Histoplanimetric Study on the Relationship between Cellular Kinetics of Epithelial Cells and Proliferation of Indigenous Bacteria in the Rat Colon

Wang-Mei QI¹⁾, Kenkichi YAMAMOTO¹⁾, Yuh YOKOO²⁾, Hidenori MIYATA²⁾,
Kankanam Gamage Sanath UDAYANGA²⁾, Junichi KAWANO²⁾, Toshifumi YOKOYAMA²⁾,
Nobuhiko HOSHI²⁾ and Hiroshi KITAGAWA^{2)*}

¹⁾Department of Bioresource and Agrobiosciences, Graduate School of Science and Technology and

²⁾Department of Bioresource Science, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan

(Received 9 December 2008/Accepted 5 February 2009)

ABSTRACT. The aim of this study was to clarify the regulatory effects of epithelial kinetics on indigenous bacterial proliferation in the large intestine. The lifespan, migration speed and proliferation rate of crypt epithelial cells in the initial 20% of the colon (proximal colon) and the 50% of the colon (middle colon) in bromodeoxyuridine-administrated rats were histoplanimetrically and chronologically compared. The proximal colon possessed well-developed mucosal folds and a large amount of indigenous bacteria which filled the crypt lumen, whereas no folds or bacteria were found to occupy the crypt lumen in the middle colon. The cell lifespans were 32.2, 42.5 and 33.6 hr in the apical and the basal parts of the mucosal folds of the proximal colon, and in the middle colon, respectively. The migration speeds were 4.2, 2.1 and 3.3 $\mu\text{m/hr}$, respectively, while the appearance frequencies of proliferating cell nuclear antigen (PCNA)-positive crypt epithelial cells were 35.0, 24.6 and 33.8%. These findings suggest that the lifespan was shortened and the migration speed increased in the most luminal mucosa of colon, contributing to the elimination of the adhered bacteria from the most luminal mucosa. By contrast, the elongation of the lifespan and deceleration of the migration of epithelial cells in the basal parts of the mucosal folds might contribute to reliable settlement of indigenous bacteria, resulting in the maintenance of a large amount of indigenous bacteria in the lumen of the proximal colon.

KEY WORDS: histology, host defense, immunohisto-chemistry, microbiological control, rat.

J. Vet. Med. Sci. 71(6): 745–752, 2009

Indigenous bacteria maintain a symbiotic relation with their hosts, and contribute to the health maintenance of hosts through interference with newly invaded pathogens, the production of short-chain fatty acids and vitamins, and the enhancement of oral immunological tolerance [5, 40]. At the same time, indigenous bacteria have been suggested to be essential in driving the inflammatory pathology in human inflammatory bowel diseases, and have been reported to play a part in the initiation of colonic cancer [16].

In healthy men, the relative amount of indigenous bacteria increases toward the distal alimentary tract and finally reaches 10^{10} – 10^{12} CFU in the colon [6, 19, 27, 36]. This tendency is the same in other mammalian species (cattle [31], dogs [12], mice [43], rats [25]). These indigenous bacteria are maintained in almost constant number under physiological conditions [36]. Intestinal indigenous bacteria are considered to be regulated by various defense factors such as the intrinsic motility of the intestinal wall, the secretion of mucus and bactericidal substances, the elimination of adhered bacteria by epithelial cells themselves and the rapid replacement of epithelial cells [22, 24, 32, 35].

Epithelial cells are generated in the intestinal crypts and predominantly migrate toward the villous apices. In their migration process, the epithelial cells are induced apoptotic

signals and finally exfoliated into the intestinal lumen in small intestine [8, 38]. A similar process is believed to occur in the large intestine [10, 15, 41, 42]. On the other hand, the fundamental settlement sites of indigenous bacteria are the apices of both the intestinal villi and domes of mucosal lymphatic follicles in the rat small intestine [11, 22, 23, 44], and around the crypt orifices of the most luminal mucosa in the rat large intestine [44]. At these sites, apoptotic epithelial cells are incessantly shed into the intestinal lumen. Therefore, changes in epithelial kinetics effectively regulate the number of adhered indigenous bacteria in the rat small intestine [35].

