FULL PAPER *Anatomy*

Histoplanimetrical Study on the Relationship between the Cell Kinetics of Villous Columnar Epithelial Cells and the Proliferation of Indigenous Bacteria in Rat Small Intestine

Wang-Mei QI[1)], Kenkichi YAMAMOTO[1)], Yuh YOKOO[2)], Hidenori MIYATA[2)], Tetsurou INAMOTO[1)], Kankanam Gamage Sanath UDAYANGA[2)], Junichi KAWANO[2)], Toshifumi YOKOYAMA[2)], Nobuhiko HOSHI[2)] 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

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ABSTRACT. The relationship between the kinetics of villous columnar epithelial cells and the expansion of colonies of indigenous bacteria from the narrow apical portions of intestinal villi was immunohistochemically and histoplanimetrically investigated in the small intestine of bromodeoxyuridine administred Wistar rats. As a result, the lifespan of villous columnar epithelial cells was slightly shorter in the distal ileum than in other portions of small intestine, accompanying the minimum height of the intestinal villi of the distal ileum in the small intestine. The migration speed of villous columnar epithelial cells was significantly decreased toward the distal small intestine. The migration speed in the distal ileum was about one-fourth of that in the duodenum. The migration speed of the villous columnar epithelial cells was greater and their lifespans were shorter in the sites with wide expansion of the indigenous bacterial colony from the narrow apical portions of the intestinal villi than that in sites with no or less expansion. Additionally, the expansion of the indigenous bacterial colony from narrow villous apices also immediately shortened the heights of the intestinal villi. These findings suggest that the migration speed of villous columnar epithelial cells might contribute to the regulation of the settlement of bacteria at the villous apices and the inevitable proliferation of indigenous bacteria at the intervillous spaces in the rat small intestine.

KEY WORDS: apotosis, enteric bacteria, gastrointestinal tract, host defence, immuno-histochemistry.

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Numerous and many species of indigenous bacteria reside and form the normal microflora in human and other mammalian alimentary tracts. These indigenous bacteria provide various benefits to the host, such as interference against newly invading pathogenic bacteria, the production of short fatty acids and vitamins, and the enhancement of oral immunological tolerance [4, 38]. The indigenous bacteria also sometimes cause opportunistic infections [5], accelerate some inflammatory bowel diseases and generate colonic cancers [12]. In healthy humans, the luminal contents contain 0–10^3 colony forming units (CFU) per m*l* in the stomach, 0–10^5 CFU/m*l* in the jejunum, 10^3–10^9 CFU/m*l* in the ileum and 10^{10}–10^{12} CFU/m*l* in the colon [5, 14, 21, 32]. This increasing tendency of indigenous bacteria toward the distal alimentary tract is similar with that in rats [20], mice [42], dogs [10] and cows [26]. The numbers of indigenous bacteria are generally maintained in the alimentary tract under a physiological condition [32], whereas the numbers are sometimes changed under the effects from foods and the environmental stress [42] or the invasion of pathogenic bacteria [12]. It has been considered that the settlement and proliferation of intestinal indigenous bacteria are complicatedly regulated by non-specific host defense mechanisms, such as motility by various contractile histological elements, the physical barrier of a thick mucous layer, the secretion of bactericidal substances, the chemical and physical elimination system of villous columnar epithelial cells themselves and the rapid replacement of their cells [17–19, 27].

In the small intestines, the villous columnar epithelial cells that are generated in the intestinal crypts migrate toward the apices of the intestinal villi. During this migration process, apoptosis is induced in the villous columnar epithelial cells, which are ultimately exfoliated into the intestinal lumen [13, 30, 34, 40, 41]. The fundamental settlement place of indigenous bacteria is the apical portions of both the intestinal villi and the domes of lymphatic follicles in Peyer's patches in the rat small intestine [8, 17]. These apices are unstable sites where apoptotic epithelial cells are incessantly shed into the intestinal lumen. The kinetics of villous columnar epithelial cells have been actively studied under steady physiological conditions in humans [24, 36], mice [6, 7, 11, 22, 23, 25, 28], rats [15, 22, 23] and chickens [16, 31, 37, 40]. On the other hand, the replacement of villous columnar epithelial cells is accelerated when bacteria are orally administrated to germ-free mice [1] and rats [2]. The replacement of villous columnar epithelial cells is also accelerated under helminth infection in germ-free rats [39]. Thus, the induction of new microorganisms and helminths changes the cell kinetics of villous columnar epithelial cells in the intestine. However, it has never been clarified how the cell kinetics of villous columnar epithelial cells concretely regulate the settlement

