [1, 8, 17, 18]. The second is a more current hypothesis in which the M cells are transformed directly from the undifferentiated crypt epithelial cells [2–4, 7, 9]. In chicken cecal tonsils, the direct transformation of undifferentiated crypt epithelial cells into the M cells has been speculated based on light microscopic observation in immunohistochemical and histoplanimetric studies [19]. However, no further demonstration of the cellular origin of M cells has been done in chickens. Most research regarding the cellular origin of M cells has employed the scanning electron microscopy [4, 9, 10, 17]. Thus, the three-dimensional clarification of details of cellular distribution is one of more powerful strategies to determine the cellular origin of the M cells in the FAE. In chickens, however, no three-dimensional study has performed regarding cellular

distribution in the FAE.

As well, little knowledge is available regarding the fate of M cells in vertebrates. In the chicken, among the small amount of information available is that the M cells disappear within 4 days after the cellular division of undifferentiated cells in the crypts [19], and light microscopic observation has not revealed the death of the M cells in the FAE of chicken cecal tonsils [20].

In the present study, based on the morphological characteristics of the apical cell surface structures of the epithelial cells, we used the scanning electron microscope to clarify the detailed distribution of all types of epithelial cells in the FAE of chicken cecal tonsils. We also observed the epithelial cells under transmission electron microscope to confirm the identification of the epithelial cell types. From these results, we demonstrated the cellular origin and discussed the fate of M cells in chicken cecal tonsils.

MATERIALS AND METHODS

Animals: A total of 8 White Leghorn chickens aged 4–6 months conventionally reared in colonies of the Tottori University were used. They were permitted free access to food and water. Artificial light was utilized between 5:00 a.m. and 9:00 p.m. Cervical exsanguination was performed under anesthesia with *i.v.* injection of pentobarbital sodium. The care and use of these animals were approved by the

Animal Research Committee in Tottori University.

Scanning electron microscopy: Cecal tonsils extracted from 5 chickens were immediately rinsed in cold RPMI-1640 with 0.1 mM dithiothreitol to remove the mucus from the intestinal surface, followed by the immersion fixation in cold 2.0% paraformaldehyde-2.5% glutaraldehyde (PF-GA) in 0.1 M phosphate buffer (pH 7.2) for 12 hr. After trimming and rinsing the tissue blocks with 0.1 M phosphate buffer, they were immersed in 1% OsO₄ in 0.1 M phosphate buffer (pH 7.2) for 2 hr at room temperature. After dehydration, the specimens were dried by the critical point drying method using carbon dioxide or by the freeze-dry method using tertial butyl alcohol, followed by sputter coating with platinum. The specimens were observed using a scanning electron microscope (Hitachi X-605, Japan) at an accelerating voltage of 25 kV.

Histoplanimetry: Cell types of 50 epithelial cells were examined at 3 small areas in the basal, the middle, and the apical regions of the FAE on scanning electron micrographs. The mean from 3 areas was calculated for each region.

Along a straight line between the orifices of intestinal crypts, the arrangement of cell types of epithelial cells were examined in the 3 regions on the FAE in well-developed cecal tonsils.

The 10 apical surface areas of each type of epithelial cell were measured on the scanning electron micrographs of the middle regions of the FAE by means of a Video Micrometer VM-31 (Olympus, Japan).

The mean areas were calculated from the 5 animals. Quantitative data were expressed as means \pm SD. Unpaired Student's *t*-test was employed for detection of statistical significances with the *P* value being less than 0.05.

Transmission electron microscopy: Cecal tonsils extracted from 3 chickens were immediately sliced and fixed in cold PF-GA in 0.1 M phosphate buffer (pH 7.2) for 12 hr, followed by postfixation with 1.0% OsO₄ in 0.1 M phosphate buffer (pH 7.2) for 2 hr at room temperature. After dehydration, small tissue blocks were embedded in an Epon-812 mixture. Ultrathin sections with both uranyl acetate and lead citrate staining were observed under a transmission electron microscope (JEOL JEM-100CX, Japan) at an accelerating voltage of 75 kV to confirm the scanning electron microscopical identification of the epithelial cell types in the FAE.