The kinetics of intestinal epithelial cells have been actively studied under steady physiological conditions in humans [30, 39], mice [7, 13, 28, 29], rats [2, 28, 29] and chickens [41]. In rodents, the proliferation site is the lower half of the intestinal crypt in the large intestine [3, 8, 9, 34]. Under physiological conditions, the lifespan of epithelial cells was 3–4 d in the mouse descending colon [8] and 4.63 d in the rabbit proximal colon [14]. The exposure of germ-free animals to conventional housing results in the acceleration of the replacement of villous columnar epithelial cells in mice [1] and rats [2]. The feeding of foods mixed with human feces results in shorter intestinal crypts and villi in germ-free rats [37]. In addition, we clarified that the lifespan of villous columnar epithelial cells is shortened and their migration speed increased at sites with hyperproliferation of indigenous bacteria compared to sites with no or less proliferation [35].

* CORRESPONDENCE TO: KITAGAWA, H., Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan.
e-mail: hkitagaw@kobe-u.ac.jp

In the rat large intestine, numerous indigenous bacteria reside in the crypt lumen and on the mucous epithelium of the proximal colon, whereas no bacteria reside in the crypt lumen, and a few on the mucous epithelium of the middle colon and rectum [44]. However, the regulation system, especially changes in epithelial kinetics, has not been clarified through the comparison of sites with different degrees of proliferation of indigenous bacteria, such as the proximal and middle colon. The present study aims to histoplanimetrically clarify the relationship between epithelial kinetics and regulation of indigenous bacteria in the rat colon under a physiological condition.

MATERIALS AND METHODS

Animals: A total of 30 male Wistar rats aged 7 weeks (Japan SLC Inc., Hamamatsu, Japan) were maintained under conventional laboratory housing conditions. They were permitted free access to food (Lab MR Stock, Nossan Corp., Yokohama, Japan) and water. The animal facility was maintained under conditions of a 12-hr light/dark cycle at $21 \pm 1^\circ\text{C}$ and 50–60% humidity. No sign of disorder was confirmed by clinical or pathological examinations in all animals. This study was approved by the Institutional Animal Care and Use Committee (Permission number: 17–04–05) and carried out according to Kobe University Animal Experimentation Regulations.

BrdU administration: Five rats each were sacrificed by *i.p.* injection of overdosed pentobarbital sodium (Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) at 18, 24, 30, 36, 42 and 48 hr after intraperitoneal injection of bromodeoxyuridine (BrdU; 50 mg/kg, Sigma-Aldrich, St. Louis, U.S.A.) in physiological saline.

Tissue preparation: The animals were intravascularly perfused with 4% paraformaldehyde-containing periodate-lysine-paraformaldehyde fixative (PLP; pH 6.2, 38°C). After perfusion, tissue blocks were removed from the initial 20% colon (proximal colon) and the 50% of the colon (middle colon). All tissue blocks were immersion-fixed in cold PLP for 24 hr at 4°C , then were snap-frozen in liquid nitrogen with reference to the embedding method described by Barthel and Raymond [3]. Sections $4\ \mu\text{m}$ in thickness were cut using a Coldtome HM505E (Carl Zeiss, Jena, Germany) and were placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan).

A section from each tissue block was stained with hematoxylin-eosin to observe the general structures of the intestinal lumen and wall.

Immunohistochemical detection of BrdU: Detection of antigens was conducted using the peroxidase-antiperoxidase (PAP) method. Briefly, after rinsing with 0.05% Tween-added phosphate buffered saline (PBS; pH 7.4), the sections were incubated in 1.0 N HCl solution for 13 min at 45°C . Then, the sections were followed by immersion in absolute methanol and 0.3% H_2O_2 solution for 30 min, respectively. After blocking with 1% normal wild bullfrog serum