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

and the proliferation of indigenous bacteria in the intestine. In this paper, the relationship between the cell kinetics of villous epithelial cells and the degree of proliferation of indigenous bacteria was histoplanimetrically investigated in the rat small intestine.

MATERIALS AND METHODS

Animals: A total of 60 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, Nosan Corp., Yokohama, Japan) and water. The animal facility was maintained under conditions of a 12-hr light/dark cycle at 21 ± 1°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 numbers: 14–4–09, 17–04–05) and carried out according to the Kobe University Animal Experimentation Regulations.

Experimental protocols: The rats were divided into the following two experiments. Experiment 1 attempted to clarify the lifespan and migration speed of villous columnar epithelial cells in each segment of the small intestine. Experiment 2 attempted to clarify the relationship between the degree of expansion of indigenous bacterial colonies and the epithelial kinetics.

In Experiment 1, 5 rats each were sacrificed by an *i.p.* injection of pentobarbital sodium (Dainippon Sumitomo Pharmaceuticals, Osaka, Japan) at 12, 18, 24, 36, 48 and 60 hr after intraperitoneal injection of bromodeoxyuridine (BrdU; 50 mg/kg, Sigma-Aldrich, St. Luis, U.S.A.) in saline. In Experiment 2, 5 rats each were sacrificed by the same method at 18, 24, 30, 36, 42 and 48 hr after *i.p.* BrdU injection.

Tissue preparation: The animals were intravascularly perfused with 4% paraformaldehyde-containing periodate-lysine-paraformaldehyde fixative (PLP; pH 6.2, 38°C). After perfusion, the small intestine was divided into 5 segments: duodenum, proximal jejunum, distal jejunum, proximal ileum and distal ileum. One tissue block was removed from each intestinal segment in Experiment 1, and 3 tissue blocks were removed from each intestinal segment in Experiment 2. 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 μm 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).

Immunohistochemistry: The detection of antigens was conducted using the peroxidase-anti peroxidase (PAP) method. Briefly, after rinsing with 0.05% Tween-added phosphate buffered saline (PBS; pH 7.4), the sections were incubated in 0.5 N HCl solution for 5 min at 45°C. Then, the sections were followed by immersion in absolute methanol and 0.5% 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, Sanbio, Uden, the Netherlands) for 18 hr at 4°C. Then the sections were incubated with anti mouse IgG rat IgG (diluted at 1:50, Jackson ImmunoResearch. Lab., West Grove, U.S.A.) for 1 hr at r.t., followed by mouse PAP-complex (diluted at 1 : 50, Seikagaku Corp., Tokyo, Japan) 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.

Histoplanimetry in Experiment 1: Intestinal villi whose central axes had been longitudinally cut were randomly chosen from each intestinal segment. The mean height of the intestinal villi was estimated from 20 villi per each intestinal segment from each of the 20 randomly selected animals. The height of each intestinal villus was measured from the upper extremity of the lowest epithelial cell nucleus to that of the highest one in the same villus. The mean depth of the intestinal crypt was estimated from 20 crypts per each intestinal segment from each of the 20 animals. The depth of each intestinal crypt was measured from the upper extremity of the lowest epithelial cell nucleus to that of the highest one in the same crypt.