RESULTS

Typing of epithelial cells in FAE: The following 4 types of epithelial cells were consistently observed scanning electron microscopically in the FAE: i) cells with flattened surface and no microvilli (Fig. 1a), ii) cells with thick and short microvilli that were arranged at densities from extremely low to moderate (Figs. 1b, c, d), iii) cells with slender and high microvilli that were densely arranged (Fig. 1e), iv) cells with small and rugged apical surfaces (Fig. 1f). These cells were transmission electron microscopically

identified as follows: The 1st and 2nd cell types were identified as M cells (M₀ and M₁, respectively); both the cell bodies and nuclei were slightly bigger than the neighboring microvillous epithelial cells (MV). The apical cytoplasm of M₀ were almost flattened and accompanied with many migrating cells at the basal surfaces (Fig. 1g). The apical cytoplasm of these cells often contained small vesicles and multivesicular bodies. The M₁ were generally columnar shaped and accompanied with a few migrating cells at the lateral surface (Fig. 1h). The 3rd cell type was identified as typical MV, which were typically columnar-shaped and possessed cytoplasmic characteristics similar to the absorptive cells of the neighboring intestinal villi (Fig. 1i). The 4th type was identified as goblet cells (G), whose apical cytoplasm contained numerous secretory mucous granules that partially protruded into the intestinal lumen (Fig. 1j). Occasionally, irregular microvilli were situated at the periphery of the apical surface.

Surface structure of the cecal tonsil: Undeveloped tonsils were composed of small and simple domes, which were separated by deep clefts. Neither fossula nor orifice of the intestinal crypts was observed in the apical to middle regions of the FAE covering each dome, whereas the intestinal crypts opened at the periphery of the dome (Fig. 2).

The developed large tonsils exhibited complicated shapes. Numerous swollen domes were demarcated by irregular clefts. Openings of the fossula occasionally existed on the apical regions of the FAE. Crypt orifices were frequently found on the lateral surface from the lower to the middle region of the FAE, in addition to the periphery of the domes (Fig. 3).

Distribution of each epithelial cell type in the FAE: The apical surface area of epithelial cells was measured in the middle region of the FAE of the developed tonsils using scanning electron microscopy. The areas were the largest in M₀ (29.7 \pm 9.2 μ m²), and significantly decreased in M₁ (24.1 \pm 4.4 μ m²) and MV (19.2 \pm 3.6 μ m²) in that order (*P*<0.05 between the cell types). The G (4.6 \pm 0.5 μ m²) was the smallest (*P*<0.001 between the G and the MV). In the central portions of the MV accumulations, the clusters of the shrunk MV (MVs; 13.9 \pm 2.3 μ m²), which were significantly smaller than the ordinary MV (*P*<0.05), were often localized (Fig. 4).

In the undeveloped tonsils, the M₀ were significantly more distributed in the basal region, whereas they were significantly less in the apical region of the FAE (*P*<0.01). Neither shrunk epithelial cells nor signs of their exfoliations were found in the M₀-occupied areas and their margins. A few M₁ were seen at the lower to the middle region, but were rare in the apical region of the FAE. The MV and G were significantly more distributed from the middle to the apical region of the FAE than in the basal region (*P*<0.05) (Table 1). The narrow zone showed a mixture of both M₀ and MV between the M₀-occupied areas and the MV-occupied area. The MV-occupied areas showed no M₀ involvement in the middle to the apical portions of the FAE. The MVs clus-