(prepared in our laboratory) for 1 hr at room temperature (r.t.), the sections were reacted with anti BrdU mouse IgG₁ (diluted at 1 : 100, Santa Cruz Biotechnol., Santa Cruz, U.S.A.) for 18 hr at 4°C . Then the sections were incubated with anti mouse IgG rat IgG (diluted at 1 : 50, Jackson ImmunoRes. Lab., West Grove, U.S.A.) for 1 hr at r.t., followed by mouse PAP-complex (diluted at 1 : 50, Serotec, Oxford, U.K.) for 1 hr at r.t. After rinsing with PBS, the sections were incubated with 3,3'-diaminobenzidine (Dojindo Lab., Kumamoto, Japan) containing 0.03% H_2O_2 , and were counterstained with hematoxylin. Control sections were incubated with non-immunized mouse serum instead of the primary antibody or with PBS.

Immunohistochemical detection of PCNA: The detection of proliferating cell nuclear antigen (PCNA) was conducted in essentially the same manner as BrdU detection. Briefly, sections from the proximal and the middle colons of 5 randomly selected animals were incubated with proteinase K (1.2 $\mu\text{g}/\text{ml}$, Sigma-Aldrich, Saint Louis, U.S.A.) solution for 13 min at 37°C . The primary antiserum was anti PCNA mouse IgG_{2a} (diluted at 1 : 100, Oncogene, Darmstadt, Germany).

Histoplanimetry: Ten intestinal crypts whose central axes had been longitudinally cut were randomly chosen from each intestinal portion of each animal. The depth of the intestinal crypt was measured from the upper extremity of the nucleus of the lowest epithelial cell to that of the highest one in the same crypt.

The mean position of the epithelial nucleus of the leading edge of the BrdU-labeled epithelial cell cluster along the entire length of the intestinal crypt was calculated from 5 animals as a percentage from the bottom of the intestinal crypt, at each time after BrdU administration. The approximate lifespan of the BrdU-labeled epithelial cells was estimated from the distribution of the mean positions of the leading edges of BrdU-labeled epithelial cell clusters at each time.

The migration speed of BrdU-labeled epithelial cells per 1 hr was calculated from the migration distance of the labeled epithelial cells during 18 to 24 hr after BrdU administration.

The appearance frequency of PCNA-positive crypt epithelial cells and the total number of epithelial cells was measured from both sides of the epithelial wall of 5 intestinal crypts of sections from each of 5 randomly selected animals.

Statistical analysis: Data are presented as means $\pm$ standard deviations. Statistical analysis was performed using ANOVA with Scheffé multiple comparison testing. *P* values of less than 0.05 were considered statistically significant.

RESULTS

General histology: The mucosal folds were well developed in the proximal colon, whereas there were no

Fig. 1. a) Numerous indigenous bacteria and no food-derived substances are visible in the spaces (small arrows) between the well-developed mucosal folds (large arrows) in the rat proximal colon. Food-derived substances (arrowheads) are visible in the chyme. Bar=100 μ m. b) High-magnification of photograph of the most luminal epithelium of the basal portion of mucosal fold in the proximal colon. Indigenous bacteria adhere to the epithelial surface (small arrows). Bar=10 μ m. c) High-magnification photograph of the crypt bottom in the basal portion of the mucosal fold in the proximal colon. Clusters of indigenous bacteria present in the lumen (small arrows). Bar=10 μ m. d) No mucosal folds are visible in the middle colon. The food-derived substances in the chyme (arrowheads) are nearby the mucosal epithelium. The chyme is demarcated by the mucus layer, which contains indigenous bacteria (small arrows). Bar=100 μ m. e) High-magnification photograph of the epithelium of the most luminal mucosa in the middle colon. Fine indigenous bacteria adhere to the epithelial surface (small arrows). Bar=10 μ m. f) High-magnification photograph of the crypt bottom in the middle colon. No indigenous bacteria are present in the lumen. Bar=10 μ m.

folds in the middle colon (Figs. 1a, d). Bacteria filled in the central luminal chyme which contained food-derived substances, and in the inter-fold spaces which contained scarcely any food-derived substances (Fig. 1a). A thick mucus layer covered the most luminal mucosa in both the proximal and middle colons. In the mucous layer, numerous indigenous bacteria were contained in the proximal colon, whereas there were a few indigenous bacteria in the middle colon (Figs. 1a, d). The indigenous bacteria adhered to the epithelium of the most luminal mucosa in both portions of the colon, and also filled the crypt lumina in the proximal

colon exclusively (Figs. 1b, c, e, f).

