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Proliferation and cellular kinetics of villous epithelial cells and M cells in the chicken caecum

TAKASHI TAKEUCHI, HIROSHI KITAGAWA, TOMOHIRO IMAGAWA AND MASATO UEHARA

Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Japan

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ABSTRACT

The proliferation sites and cellular kinetics of villous epithelial cells and M cells in the intestine of the adult chicken have never been clarified. In this study, we determined the proliferation sites in the chicken caecum using colchicine treatment and detection of proliferative cell nuclear antigen (PCNA). The cellular kinetics of these cells were also studied using bromodeoxyuridine (BrdU) as a tracer. Enterocytes in their mitotic period were observed along the entire length of the intestinal crypt of the caecum, with a denser distribution in the middle portion of the crypt, except for the caecal tonsil. The centres of distributions were at 49% of the distance from the bottom of the crypt in the base and 41% in the apex of the caecum. In the caecal tonsil, the centres of distributions were at 64% in the long type of crypt from the bottom of the crypt and at 44% in the short type of crypt. On the other hand, the PCNA-positive enterocytes were distributed more densely at the bottom of the crypt, except for the caecal tonsil. The centres of distributions were at 36% in the base from the bottom of the crypt, 37% in the body, and 34% in the apex. In the caecal tonsil, they were at 54% in the long type of crypt and 44% in the short type. The BrdU-labelled enterocytes reached to the basement of the intestinal villi in all caecal portions at 1 d after the BrdU administration. The leading edge of the labelled enterocytes disappeared from the villous tips at 4 d in the base and the body and 3 d in the apex. In the caecal tonsil, the BrdU-labelled microvillous epithelial cells and the M cells appeared near the orifice of the crypt at 1 d, and BrdU-labelled M cells were not observed in the crypt. Thereafter, almost all of these cells disappeared at 5 d from the follicle associated epithelium (FAE). These results suggest that M cells are transformed from their precursors within 1 d, and the turnover time for M cells occurs within 4 d after the cell division of the precursors.

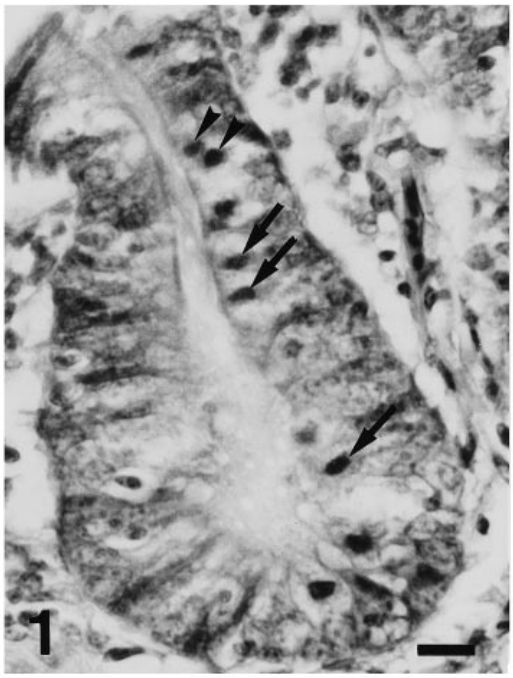
Key words: Gastrointestinal tract; large intestine, lymphoid tissue.