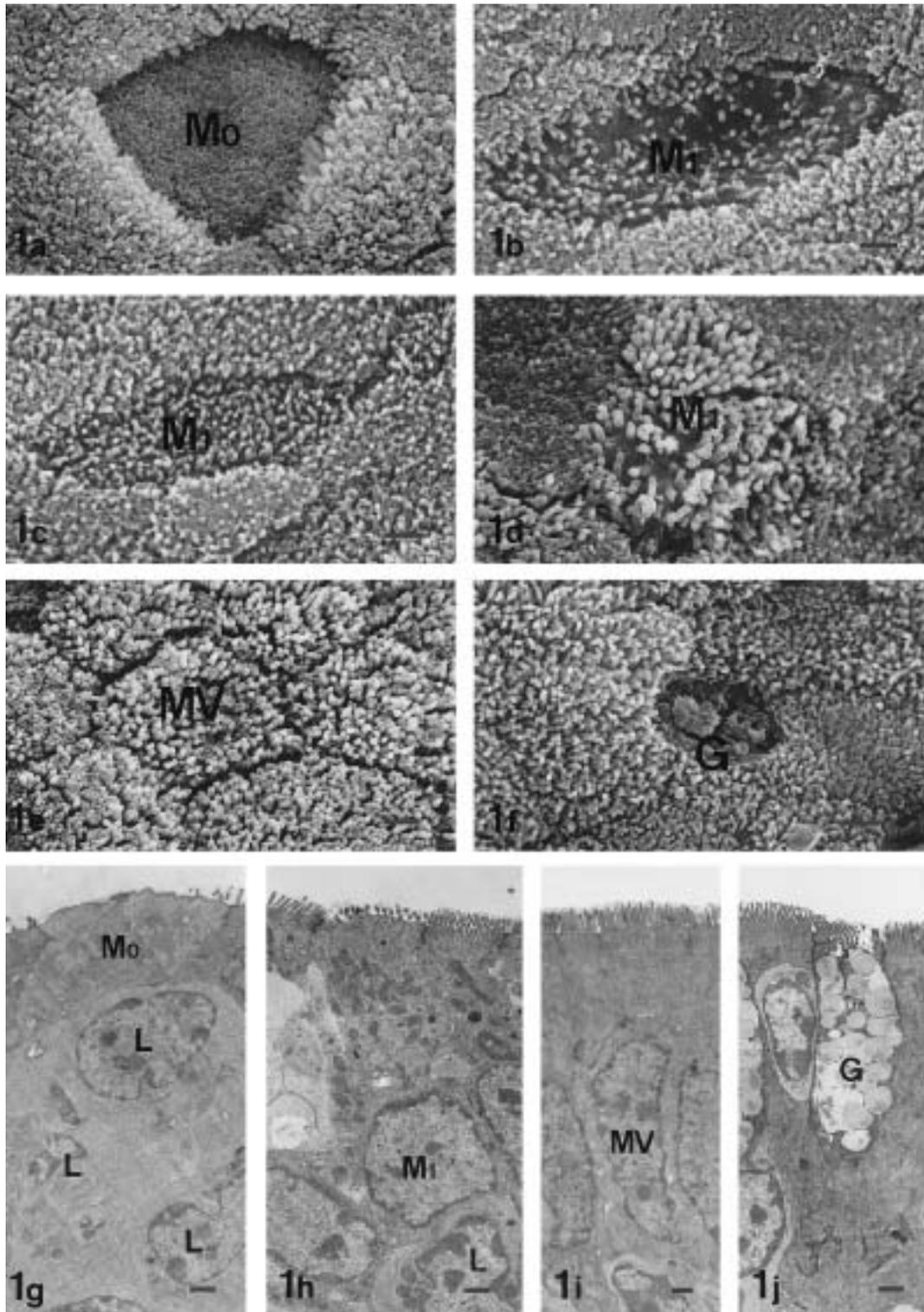


Fig. 1. Scanning (a-f) and transmission (g-i) electron micrographs of 4 main cell types in the FAE of cecal tonsils. a) M cell (M_0) with no microvilli. Numerous pits are visible on the luminal surface. b-d) M cell (M_1) with slightly thick and short microvilli on the cell surface. The densities of microvilli are gradually higher in (b), (c), and (d) in that order. e) Microvillous epithelial cells (MV) with slender and high microvilli densely arranged. f) Goblet cell (G) with small and rugged apical surface. g) A M_0 with a flattened cell surface. Lymphocytes (L) harbor beneath the flattened apical cytoplasm. h) M_1 with short and thick microvilli on their cell surfaces. A lymphocyte harbors on the lateral surface of M_1 . i) MV with densely arranged microvilli. j) G with numerous secretory granules in the apical cytoplasm. The cell surface is protruded toward the lumen by the secretory granules. Bar = 1 μ m.