The intestinal crypts were significantly deeper in the apical parts of the mucosal folds in the proximal colon than in the middle colon; the deepest crypts were in the basal parts of the mucosal folds (Fig. 2).

Epithelial kinetics: At 18 hr after BrdU administration, the leading edge of the BrdU-labeled epithelial cell cluster reached $64.5 \pm 4.5\%$, $60.9 \pm 2.3\%$ and $58.2 \pm 5.4\%$ levels from the crypt bottom in the apical and basal parts of the mucosal folds of the proximal colon and in the middle colon, respectively (Figs. 3a, 4a, 5a). Thereafter, the

Fig. 2. Depth of the intestinal crypts in the proximal and middle colon. *, column is significantly different from the other columns ($P < 0.01$). Each value represents mean $\pm$ S.D.

epithelial cells of the leading edge gradually migrated toward the crypt orifice (Figs. 3b, c, 4b-e, 5b, c). At 36, 48 and 36 hr after BrdU administration, the epithelial cells of the leading edge had been exfoliated into the intestinal lumen in the apical and the basal parts of the mucosal folds and in the middle colon, respectively (Figs. 3d-f, 4f, 5d-f). The lifespan was markedly longer in the basal parts of the mucosal folds than in the apical parts of the mucosal folds and in the middle colon (Fig. 6, Table 1). The migration speed per hour was also smaller in the basal parts of the mucosal folds than in the apical parts of the mucosal folds in the proximal colon; in the middle colon was between these two (Table 1).

The total number of crypt epithelial cells was significantly smaller in the basal parts of the mucosal fold than in the apical parts of the mucosal folds of the proximal colon; the middle colon was between these two. The number of PCNA-positive crypt epithelial cells was significantly smaller in the basal than in the apical parts of the mucosal folds and in the middle colon. The ratio of PCNA-positive cells showed a similar tendency (Fig. 7).

DISCUSSION

The fundamental settlement places of indigenous bacteria under physiological conditions have been identified as the apical portions of both the intestinal villi and dome of mucosal lymphatic follicles in rat small intestine [11, 44]. Indigenous bacteria contained in the chyme and the deep spaces of the mucosa are provided from these fundamental settlement sites [44]. The apices of both the intestinal villi and the dome of mucosal lymphatic follicles also are the shedding sites of effete epithelial cells at the late apoptotic stage [33]. Therefore, the stable settlement of indigenous bacteria is considered to be maintained by the delicate balance of the proliferation rate of indigenous bacteria over the exfoliation rate of effete apoptotic epithelial cells. On the other hand, the replacement of villous columnar

epithelial cells is accelerated in germ-free mice and rats which are reared in conventional conditions [1, 2]. In addition, our previous study clarified that the lifespan of villous columnar epithelial cells is shorter at sites with good proliferation of indigenous bacteria than at sites with no or less bacterial proliferation in the rat small intestine [35]. Therefore, the present finding that the lifespan of epithelial cells in the apical parts of the mucosal folds of the proximal colon and in the most luminal mucosa of the middle colon was shorter than in the basal parts of the mucosa of proximal colon suggests that the short lifespan might contribute to the expelling of indigenous bacteria which adhere to the apoptotic epithelial cells. However, the lifespan of epithelial cells is also changed by the length from the proliferation site to the exfoliation site. Therefore, the change of lifespan of epithelial cells might not be accurately reflected in the acceleration/deceleration of the epithelial kinetics, when the length of the migration distance is changed by the acceleration of the epithelial expelling. Our previous study clarified that the migration speed of villous columnar epithelial cells contributes to the regulation of proliferation and settlement of indigenous bacteria in the rat small intestine [35]. The migration speed of epithelial cells is considered to be more reflected in the changes of epithelial kinetics caused by the expansion of bacterial colonies, because the migration speed is determined by the migration distance during a unit time. Therefore, the present finding that the migration speed of epithelial cells in the apical parts of the mucosal folds of proximal colon and in the most luminal mucosa of middle colon was greater than in the basal parts of the mucosa in proximal colon, suggests that the acceleration of epithelial cell migration might represent an increase of the exfoliation rate of apoptotic epithelial cells with indigenous bacteria in the large intestine.