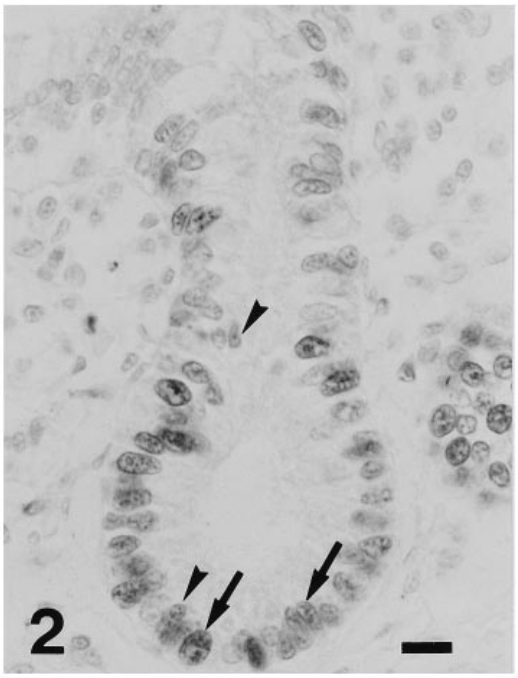
INTRODUCTION

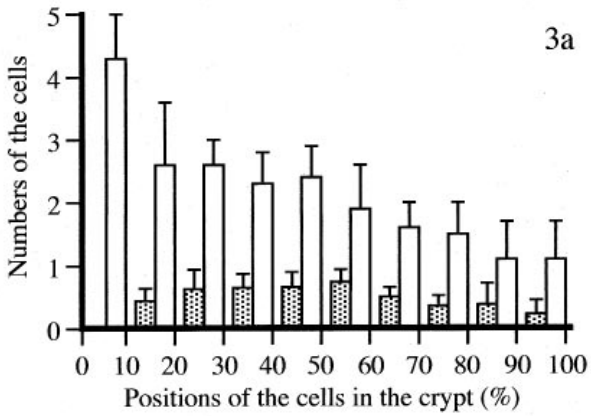
The proliferation sites and cellular kinetics for enterocytes in the murine small intestine have been established. The proliferation sites are in the middle portion of the crypt in the small intestine (Pozharisski et al. 1980; Loeffler et al. 1986; Potten & Loeffler, 1987; Chen et al. 1988) and in the lower half of the crypt in the large intestine (Chang & Leblond, 1971; Chang & Nadler, 1975; Pozharisski et al. 1980; Appleton et al. 1983). The turnover times are 3 or 4 d in the small and large intestines, respectively (Chang & Leblond, 1971, 1974). In the chicken, however, the epithelial cellular kinetics of the intestine have been studied only in newly-hatched or young birds by using H^3-thymidine (Imondi & Bird, 1965; Rodak & Prochazka, 1971; Spielvogel et al. 1972). Therefore, in the adult chicken, the proliferation sites and the cellular kinetics of the enterocytes are still unknown.

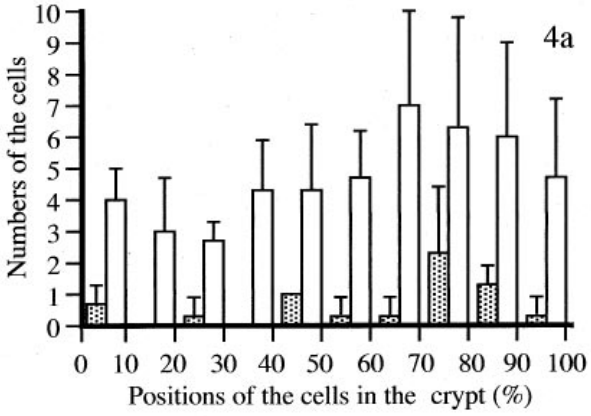
M cells, which are specialised luminal-antigen sampling cells in the follicle associated epithelium (FAE), have been reported in the mammalian gut associated lymphoid tissues (GALT) (Gebert et al. 1996). Until now, 2 hypotheses have been proposed regarding their origin. The first is that the M cells differentiate from the mature enterocytes in the FAE (Bhalla & Owen, 1982; Sicinski et al. 1986; Smith & Peacock, 1980, 1982, 1992). The second is

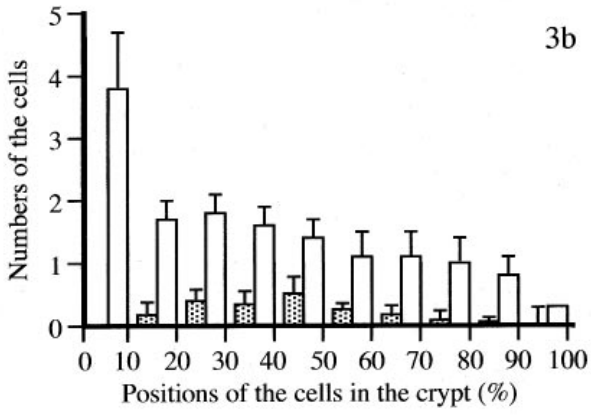
Correspondence to Associate Professor Hiroshi Kitagawa, Department of Veterinary Anatomy, Faculty of Agriculture, Tottori University, Tottori 680, Japan.