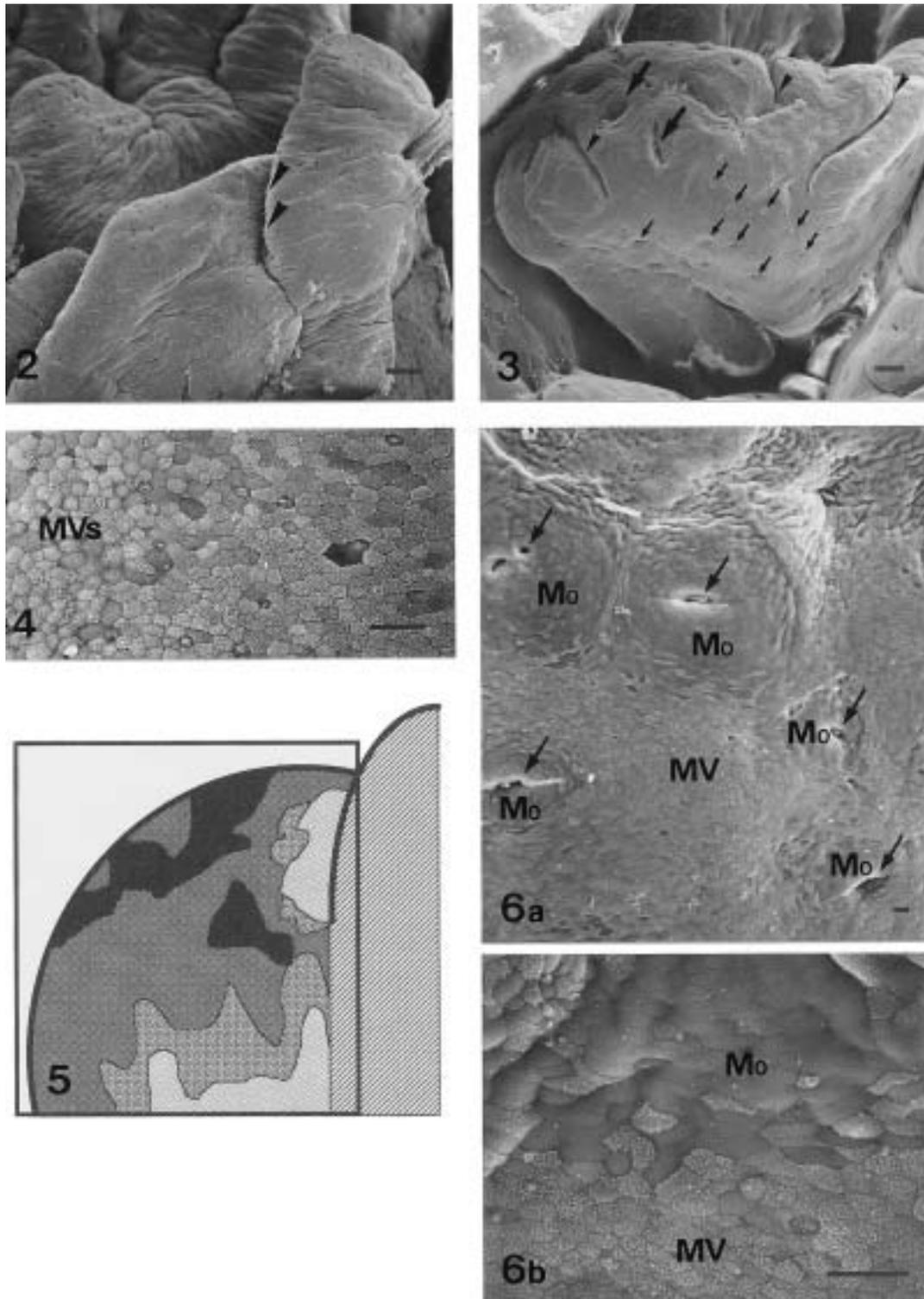


Fig. 2. Scanning electron micrograph of a typical undeveloped cecal tonsil. A cleft (arrow) incompletely separates the 2 domes. Neither fossula nor intestinal crypt is evident on the domes. Bar=100 μ m.

Fig. 3. Scanning electron micrograph of a typical developed cecal tonsil. The fossula (large arrows) and the clefts (arrowheads) open on the apical region of the FAE. The orifices of intestinal crypts (small arrows) are seen on the lateral FAE. Bar=100 μ m.

Fig. 4. Scanning electron micrograph of an intermediate region among the orifices of the intestinal crypts. The shrunk MV (MVs) with small cell surface area are clustered more on the left half than in the right half of this photograph. Bar=10 μ m.

Table 1. Distribution of epithelial cells in the FAE of the undeveloped cecal tonsil of chicken

Region in FAE	Type of epithelial cell			
	M ₀	M ₁	MV	G
				(%)