In general, the tendency of indigenous bacteria to increase toward the lower alimentary tract has been confirmed in various animal species; healthy humans [6, 19, 27, 36], dogs [12], cattle [31], mice [43] and rats [25, 44]. To the contrary, the migration speed of villous columnar epithelial cells decreases toward the ileum in rat small intestine [21, 35]. In the rat large intestine, a large amount of indigenous bacteria reside in the inter-mucosal fold spaces of the initial 30% portion and invade into the intestinal crypt lumen, whereas no indigenous bacteria invade into the intestinal crypt lumen of the distal 70% portion [44]. In the present study, a large amount of indigenous bacteria and lack of chyme were confirmed in the inter-mucosal fold spaces and their intestinal crypt lumen. Further, the migration speed was less and the lifespan of epithelial cells was greater in the basal parts of mucosal folds than in the apical parts of the mucosal folds of the proximal colon and in the middle colon. Therefore, the elongation of the lifespan and the deceleration of migration of epithelial cells might contribute to the reliable settlement of indigenous bacteria in the basal parts of the mucosal folds, enabling the maintenance of a large amount of

Figs. 3–5. BrdU-labeled epithelial cells in the apical (Fig. 3) and basal portions (Fig. 4) of mucosal folds in the proximal colon and in the middle colon (Fig. 5) at 18 (a), 24 (b), 30 (c), 36 (d), 42 (e) and 48 hr (f) after BrdU administration. The arrows represent the epithelial cell of the leading edge of BrdU-labeled epithelial cell cluster. Bar=10 μ m.

indigenous bacteria in the proximal colon.

In the small intestine, epithelial cells proliferate from the undifferentiated epithelial cells in the intestinal crypts, undergo the induction of apoptosis in the migration process,

and finally exfoliate into the intestinal lumen [8, 10, 18, 33, 38, 41]. The villous length is considered to be maintained by the balance between epithelial cell generation and epithelial exfoliation. In the large intestine, the depth of the

Table 1. Lifespan and migration speed of the crypt epithelial cells in the proximal and middle colon of rats

	Proximal colon		Middle colon
	Apical portion of mucosal fold	Basal portion of mucosal fold	
Lifespan of epithelial cells (hr)	32.2	42.5	33.6
Migration speed of epithelial cells ($\mu\text{m/hr}$) ^{a)}	4.2	2.1	3.3

a) Migration speed of BrdU-labeled epithelial cells per 1 hr was calculated from the migration distance between the mean position of BrdU-labeled epithelial cells at 18 hr and that at 24 hr.

Fig. 6. Cell kinetics of the BrdU-labeled epithelial cells in the proximal and middle colon. Black dots, apical portion of mucosal fold in the proximal colon; white dots, basal portion of mucosal fold in the proximal colon; white squares, middle colon. Each dot expresses the mean position of the leading edges of BrdU-labeled epithelial cells at each time after BrdU administration. Each value represents mean $\pm$ S.D.

Fig. 7. Number of total epithelial cells (light columns) and PCNA-positive epithelial cells (middle gray columns) per intestinal crypt in histological section. The black columns represent the ratio of PCNA-positive epithelial cells to total epithelial cells. *, portion is significantly different from the other portion ($P < 0.01$). Each value represents mean $\pm$ S.D.