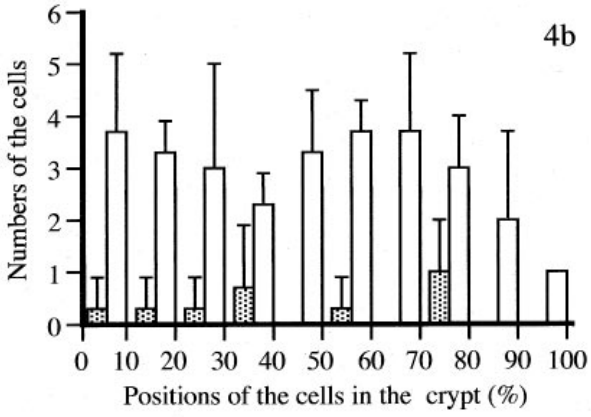












Figs 1–4. For legend see opposite.

that the M cells are transformed directly from the undifferentiated cells in the crypt (Bye et al. 1984; Jepson et al. 1993*a*, *b*; Gebert et al. 1996). In terms of kinetics, the immature M cells have appeared in the FAE 24 h after the division in the mouse ileal Peyer's patches (Bye et al. 1984). In the avian GALT, the existence and function of M cells have been reported (Bockman & Cooper, 1973; Befus et al. 1980; Burns, 1982; Kitagawa et al. 1996), but their derivation and kinetics have never been studied.

In this study, the proliferation sites and cellular kinetics of both villous epithelial cells and M cells were investigated in the caecum of the adult chicken by means of colchicine and antiproliferating cell nuclear antigen (PCNA) antibody. Furthermore, the cellular kinetics of the villous epithelial cells and the M cells were examined using bromodeoxyuridine (BrdU) as a tracer.

MATERIALS AND METHODS

Animals

Thirty-five White Leghorn chickens (aged more than 6 mo) conventionally reared in our laboratory were used from March to June.

Distribution of mitotic enterocytes

At 1 h after i.p. administration of colchicine (Wako, Japan; 1.7 mg/kg), 5 chickens were killed by cervical exsanguination under anaesthesia with an i.v. injection of pentobarbital sodium around noon. Both the base, including the caecal tonsil, and the apex of the caecum were fixed in 10% formalin and embedded in paraffin. The serial sections were cut longitudinally at 4 μm and stained with haematoxylin and eosin. The distributions of the mitotic enterocytes were observed in 20 crypts of the base and the apex of the caecum per chicken by means of Video Micrometer VM-31 (Olympus, Japan). In the caecal tonsil, the distributions were observed in 1 crypt per chicken. The relative positions of the mitotic enterocytes along the entire length of the crypt were calculated as a percentage from the bottom of the crypt. The mean number of mitotic enterocytes per each 10% of the entire length of the crypt was represented. The mean depths of the crypts were estimated from 10 crypts per caecal portion from each of the 5 chickens. The crypts, which were cut longitudinally through their centres, were randomly selected. In the caecal tonsil, the depths of the long crypts were estimated by combining section thickness and number of serial sections. A paired Student's *t* test was employed throughout and the statistical significance was accepted at the 5% level.

Distribution of proliferating enterocytes

The base, including the caecal tonsil, the body, and the apex of the caecum from 5 chickens were fixed in 70% ethanol for 24 h at 4 °C, and then paraffin sections were prepared. After blocking of the endogenous peroxidase with 99% methanol and 0.1% H_2O_2 for 30 min, respectively, the sections were treated with 1% normal goat serum for 1 h, followed by incubation with antiPCNA mouse monoclonal antibody (Oncogene Science, USA; diluted 1:500) for 18 h at 4 °C. After incubation with antimouse IgG+IgM goat serum (American Qualex, USA; diluted 1:100) for 2 h at 4 °C, the sections were incubated with mouse PAP (Wako, Japan; diluted 1:100) for 1 h at 4 °C. The sections were finally incubated with 3,3′-diaminobenzidine containing 0.03% H_2O_2 (DAB) and then counterstained with methyl green. The distribution of the PCNA-positive enterocytes was observed as described above.

Cellular kinetics of enterocytes

At 1, 2, 3, 4 and 5 d after i.p. administration of BrdU (Sigma, USA; 50 mg/kg), 5 chickens were killed by

Fig. 1. Longitudinal section of the crypt in the base of the caecum after colchicine treatment. The mitotic enterocytes (arrows) and lymphocytes (arrowheads) in which mitosis was arrested by colchicine, are visible. Haematoxylin and eosin. Bar, 10 μm.