Apical	0.0 ± 0.0 ^{a)}	3.3 ± 1.9	87.3 ± 3.8 ^{c)}	9.3 ± 1.8 ^{d)}
Middle	0.7 ± 0.9 ^{b)}	9.3 ± 3.7	81.3 ± 2.5	8.7 ± 3.4
Basal	27.3 ± 9.0 ^{a,b)}	13.3 ± 6.2	56.0 ± 14.0 ^{c)}	3.3 ± 1.9 ^{d)}

M₀, M cells with no microvilli; M₁, M cells with microvilli; MV, microvillous epithelial cells; G, goblet cells; a), $P < 0.01$; b) c) and d) $P < 0.05$.

Table 2. Distribution of epithelial cells in the FAE of the developed cecal tonsil of chicken

Region in FAE	Type of epithelial cell			
	M ₀	M ₁	MV	G
				(%)
Apical	4.0 ± 5.7 ^{a)}	2.0 ± 2.8	80.7 ± 8.1 ^{c)}	13.3 ± 7.5 ^{d)}
Middle	26.0 ± 2.8 ^{b)}	20.7 ± 15.0	47.3 ± 9.4	6.0 ± 4.9
Basal	51.3 ± 13.6 ^{a,b)}	12.0 ± 9.8	32.7 ± 7.7 ^{c)}	4.0 ± 0.0 ^{d)}

M₀, M cells with no microvilli; M₁, M cells with microvilli; MV, microvillous epithelial cells; G, goblet cells; a) $P < 0.01$; b) c) and d) $P < 0.05$.

tered in the transitional regions from the lateral to the roof of the apical region of the FAE (Fig. 5).