The distribution of the BrdU-labeled villous columnar epithelial cells, except for the labeled intraepithelial migrating cells, was measured in 20 randomly selected longitudinal sections of intestinal villi per each intestinal segment from each of 5 animals. The mean nuclear position of the leading edge of the BrdU-labeled epithelial cell cluster along the entire length of the crypt-villus axis was calculated as a percentage from the bottom of the intestinal crypt. The approximate lifespan of the BrdU-labeled villous columnar epithelial cells was estimated from the distribution of the positions of the leading edges of positive columnar epithelial cell clusters at each time after BrdU administration.

The migration speed of BrdU-labeled villous columnar epithelial cells per 1 hr was calculated from the migration distance of the labeled villous columnar epithelial cells, that is, the difference between the mean position of BrdU-labeled villous columnar epithelial cells at 18 hr after BrdU administration and that at 24 hr.

Histoplanimetry in Experiment 2: To clarify the relationship between the degree of indigenous bacterial proliferation and epithelial cell kinetics, sites with various degree of indigenous bacterial proliferation were preliminarily detected in hematoxylin-eosin stained sections from all specimens at each time after BrdU administration. An insufficient number of sites with a high degree of indigenous bacterial proliferation were found in each intestinal segment from the duodenum to the proximal ileum at each time after BrdU administration. Therefore, the

relationship between the degree of indigenous bacterial proliferation and the epithelial cell kinetics was investigated in the distal ileum. At first, the similarities with Experiment 1 was confirmed in the lifespan of BrdU-labeled villous columnar epithelial cells, the height of intestinal villi and the depth of intestinal crypts.

The degree of proliferation of bacterial colonies was estimated as the relative position of the lowest edge of the bacterial colony which diffusely expanded from the villous apex into the intervillous space, from the villous apex in the entire length of the intestinal villus in the distal ileum. The migration speed of villous columnar epithelial cells was estimated from the migration distance during the initial 18 hr after BrdU administration in 10 intestinal villi, in which the lowest edge of indigenous bacterial colony reached almost the same position within each 10% portion of the height of the intestinal villi. The height of the intestinal villi and the depth of the intestinal crypts were also measured from the same the intestinal villi in each 10% portion of the lowest edges of indigenous bacterial colonies.

The lifespan of villous columnar epithelial cells was estimated from the mean positions of BrdU-labeled villous columnar epithelial cells from 10 intestinal villi, at sites with indigenous bacterial colonies reaching only the 0–20% position of intervillous spaces (distance from the apex=20% of villi height; low or no proliferation) and at sites reaching the 70–90% position of intervillous spaces (high proliferation), at each time after BrdU administration.

Statistical analysis: Data are presented as means ± standard deviation. The Kruskal-Wallis test was used to compare the means, followed by Steel-Dwass multiple comparisons. *P* values of less than 0.05 were considered statistically significant.

RESULTS

Differences of epithelial cell kinetics among intestinal segments: The height of intestinal villi gradually decreased from the duodenum toward the distal ileum, whereas the depth of intestinal crypts was almost the same in intestinal segments except for in the duodenum (Fig. 1).

In Experiment 1, BrdU-labeled epithelial cells appeared at the bases of intestinal villi from the intestinal crypts at 12 hr after BrdU administration (Fig. 2a). During the period 24–36 hr after BrdU administration, the labeled villous columnar epithelial cells migrated toward the villous apex. The epithelial cells of the leading edge of BrdU-labeled epithelial cell clusters had already exfoliated into the intestinal lumen in the proximal jejunum and the distal ileum at 36 hr, and in the duodenum, distal jejunum and proximal ileum at 48 hr (Fig. 2b). The lifespans of villous columnar epithelial cells were almost the same: 38.2, 36.0, 39.4 and 38.9 hr in the duodenum, proximal and distal jejunum, and proximal ileum, respectively; the exception was the slightly shorter 34.4 hr lifespan in the distal ileum.

The migration speed of BrdU-labeled epithelial cells per 1 hr was significantly decreased from the duodenum to the distal ileum. The migration speed in the distal ileum was about one-fourth that in the duodenum (Table 1).