Fig. 2. Longitudinal section of the crypt in the base of the caecum with anti-PCNA treatment. The enterocytes located in the bottom of the crypt express strong immunoreactivity (arrows), but in the upper portion of the crypt they express only weak immunoreactivity. The PCNA-positive lymphocytes (arrowheads) are also visible. Methyl green counterstain. Bar, 10 μm.

Fig. 3. Distributions of the mitotic (dotted columns) and PCNA-positive (open columns) enterocytes in the crypts of the base (*a*) and apex (*b*) of the caecum. The relative positions of the enterocytes to the entire length of the crypt were calculated as a percentage from the bottom of the crypt, and each bar expresses the mean number of mitotic or PCNA-positive enterocytes per every 10% of the entire length of the crypt.

Fig. 4. Distributions of the mitotic (dotted columns) and PCNA-positive (open columns) enterocytes in the long (*a*) and short (*b*) crypts in the caecal tonsil. The relative positions of the enterocytes to the entire length of the crypt were calculated as a percentage from the bottom of the crypt, and each bar expresses the mean number of mitotic or PCNA-positive enterocytes per each 10% of the entire length of the crypt.

cervical exsanguination and anaesthetised with pentobarbital sodium around noon. The base, body, and apex portions of the caecum were extracted and fixed in 70% ethanol for 24 h at 4 °C. Paraffin sections were pretreated with 1N-HCl for 4 min at 60 °C. After blocking the endogenous peroxidase with 99% methanol and 0.1% H_2O_2 for 30 min, respectively, the sections were treated with 1% normal goat serum for 1 h. Thereafter, they were incubated with anti-BrdU mouse monoclonal antibody (Sanbio BV, Netherlands; diluted 1:100). The distribution of the BrdU-positive enterocytes was observed as described above. Ten longitudinal sections from each caecal portion were used for detection of the BrdU-labelled nuclei of the enterocytes. The mean heights of the villi were estimated from 10 villi per caecal portion from each of the 5 chickens, respectively. The centres of villi which had been cut longitudinally were selected randomly. A paired Student's *t* test was employed throughout and the statistical significance was accepted at the 5% level.

Identification of M cells

To identify M cells more precisely, the negative lectin-binding properties for wheat germ agglutinin (WGA) exhibited only by the luminal surfaces of M cells in the chicken caecal tonsil (Kitagawa et al. unpublished data), were utilised. Briefly, the sections which were treated with the procedure to detect BrdU-labelled enterocyte nuclei were incubated simultaneously with 0.5 μg/ml-biotinylated WGA (Sigma, USA) for 18 h at 4 °C. Thereafter, these sections were incubated with streptavidin-biotin peroxidase complex (Dako, USA) for 30 min at room temperature and finally visualised with DAB.

RESULTS

Distribution of mitotic enterocytes

The mean depths of the intestinal crypts were 131 ± 23 μm (n = 5) in the base, 94 ± 14 μm (n = 5) in the body, and 113 ± 9 μm (n = 5) in the apex of the caecum. There were significant differences in the mean depths of the crypts between these 3 portions ($P < 0.01$). After the colchicine treatment, enterocyte mitosis was seen in the intestinal epithelial lining throughout the crypt (Fig. 1). Mitotic enterocytes were more densely distributed in the middle portion of the crypt. The centres of distribution were at 49 ± 5% (n = 5) in the base and 41 ± 9% (n = 5) in the apex (Fig. 3). There was no significant difference between these portions.

There were both long and short types of crypts in the caecal tonsil, and their mean depths were 727 ± 125 μm (n = 3) and 246 ± 84 μm (n = 3), respectively. The mean depth of the long type of crypt was clearly different from that of the other caecal portions ($P < 0.01$). Mitotic enterocytes were distributed more densely in the upper half of the long type of crypt, but in the short type of crypt, mitotic enterocytes were distributed along the entire length of the crypts. The centres of distribution were at 64 ± 5% (n = 3) in the long type and 44 ± 7% (n = 3) in the short type (Fig. 4).