In well-developed tonsils, the FAE were more complicatedly arranged showing 4 types of epithelial cells type in comparison with those in the undeveloped tonsils. In general, the M₀ were more frequently distributed in the basal, the middle, and the apical regions in that order ($P < 0.05$: between the apical and the middle, $P < 0.01$: between the apical and the basal). A few M₁ were dispersed in both the basal and the middle regions, whereas they were rare in the apical region. The MV were more predominant in the apical region and decreased toward the basal region ($P < 0.05$: between the apical and the middle, $P < 0.01$: between the apical and the basal). The G were fewer in the basal region and slightly more numerous in apical regions ($P < 0.05$: between the basal and the apical) (Table 2). Viewing over the FAE, the localization of M₀ was restricted in the small areas around the crypt orifices. The narrow zones around the M₀-occupied areas consisted of both M₀ and MV. No signs of epithelial exfoliation were found in the M₀-occupied areas and the mixed areas of M₀ and M₁. A few M₁ were dispersed in the MV accumulations, but not in their central portions among the crypt orifices (Figs. 6, 7). The MVs were often clustered in the central regions among the orifices in addition to the roof of the FAE. The crypt

orifices were often biased in the M₀-occupied areas. The distances between the M₀-occupied areas and the neighboring clusters of MVs were various; i.e., in the cases of the shortest distance, the M₀ accumulations were directly adjacent to the MVs accumulation (Fig. 8).

DISCUSSION

The chicken cecal tonsil is an accumulation of a basic unit, the "nodular unit", in which a fossula is located centrally [12]. In the nodular unit, M cells are located in the epithelium of fossulae and the lateral FAE [11]. In present study, the localization of M cells was three-dimensionally confirmed in chicken cecal tonsils.

In rabbit Peyer's patches and appendices [9, 10, 21] and Peyer's patches of mouse [15] and human [4], M cells were also distributed more in the lateral FAE rather than in the apical FAE. The FAE of the human Peyer's patch is obscured by adjacent overhanging villi [14]. M cells function as the specialized luminal-antigen sampling cells in the FAE in mammalian [7] and chicken gut-associated lymphoid tissues [13]. It seems difficult for M cells to directly contact the luminal particulates or macromolecules on the lateral surface of the FAE, at which the M cells reside in chickens and mammals. Therefore it is considered that

Fig. 5. Schematic drawing of the cellular distribution on a dome of the undeveloped cecal tonsil. Light gray areas, the M₀ occupied areas; moderate gray areas, the areas mixed with M₀ and MV; dark gray areas, the MV occupied areas without M₀; black areas, the shrunk MV-clustered areas; diagonal areas, non-observed areas.

Fig. 6. a) Distribution of 5 M₀-accumulations around the orifices of intestinal crypts (arrows) on the lateral FAE of a well-developed cecal tonsil. A large accumulation of microvillous epithelial cells (MV) is visible in the central region among the crypt orifices. Bar=100 μ m. b) High magnification of the periphery of the M₀-occupied area. The accumulations of both epithelial cells were clearly demarcated. Bar=10 μ m.