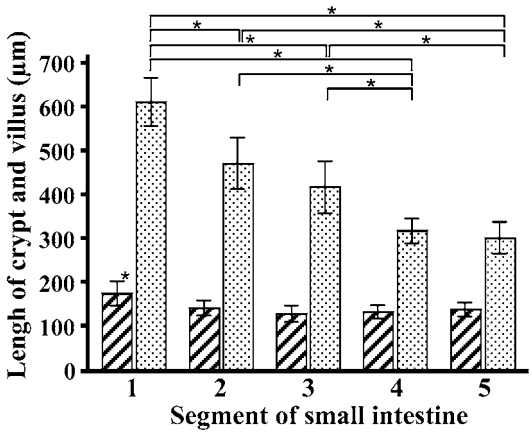

Fig. 1. The height of intestinal villi (dotted columns) and the depth of intestinal crypts (oblique columns) in the rat small intestine. (1) duodenum; (2) proximal jejunum; (3) distal jejunum; (4) proximal ileum; (5) distal ileum. Asterisks: $P<0.01$. Each value represents mean ± S.D.

In all intestinal segments, no Paneth cells were labeled in the bases of intestinal crypts during the time course.

Changes of epithelial cell kinetics under bacterial proliferation: In Experiment 2, the lifespan of the villous columnar epithelial cells, the height of intestinal villi and the depth of intestinal crypts in each intestinal segment were very similar with those in Experiment 1 (data not shown). At 18 hr after BrdU administration, the position of the leading edge of the labeled epithelial cell cluster in the distal ileum differed according to the degree of expansion of the indigenous bacterial colonies associated with the villi, regardless of the morphological differences and the densities of indigenous bacteria (Fig. 3a-d).

The lifespan of villous columnar epithelial cells was 26.3 hr in the sites with an indigenous bacterial colony reaching 70–90% the height of the villus in the intervillous space (high proliferation), whereas it was 34.7 hr in sites with an indigenous bacterial colony reaching the 20% position (low proliferation) (Fig. 4a).

The migration speed of the villous columnar epithelial cells was the lowest in the sites with indigenous bacterial colony reaching the 0–10% position (low proliferation), whereas the migration speed was slightly greater in the sites with indigenous bacterial colony reaching the 20–50% positions from the villous tip. The maximum migration speed on columnar epithelial cells was reached at sites with indigenous bacterial colonies reaching the 50–90% position of intervillous space from the villous tip. No indigenous bacterial colony reaching the 90–100% position of intervillous space from the villous tip was observed in this study (Fig. 4b).

The depth of intestinal crypts was almost constant regardless of the expansion of the indigenous bacterial