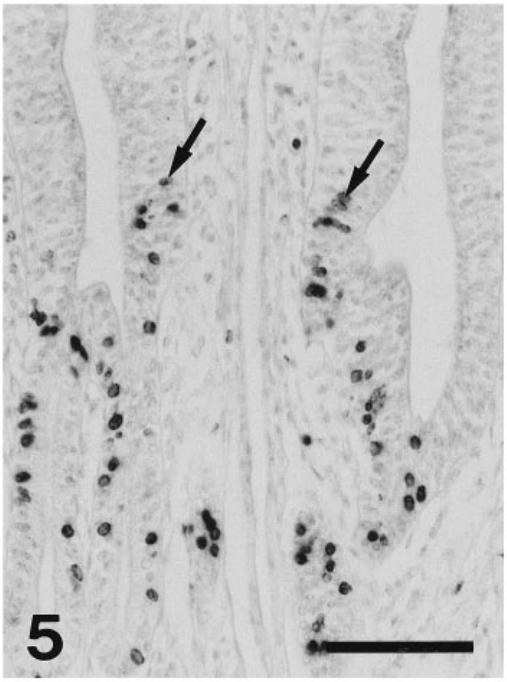
Distribution of proliferating enterocytes

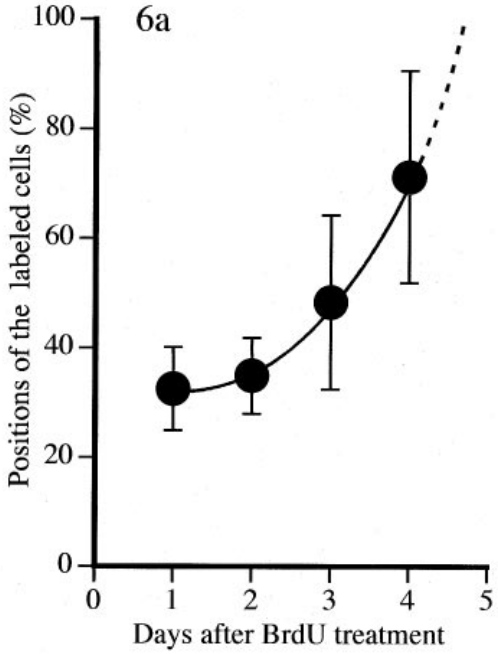
The number of PCNA-positive enterocytes was maximum at the bottom of the crypt and then decreased towards the orifice. The PCNA-positive enterocytes at the bottom were heavily stained, but were only weakly stained in the upper portion of the crypt (Fig. 2). The centres of distribution were at 36 ± 6% (n = 5) in the base, 37 ± 2% (n = 5) in the body, and 34 ± 3% (n = 5) in the apex (Fig. 3).

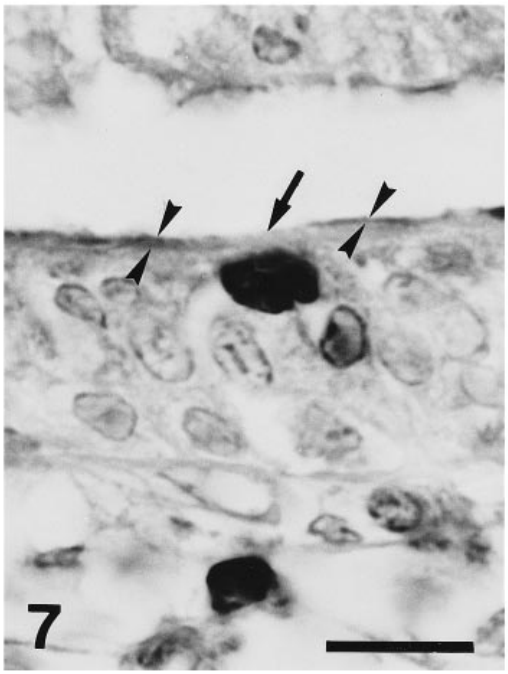
In the caecal tonsil, PCNA-positive enterocytes were distributed more densely in the upper half of the long type of crypt. In the short type of crypt, however, the PCNA-positive enterocytes expressed 2 peaks. One was for the bottom of the crypt, as with the crypts of the base, the body, and the apex of the caecum. The other was for the upper half of the crypt, as with the long type of crypt. The centres of distribution were at 54 ± 7% (n = 3) in the long type, and 44 ± 2% (n = 3) in the short type (Fig. 4).