intestinal crypts is also considered to be maintained by the balance between epithelial expelling and production. In 7-week-old Wistar rats, the amount of indigenous bacteria increased toward the distal small intestine, accompanying the decrease in the height of the intestinal villi [35, 44]. In the colon, the depth of the intestinal crypts was less in the proximal colon than in the distal colon in 3-month-old Sprague-Dawley rats [17]. The rearing of germ-free rats in conventional conditions, makes the depth of intestinal crypts longer in the middle colon [2]. The administration of human feces to germ-free rats decreases the depth of intestinal crypts of the proximal colon [37]. In the present study, the crypt depth, total number of crypt epithelial cell and number of PCNA-positive epithelial cell were significantly larger in the apical portions of mucosal folds in the proximal colon and in the middle colon than in the basal portions of mucosal folds of the proximal colon. Therefore, up-/down-regulation of indigenous bacteria in the most luminal mucosa and the deep mucosa is also confirmed by these structural differences in the intestinal crypts of the rat colon.

Proliferation of intestinal indigenous bacteria is regulated by the secretion of various soluble factors and the elimination system in the intestinal walls themselves, in addition to the regulation of epithelial cell kinetics under physiological conditions [22–24, 32, 35]. The rearing of germ-free rats in conventional condition, decreases the neutral mucins of goblet cells in the proximal colon [37]. Goblet cells are decreased in specific pathogen free mice in comparison with germ-free ones. Therefore, the formation of intestinal microflora leads to a decrease in the mucin secretion [20]. Thus, the difference in regulation of indigenous bacteria between the basal portions of the mucosal folds in the proximal colon and the middle colon might be caused by other defense factors such as the regulation of the host's mucin secretion, in addition to the regulation of epithelial kinetics.

ACKNOWLEDGMENT. This work was financially supported in a part by Grant-in-Aid for Scientific Research (no. 19580339) from the Japan Society for the Promotion of Science.