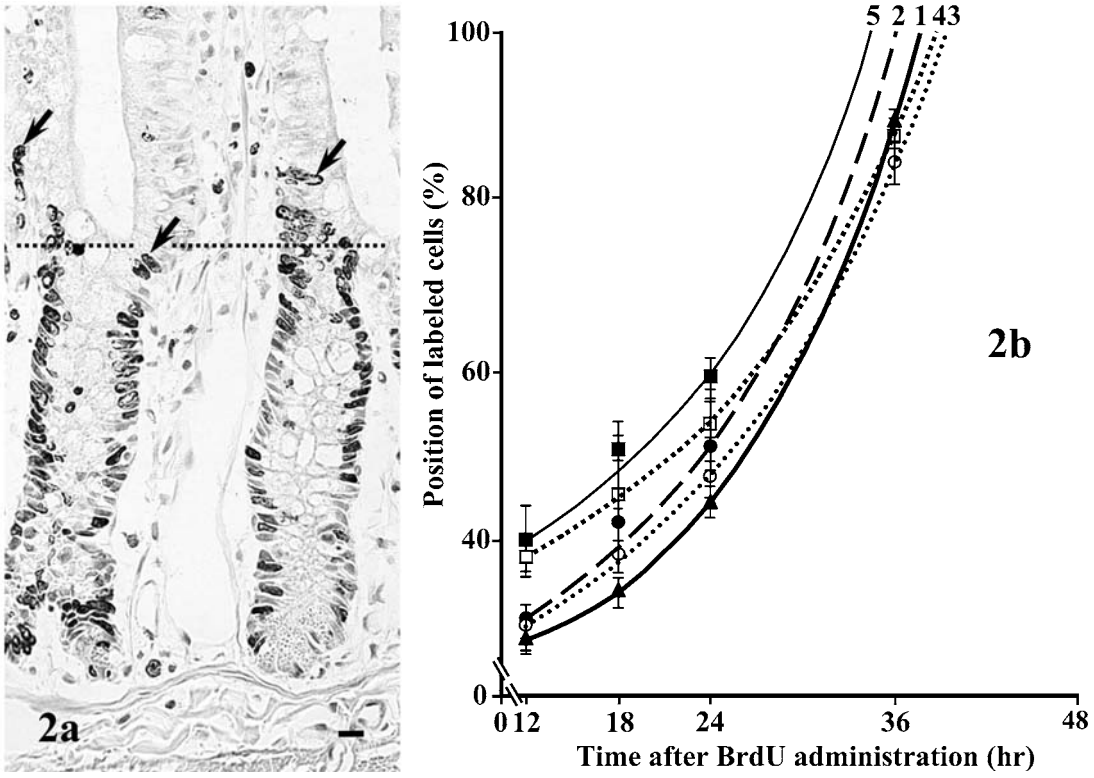

Fig. 2. a) Basal regions of intestinal villi and intestinal crypts in distal ileum at 12 hr after BrdU administration. Arrows, the leading edge of the BrdU-labeled villous columnar epithelial cells; dotted lines, orifices of intestinal crypts. Counterstained by hematoxylin. Bar=10 μm. b) Cell kinetics of the BrdU-labeled villous columnar epithelial cells in the small intestine. (1, ▲) duodenum; (2, ●) proximal jejunum; (3, ○) distal jejunum; (4, □) proximal ileum; (5, ■) distal ileum. Each dot expresses the mean position of the leading edges of BrdU-labeled villous columnar epithelial cells at each time after BrdU administration. Each value represents mean ± S.D.

Table 1. Migration speed of the BrdU-labeled villous columnar epithelial cells in rat small intestine

	Duodenum	Proximal jejunum	Distal jejunum	Proximal ileum	Distal ileum
Migration speed of epithelial cells (μm/hr)[a)]	13.4	12.5	9.1	6.8	3.7

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.

colony in the distal ileum, while the height of the intestinal villi decreased sharply in sites with indigenous bacterial colonies over the 10% position of intervillous space from the villous tips and was maintained at the shortest level at sites with indigenous bacterial colonies reaching the 10–90% position of intervillous space from villous tips (Fig. 4c, d).

DISCUSSION

In the present study, the relationship between the kinetics of villous columnar epithelial cells and the expansion of colonies of indigenous bacteria was immunohistochemically and histoplanimetrically clarified in the small intestine of BrdU-administered Wistar rats.

In general, the lifespans of villous columnar epithelial cells are 42.7, 49.4 and 39.4 hr in the duodenum, jejunum and ileum, respectively, after ^{3}H-thymidine administration to 27-months-old Fisher 344 strain of rats [15]. In 4- to 5-month-old Fisher 344 rats, the lifespan of the villous columnar epithelial cells were 47.6, 52.8 and 32.8 hr in the duodenum, jejunum and ileum, respectively [15]. In the present study, the lifespan of villous columnar epithelial cells are 38.2, 36.0, 39.4, 38.9 and 34.4 hr in the duodenum,