Cellular kinetics of enterocytes

The height of the intestinal villi became shorter from the proximal to the distal portion of the caecum. The mean heights of the villi were 858 ± 97 μm (n = 5) at the base, 250 ± 77 μm (n = 5) at the body, and 103 ± 17 μm (n = 5) at the apex of the caecum. There were significant difference between the heights ($P < 0.001$). The leading edge of the BrdU-labelled enterocytes reached the base of the villi at 1 d (Fig. 5). A row of the BrdU-labelled enterocytes then gradually migrated towards the villous tips, but their migration velocity became faster at 3 d after BrdU administration compared with that at 1 and 2 d. Almost all of the BrdU-labelled enterocytes disappeared from







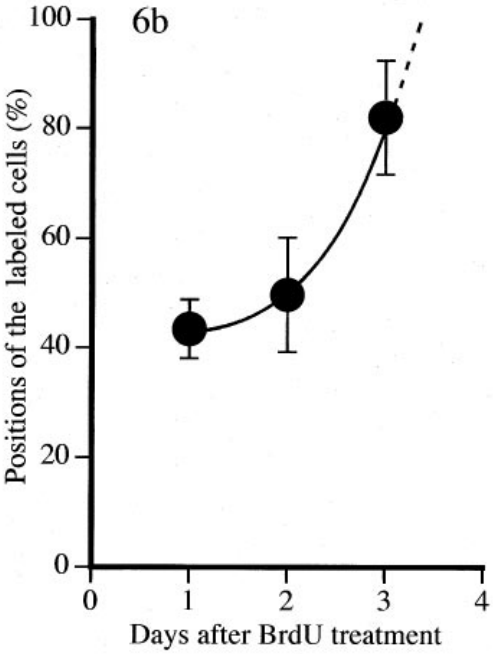


Fig. 5. The mucosa of the base of the caecum at 1 d after BrdU administration. The leading edge (arrows) of the BrdU-labelled enterocytes is visible at the base of the villi. Methyl green counterstained. Bar, 50 μm.

Fig. 6. Cellular kinetics of the BrdU-labelled enterocytes in the base (*a*) and apex (*b*) of the caecum. The positions are shown as percentages of the total length from the bottom of the crypts to the tip of the villi. Each circle expresses the mean positions of the BrdU-labelled nuclei of the enterocytes each day after BrdU administration.

Fig. 7. Microvillous epithelial cells and an M cell in the caecal tonsil at 1 d after BrdU administration. The microvillous epithelial cells, which are positive for WGA (arrowheads), and the BrdU-labelled M cell, which is negative for WGA (arrow), are visible. Methyl green counterstain. Bar, 1 μm.

the villous tips at 5 d in the base and the body and at 4 d in the apex, so that the turnover time for the enterocytes was estimated to be 4 d and 3 d, respectively (Fig. 6). In the caecal tonsil, the BrdU-labelled microvillous epithelial cells were seen near the orifices of the crypt in the FAE at 1 d after BrdU adminis-

tration. Thereafter, they disappeared 5 d following their spread over the FAE. The BrdU-labelled M cells were not observed along the crypt. At 1 d after BrdU administration, the M cells were seen near the orifices of the crypt and then disappeared from the FAE at 5 d (Fig. 7).

DISCUSSION

The proliferation sites for the enterocytes are mainly situated in the middle portion of the crypt in the small intestine of the mouse (Appleton et al. 1983; Loeffler et al. 1986; Potten & Loeffler, 1987; Chen et al. 1988) and the rat (Pozharisski et al. 1980; Chen et al. 1988), and in the lower half of the crypt in the large intestine of the mouse (Chang & Leblond, 1971; Chang & Nadler, 1975; Appleton et al. 1983) and the rat (Pozharisski et al. 1980). In this study, the distribution of mitotic enterocytes at the base and apex of the caecum was similar to the murine small and large intestine, respectively. There are typical intestinal villi in the base of the chicken caecum as in the murine small intestine. They gradually become lower towards the apex, and they never present a typical appearance at the apex (Kitagawa et al. 1996). The mucosa of the apex resembles the murine large intestine rather than the small intestine. Therefore, mitotic enterocytes may be located in the lower portion of the crypt at the apex in contrast to those at the base of the chicken caecum.