REFERENCES

- Abrams, G. D., Bauer, H. and Sprinz, H. 1963. Influence of the normal flora on mucosal morphology and cellular renewal in the ileum. A comparison of germ-free and conventional mice. *Lab. Invest.* **12**: 355–364.
- Alam, M., Midtvedt, T. and Uribe, A. 1994. Differential cell kinetics in the ileum and colon of germfree rats. *Scand. J. Gastroenterol.* **29**: 445–451.
- Appleton, D. R., Al-Dewachi, H. S., Morley, A. R., de Rodriguez, M. S., Sunter, J. P., Watson, A. J. and Wright, N. A. 1983. Autoradiographic investigations of cell proliferation in the small and large bowel of the mouse, and the jejunal response to some abnormal conditions. *Acta Histochem.* **27**: 185–194.
- Barthel, L. K. and Raymond, P. K. 1990. Improved method for obtaining 3-microns cryosections for immunocytochemistry. *J. Histochem. Cytochem.* **38**: 1383–1388.
- Batt, R. M., Rutgers, H. C. and Sancak, A. A. 1996. Enteric bacteria: friend or foe? *J. Small Anim. Pract.* **37**: 261–267.
- Berg, R. D. 1996. The indigenous gastrointestinal microflora. *Trends Microbiol.* **4**: 430–435.
- Bhalla, D. K. and Owen, R. L. 1982. Cell renewal and migration in lymphoid follicles of Peyer's patches and cecum—an autoradiographic study in mice. *Gastroenterology* **82**: 232–242.
- Chang, W. W. L. and Leblond, C. P. 1971. Renewal of the epithelium in the descending colon of the mouse. I. Presence of three cell populations: Vacuolated-columnar, mucous and argentaffin. *Am. J. Anat.* **131**: 73–100.
- Chang, W. W. L. and Nadler, N. J. 1975. Renewal of the epithelium in the descending colon of the mouse. IV. Cell population kinetics of vacuolated- columnar and mucous cells. *Am. J. Anat.* **144**: 39–56.
- Cheng, H. and Leblond, C. P. 1974. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine I. Columnar cell. *Am. J. Anat.* **141**: 461–480.
- Chin, K., Onishi, S., Yuji, M., Inamoto, T., Qi, W.-M., Warita, K., Yokoyama, T., Hoshi, N. and Kitagawa, H. 2006. Differentiation of epithelial cells to M cells in response to bacterial colonization on the follicle-associated epithelium of Peyer's patch in rat small intestine. *J. Vet. Med. Sci.* **68**: 1023–1028.
- Davis, C. P., Cleven, D., Balish, E. and Yale, C. E. 1977. Bacterial association in the gastrointestinal tract of beagle dogs. *Appl. Environ. Microbiol.* **34**: 194–206.
- Gordon, J. I. 1989. Intestinal epithelial differentiation: New insights from chimeric and transgenic mice. *J. Cell Biol.* **108**: 1187–1194.
- Grant, T. D. and Specian, R. D. 2001. Epithelial cell dynamics in rabbit cecum and proximal colon P1. *Anat. Rec.* **246**: 427–437.
- Grossmann, J., Walter, K., Artinger, M., Rümmele, P., Woenckhaus, M. and Schölmerich, J. 2002. Induction of apoptosis before shedding of human intestinal epithelial cells. *Am. J. Gastroenterol.* **97**: 1421–1428.
- Guarner, F. and Malagelada, J. R. 2003. Gut flora in health and disease. *Lancet* **361**: 512–519.
- Hammann, A., Arveux, P. and Martin, M. 1992. Effect of gut-associated lymphoid tissue on cellular proliferation in proximal and distal colon of the rat. *Dig. Dis. Sci.* **37**: 1099–1104.
- Han, H., Iwanaga, T. and Fujita, T. 1993. Species-differences in the process of apoptosis in epithelial cells of the small intestine: An ultrastructural and cytochemical study of luminal cell elements. *Arch. Histol. Cytol.* **56**: 83–90.
- Hao, W. L. and Lee, Y. K. 2004. Microflora of the gastrointestinal tract: a review. *Methods Mol. Biol.* **268**: 491–502.
- Hill, R. R. and Cowley, H. M. 1988. Effects of bacterial colonization on mucosal structure of the large bowel of neonatal rats. *Acta Anat.* **132**: 230–233.
- Holt, P. R., Kotler, D. P. and Pascal, R. R. 1983. A simple method for determining epithelial cell turnover in small intestine. Studies in young and aging rat gut. *Gastroenterology* **84**: 69–74.
- Inamoto, T., Kawata, Y., Qi, W.-M., Yamamoto K., Warita, K., Kawano, J., Yokoyama, T., Hoshi, N. and Kitagawa, H. 2008. Ultrastructural study on the epithelial responses against attachment of indigenous bacteria to epithelial membranes in Peyer's patches of rat small intestine. *J. Vet. Med. Sci.* **70**: 235–241.
- Inamoto, T., Namba, M., Qi, W.-M., Yamamoto, K., Yokoo, Y., Miyata, H., Kawano, J., Yokoyama, T., Hoshi, N. and Kitagawa, H. 2008. An immunohistochemical demonstration of the contribution of actin and myosin to physical elimination of adhered indigenous bacteria from mucous epithelial cells in Peyer's patches and intestinal villi of the rat jejunioileum. *J. Vet. Med. Sci.* **11**: 1153–1158.
- Insoft, R. M., Sanderson, I. R. and Walker, W. A. 1996. Development of immune function in the intestine and its role in neonatal diseases. *Pediatr. Clin. North Am.* **43**: 551–571.
- Itoh, K., Tamura, H. and Mitsuoka, T. 1989. Gastrointestinal flora of cotton rats. *Lab. Anim.* **23**: 62–65.
- Jacobs, L. R. and Schneeman, B. O. 1981. Effects of dietary wheat bran on rat colonic structure and mucosal cell growth. *J. Nutr.* **70**: 798–803.
- Kararli, T. T. 1995. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* **16**: 351–380.
- Klein, R. M. and Mckenzie, J. C. 1983. The role of cell renewal in the ontogeny of the intestine. I. Cell proliferation patterns in adult, fetal, and neonatal intestine. *J. Pediatr. Gastroenterol. Nutr.* **2**: 10–43.
- Klein, R. M. and Mckenzie, J. C. 1983. The role of cell renewal in the ontogeny of the intestine. II. Regulation of cell proliferation in adult, fetal, and neonatal intestine. *J. Pediatr. Gastroenterol. Nutr.* **2**: 204–228.
- Lipkin, M., Sherlock, P. and Bell, B. 1963. Cell proliferation kinetics in the gastrointestinal tract of man. II. Cell renewal in stomach, ileum, colon and rectum. *Gastroenterology* **45**: 721–729.
- Maki, L. R. and Picard, K. 1965. Normal intestinal flora of cattle fed high-roughage rations. *J. Bacteriol.* **89**: 1244–1249.
- Mantis, N. J., Frey, A. and Neutra, M. R. 2000. Accessibility of glycolipid and oligosaccharide epitopes on rabbit villus and follicle-associated epithelium. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**: G915–G923.
- Onishi, S., Miyata, H., Inamoto, T., Qi, W.-M., Yamamoto, K., Yokoyama, T., Warita, K., Hoshi, N. and Kitagawa, H. 2007. Immunohistochemical study on the delayed progression of epithelial apoptosis in follicle-associated epithelium of rat Peyer's patch. *J. Vet. Med. Sci.* **69**: 1123–1129.
- Pozharisski, K. M., Klimashevski, V. F. and Gushchin, V. A. 1980. Study of kinetics of epithelial cell populations in normal tissues of the rats intestines and in carcinogenesis. I. A comparison of enterocyte population kinetics indifferent segments of the small intestine and colon. *Exp. Pathol.* **18**: 387–406.