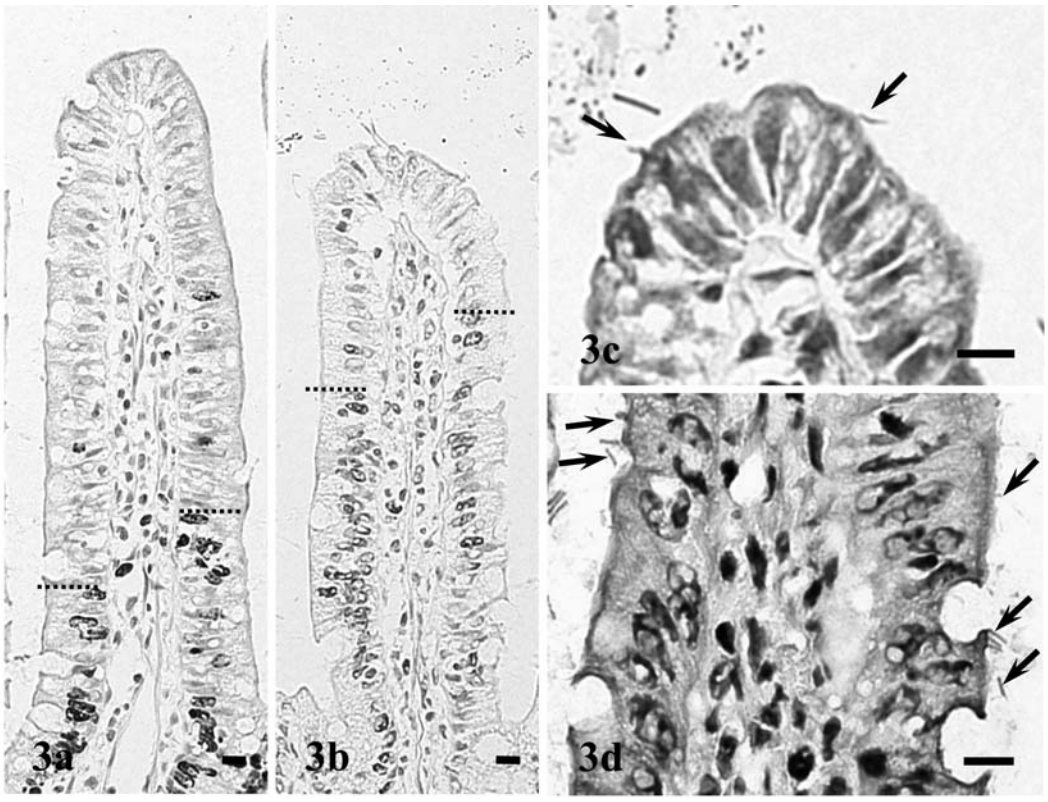

Fig. 3. a,b) Intestinal villi in distal ileum at 18 hr after BrdU administration. The leading edges (dotted lines) of BrdU-labeled villous columnar epithelial cell clusters are situated at a lower level of an intestinal villus at a site with no expansion of indigenous bacterial colony (a) compared to a site with an expanding colony (b). The intestinal villus in (b) is markedly shorter than that in (a). Counterstained by hematoxylin. Bar=10 μm. c) High-magnification photograph of the apex of intestinal villus at a site with no expansion of the indigenous bacterial colony. A serial section of (a). d) High-magnification photograph of the basal portion in the intestinal villus. A serial section of (b). In (c) and (d), the bacilli (arrows) attach to the epithelial surface. Hematoxylin-eosin staining. Bar=10 μm.

proximal and distal jejunum and proximal and distal ileum, respectively, in 7-week-old Wistar rats. From these findings, the lifespan of the villous columnar epithelial cells in the ileum, particularly the distal ileum, is by far the shortest in the rat small intestine, regardless of age or strain. In addition, the present study found an apparently shorter lifespan of villous columnar epithelial cells in well proliferating sites of indigenous bacteria than in less proliferating ones. On the other hand, the ileum, which is considered a transition zone between the relatively sparse microflora of the upper intestine and the tremendous numbers of bacteria found in the large intestine, maintains a more diverse microflora and higher bacterial populations [5]. Therefore, the shortened lifespan of villous columnar epithelial cells in the distal ileum might be a response to the increased total quantity of indigenous bacteria in the transition zone of the intestine.