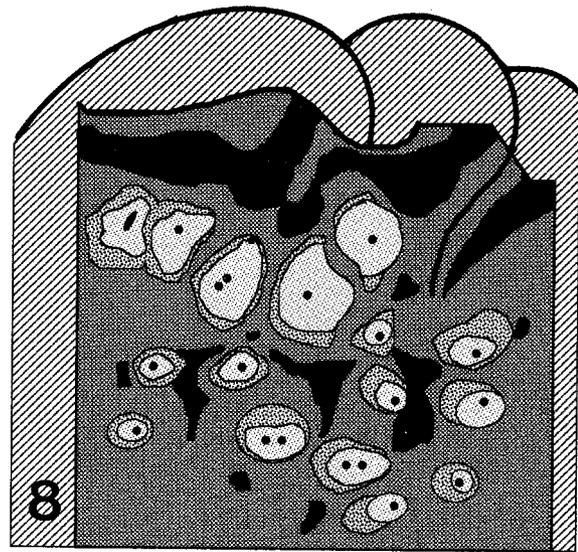
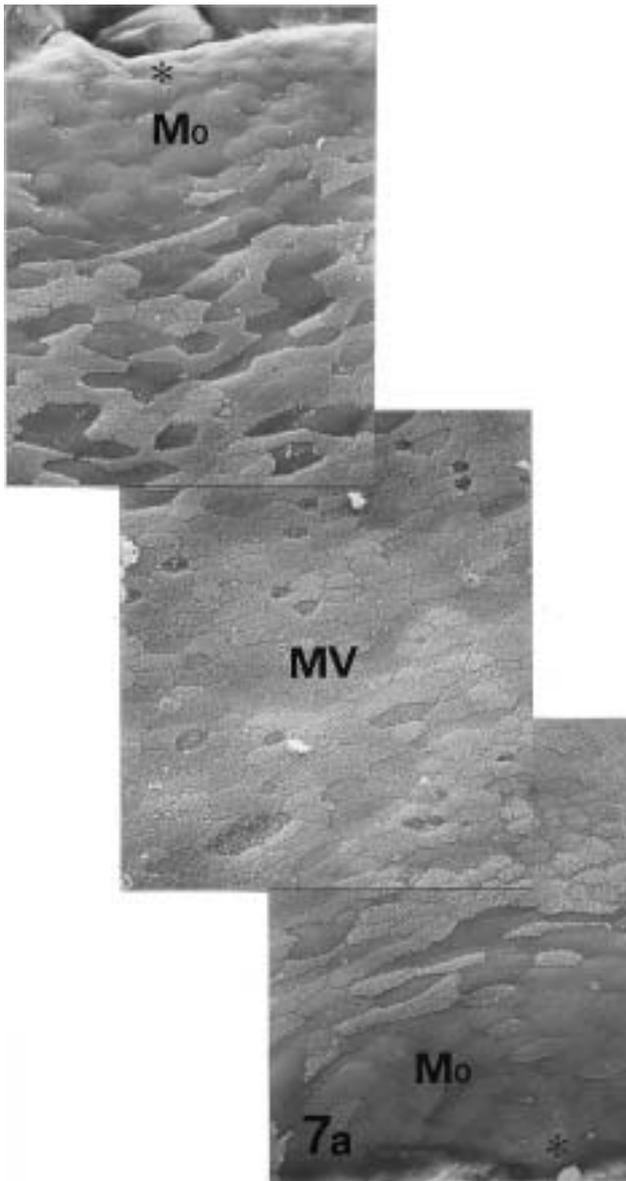
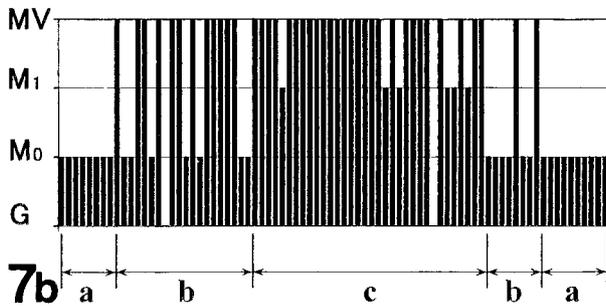


Fig. 7. Typical cell arrangement between the crypt orifices in the FAE of a well-developed cecal tonsil. a) Scanning electron micrograph of an inter-crypt region. Asterisks represent the base points in the measurement. A cellular arrangement on a straight line between the base points is presented in (b). Bar=10 μ m. b) Each cell type is dotted as a bar with different length. G, goblet cells; M₀, M cells of M₀ type; M₁, M cells of M₁ type; MV, microvillous epithelial cell. a, the M₀ occupied areas; b, the areas mixed with M₀ and MV; c, the MV occupied areas without M₀.

Fig. 8. Schematic drawing of the cellular distribution on a dome of a well-developed cecal tonsil. Light gray areas, the M₀ occupied areas; moderate gray areas, the areas mixed with M₀ and MV; dark gray areas, the MV occupied areas without M₀; black areas, the shrunk MV-clustered areas; small black-dots, the orifices of intestinal crypts; diagonal areas, non-observed areas.



the M cells ordinarily absorb the diffused low molecular antigenic peptides or the local microflora on the FAE, and that the M cells might absorb the luminal particulates or

macromolecules during the expansion of the intestinal wall by the luminal contents.