35. Qi, W.-M., Yamamoto, K., Yokoo, Y., Miyata, H., Inamoto, T., Udayanga, K. G., Kawano, J., Yokoyama, T., Hoshi, N. and Kitagawa, H. 2009. Histoplanimetric study on the relationship between the cell kinetics of villous epithelial cells and the proliferation of indigenous bacteria in rat small intestine. *J. Vet. Med. Sci.* **71**: 463–470.
36. Salminen, S., Isolauri, E. and Onnela, T. 1995. Gut flora in normal and disordered states. *Chemotherapy* **41**: 5–15.
37. Sharma, R., Schumacher, U., Ronaasen, V. and Coates, M. 1995. Rat intestinal mucosal responses to a microbial flora and different diets. *Gut* **36**: 209–214.
38. Shibahara, T., Sato, N., Waguri, S., Iwanaga, T., Nakahara, A., Fukutomi, H. and Uchiyama, Y. 1995. The fate of effete epithelial cells at the villus tips of the human small intestine. *Arch. Histol. Cytol.* **58**: 205–219.
39. Shorter, R. G., Moertel, C. G., Titus, J. L. and Reitemeier, R. J. 1964. Cell kinetics in the jejunum and rectum of man. *Am. J. Dig. Dis.* **9**: 760–763.
40. Sudo, N., Sawamura, S., Tanaka, K., Aiba, Y., Kubo, C. and Koga, Y. 1997. The requirement of intestinal bacterial flora for the development of an IgE production system fully susceptible to oral tolerance induction. *J. Immunol.* **159**: 1739–1745.
41. Takeuchi, T., Kitagawa, H., Imagawa, T. and Uehara, M. 1998. Proliferation and cellular kinetics of villous epithelial cells and M cells in the chicken caecum. *J. Anat.* **193**: 233–239.
42. Takeuchi, T., Kitagawa, H., Imagawa, T. and Uehara, M. 1999. Apoptosis of villous epithelial cells and follicle-associated epithelial cells in chicken cecum. *J. Vet. Med. Sci.* **61**: 149–154.
43. Tannock, G. W. and Savage, D. C. 1974. Influences of dietary and environmental stress on microbial populations in the murine gastrointestinal tract. *Infect. Immun.* **9**: 591–598.
44. Yamamoto, K., Qi, W.-M., Yokoo, Y., Miyata, H., Udayanga, K. G. S., Kawano, J., Yokoyama, T., Hoshi, N. and Kitagawa, H. 2009. Histoplanimetric study on the spatial relationship of indigenous bacteria and mucosal lymphatic follicles throughout alimentary tract of rat. *J. Vet. Med. Sci.* **71** (5) (in press).