The intestinal villi significantly reduce their heights from the duodenum to ileum in 4- to 5-month-old Fisher 344 rats [15]. In the 7-week-old Wistar rats used in the present study, the heights of intestinal villi also decreased significantly from the proximal small intestine to the distal ileum, in spite of uniform depths of intestinal crypts except for the duodenum. Particularly, the height of intestinal villi in the distal ileum was about one-half that of duodenal villi. Rearing of germ-free rats in the conventional condition makes the intestinal villi shorter, but does not affect the depth of intestinal crypts [2]. Oral administration of human feces to germ-free rats reduces the heights of both the intestinal villi and the intestinal crypts [33]. In the present study, the heights of intestinal villi were apparently shortened when the indigenous bacteria began proliferating toward the deep intervillous spaces in the distal ileum. In general, the villous columnar epithelial cells are produced in intestinal crypts, migrate to the apices, induced the stimulation of apoptosis in their migration processes and finally exfoliate from the apices of the intestinal villi. The intestinal villi are maintained by the balance between epithelial generation and epithelial exfoliation [13, 34]. In the present observation, the intestinal villi have markedly reduced heights despite a constant intestinal crypt depth, under the condition of indigenous bacterial expansion into the deep intervillous spaces. From these findings, the hyperproliferation of indigenous bacteria into the