The epithelial cells of the cecal tonsils and the intestinal villi are newly generated in the intestinal crypts in the chicken cecum. The intestinal crypts are disposed at the peripheries of the lymphoid nodules of all cecal tonsils, and also on the lateral FAE in well-developed tonsils [19]. Apoptosis is induced in epithelial cells as they migrate from the crypts, and they are finally exfoliated from the villous tips or the transitional regions from the lateral to the apical FAE [19, 20]. One of the typical apoptotic signs of chicken intestinal epithelial cells is cell shrinkage [19]. This sign is possible to detect by a scanning electron microscope. In this study, significantly shrunk MVs were three-dimensionally detected in the transitional portion from the lateral surface to the roof and also the intermediate portions among the crypt orifices in the FAE of chicken cecal tonsils. Therefore, these findings suggest that the main streams of epithelial cells are oriented toward the apical region in the FAE as

well as in the ordinary intestinal villi, and that the exfoliation of epithelial cells occurs at the transitional regions from the lateral surface to the roof or at the intermediate regions among the crypt orifices, where the epithelial cells might stagnate. Thus, the disappearance sites of the epithelial cells are closely associated with the situation of crypt orifices and with the patterns of the epithelial flux in the FAE.

The most influential hypotheses regarding the cellular origin of mammalian M cells are that M cells are also derived directly from the undifferentiated cells in the intestinal crypts [5, 7]. This hypothesis is supported by the phenomena that the majority of M cells at the periphery of the FAE are adjacent to the crypts in the intestinal lymphoid follicles of rabbits [6, 9, 10], mice [2, 3], human [4], and that immature M cells are labeled 24 hr after the injection of ^3H -thymidine in murine ileal Peyer's patches [2]. In chicken cecal tonsils, M cells appear on the FAE from the crypts within 1 day [19], and that M cells are significantly more distributed the basal portions of FAE and are especially limited in the small areas around the crypt orifices as seen in this study. These findings clearly demonstrate that the M cells differentiate directly from undifferentiated crypt epithelial cells in chicken cecal tonsils.

The other hypothesis, in which M cells differentiate from mature enterocytes, is based on the results of detailed three-dimensional observations that M cells are distributed at the higher region of the FAE of mouse Peyer's patch and that no M cells exist in the intestinal crypts of the FAE [17, 18]. This hypothesis has been supported by the correlation of ultrastructural changes of mature enterocytes into M cells with the intraepithelial location of the lymphocytes [8], although Sicinski *et al.* [16] present several types of evidences that contradict this hypothesis, and the observations of the present study also deny the validity of this hypothesis in chicken cecal tonsils. However, we also found small lymphoid follicles in the middle portions of the intestinal villi of chicken jejunum (data not shown). The appearance of the lymphoid follicles in the intestinal villi might also show the possibility of M cell generating from mature enterocytes in some physiological conditions.

In the mouse ileal Peyer's patches, the synchronous exfoliation of the epithelial cells of the FAE and the intestinal villi indicates that the turnover time for M cells is no longer than that of enterocytes of the villi [2]. In chicken cecal tonsils, BrdU-labeled M cells disappeared within 4 days from the FAE as well as did villous epithelial cells in the cecal villi [19]. In addition, the apoptotic MV with DNA fragmentation exfoliate from the villous tips and the apical FAE, but no M cells light microscopically exhibited apoptotic signs and do the exfoliation [20]. In the present study, cell shrinkage, a typical apoptotic features, was found in MV exclusively. Furthermore, the cell shrinkage and the exfoliation were never seen in M_0 . In addition to this, the densities of the microvilli on the M_1 were from extremely low to moderate, being suggestive of the transforming processes from the M_0 to the MV. These findings suggest

that the M cells never kill themselves by apoptosis and further differentiate into mature MV in chicken cecal tonsils.

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