It has been suggested that the crypt of the mouse small intestine can be divided into a stem cell zone and a proliferative zone. The stem cell zone is located near the bottom of the crypt, and the proliferative zone at the middle position (Loeffler et al. 1986; Potten & Loeffler, 1987). It has also been suggested that the turnover time for stem cells is longer than that for enterocytes in the proliferative zone of the small intestine of the rat and the mouse and the large intestine of the rat (Pozharisski et al. 1980; Loeffler et al. 1986; Potten & Loeffler, 1987; Chen et al. 1988). On the other hand, the number of enterocytes which are labelled with H^3-thymidine is more abundant lower in the crypt at 1 h after H^3-thymidine treatment in the mouse descending colon (Chang & Leblond, 1971; Chang & Nadler, 1975). In this study, the distribution of the mitotic enterocytes, most of which were observed near the middle portion of the crypt, was consistent with that seen in the mouse small intestine. In contrast, the distribution of PCNA-positive enterocytes, which was maximum at the bottom and then decreased towards the orifice of the crypt, was consistent with that observed in the mouse large intestine. To explain this discrepancy, we might consider a hypothesis that the mitotic stem cells at the bottom of the crypt migrate up towards the middle portion of the crypt and then migrate back towards the bottom after mitotic division.

The turnover times for the enterocytes was 3.3 d in the duodenum, 3.4 d in the jejunum and 3.0 to 4.0 d in the mouse descending colon (Chang & Leblond, 1971; Cheng & Leblond, 1974). In the duodenum of 7 and 21-d-old chickens, the H^3-thymidine labelled enterocytes reach the villous tips at 72 h after the injection of H^3-thymidine (Rodak & Prochazka, 1971). In this study, the turnover times for the enterocytes were estimated at 4 d in the base and the body and 3 d in the apex. It has been reported that infection with intestinal bacteria stimulates the epithelial turnover time in the rat jejunum (Symons, 1965) and that, due to the influence of the luminal microflora, rate of turnover in the germ-free animals is significantly lower than that for conventional animals, i.e. mouse ileum (Abrams et al. 1963) and rat ileum and colon (Alam et al. 1994). The chicken caecum plays a special role in that uric acid which is taken back from the cloaca by antiperistalsis into the caecum, is absorbed after degradation to volatile fatty acids and ammonia by bacteria (Braun & Campbell, 1989). This role is primarily carried out in the body and apex of the caecum; therefore, the turnover time for the enterocytes is thought to be shorter toward the apex.

There are 2 hypotheses regarding the origin of M cells in mammals. The first is that M cells differentiate from mature enterocytes in the upper portion of the FAE. This hypothesis is based on the phenomenon where M cells in the Peyer's patches of the mouse ileum are found to be distributed throughout the higher regions of the FAE, and no M cells are found in the intestinal crypts of the FAE (Sicinski et al. 1986; Smith & Peacock, 1980, 1982, 1992), nor are they labelled until 3 d after the H^3-thymidine administration (Bhalla & Owen, 1982). The second hypothesis is that the M cells are derived directly from the undifferentiated cells in the crypt (Gebert et al. 1996). This is based on studies showing that the majority of M cells at the periphery of the FAE are located adjacent to the crypt in the ileal Peyer's patches and the appendix of the rabbit (Jepson et al. 1993*a*, *b*) and are labelled 24 h after the injection of H^3-thymidine in the ileal Peyer's patches of the mouse (Bye et al. 1984). In this study, the BrdU-labelled M cells appeared near the orifices of the crypt at 1 d after the BrdU administration. Therefore, it was speculated that the M cells are derived directly from the undifferentiated cells at the crypt in the chicken caecal tonsil.

In the mouse ileal Peyer's patches, H^3-thymidine labelled enterocytes exfoliated synchronously both from the follicular domes and intestinal villi, and the lack of labelled enterocytes in the FAE after loss from the villi indicates that the turnover time for M cells is no longer than that of the enterocytes of the villi (Bye et al. 1984). In this study, almost all of the BrdU-labelled enterocytes also disappeared from the FAE at 5 d with those in the villi. Therefore, it appears that the turnover time for M cells in the chicken cecal tonsil seems to be within 4 d.

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