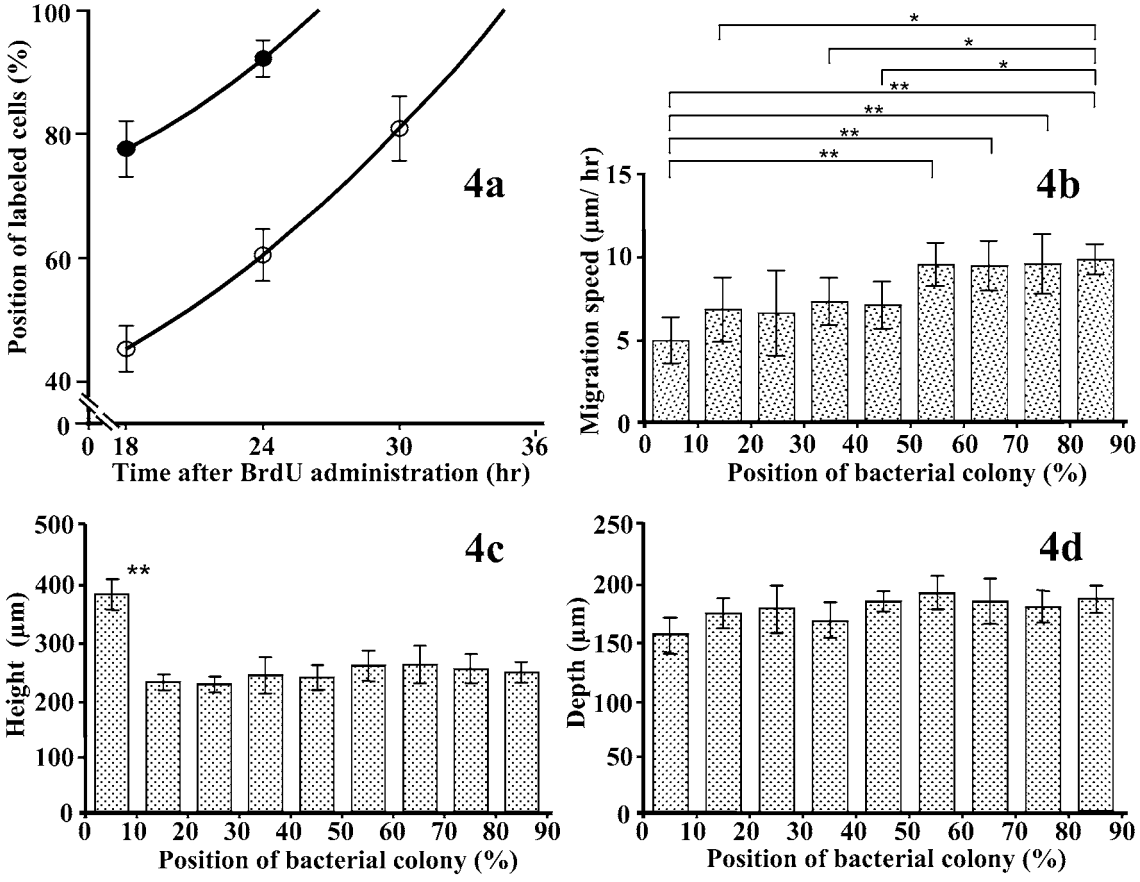

Fig. 4. a) Relationship between the lifespan of BrdU-labeled villous columnar epithelial cells and the degree of expansion of the associated indigenous bacterial colony in the distal ileum. Closed circles indicate the mean positions of the leading edge of the BrdU-labeled villous columnar epithelial cells at sites at which the indigenous bacterial colony reached the 70–90% position of the intervillous spaces from the villous tip (high proliferation). Open circles indicate the mean position of the leading edge of the BrdU-labeled villous columnar epithelial cells at sites with indigenous bacterial colonies within the 20% apical position of the intestinal villi (low proliferation). b) Relationship between the migration speed of BrdU-labeled villous columnar epithelial cells during the initial 18 hr after BrdU administration and the degree of expansion of the indigenous bacterial colony in the distal ileum. Asterisks, $P<0.05$; double asterisks, $P<0.01$. c,d) Relationship between the degree of expansion of the indigenous bacterial colony and the height of intestinal villus (c) and the depth of intestinal crypt (d) in the distal ileum. Double asterisks indicate a significant difference from other columns at the $P<0.01$ level. In (b-d), the horizontal axes indicate the relative position of the lowest edge of the indigenous bacterial colony from the villous tip. Each value represents mean ± S.D.

intervillous spaces is associated with the reduction of the heights of intestinal villi, owing to probable acceleration of the apoptotic process in the villous columnar epithelial cells.

The migration speed of villous columnar epithelial cells has been reported as 14.6, 11.3 and 8.7 μm/hr in the duodenum, jejunum and ileum of 4- to 5-month-old Fisher 344 rats, respectively [15]. In the present study, it was 13.4, 12.5, 9.1, 6.8 and 3.7 μm/hr in the duodenum, proximal and distal jejunum and proximal and distal ileum in 7-week-old Wistar rats, respectively. Thus, the migration speed of villous columnar epithelial cells in the distal ileum is the smallest in the rat small intestine. In addition, from the present study, the expansion of indigenous bacterial colonies to occupy half the height of the intervillous spaces resulted in increased migration speed of villous columnar epithelial cells compared with those in environments without development of indigenous bacterial colonies. The indigenous bacteria preferentially settle on the epithelial cells of the narrow apical portions of both the intestinal villi and the domes of mucosal lymphatic follicles [8, 9, 17]. The passing time of apoptotic epithelial cells with indigenous bacteria through the narrow spaces of the apical portions of intestinal villi is calculated to be within several hours from both the villous length and the lifespan of the BrdU-labeled villous columnar epithelial cells in Experiment 1. During this short time, the indigenous bacteria which adhere to epithelial cells incessantly proliferate and are in turn expelled by the physical and chemical activities of villous columnar epithelial cells [17, 18]. From these findings, the lowest migration speed of the epithelial cells, that is, the

longest "stay" of the adhered indigenous bacteria on the epithelial surface, might intensify the settlement of indigenous bacteria on the mucosal epithelium. Consequently, the migration speed is considered to be one of important regulatory factors against the settlement and inevitable proliferation of indigenous bacteria which adhere to the apoptotic epithelial cells in the apices of both intestinal villi and the domes of mucosal lymphatic follicles.

Recently, it has become widely known that Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) to induce the activation of innate immunity [29]. In swine, sTLR9, which recognizes bacterial DNA and induces the immune response, is expressed on the epithelial cells in the small intestine [35]. In the present study, the close relationship between indigenous bacterial proliferation and the change in the kinetics of villous columnar epithelial cells allows us to speculate that the villous columnar epithelial cells recognize the degree of indigenous bacterial proliferation through receptors such as TLRs. The expression of TLRs that contribute to the recognition of indigenous bacterial proliferation should be studied in the future.

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