FULL PAPER *Anatomy*

Peculiar Composition of Epithelial Cells in Follicle-Associated Intestinal Crypts of Peyer's Patches in the Rat Small Intestine

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ABSTRACT. The epithelial cell composition was investigated in the follicle-associated intestinal crypt (FAIC) of rat Peyer's patches. The epithelium of the FAIC mainly consisted of columnar epithelial cells, goblet cells and Paneth cells. The characteristics of secretory granules in Paneth cells and goblet cells of both the FAIC and ordinary intestinal crypts (IC) were almost the same in periodic acid-Schiff (PAS) reaction, Alcian blue (AB) staining and the immunohistochemical detection of lysozymes and soluble phospholipase A2. Both goblet cells and Paneth cells were markedly less frequent on the follicular sides than on the anti-follicular sides of the FAIC. Goblet cells were also markedly less frequent in the follicle-associated epithelium (FAE) than in the ordinary intestinal villi (IV). Indigenous bacteria were more frequently adhered to FAE than to follicle-associated intestinal villi or IV. These findings suggest that the host defense against indigenous bacteria is inhibited on the follicular sides of FAIC, which might contribute to the preferential settlement of indigenous bacteria on the FAE; they also suggest that differentiation into secretory cells is inhibited in the epithelium of the follicular sides of FAIC, so that differentiation into M cells might be admitted in the FAE of rat Peyer's patches. Furthermore, intermediate cells possessing characteristics of both Paneth cells and goblet cells were rarely found in the FAIC, but not in the IC. This finding suggests that the manner of differentiation into Paneth cells in the FAIC differs from that in the IC.

KEY WORDS: cell differentiation, goblet cell, Paneth cell, Peyer's patch, rat.

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Numerous indigenous bacteria settle in the alimentary tract of animals. The settlement of indigenous bacteria is regulated by specific and non-specific host defense. Non-specific host defense includes physical and chemical elimination from the epithelial cells themselves [11, 12], a thick mucus layer, digestive enzymes [3, 8], the secretion of several bactericidal substances [31, 32] and the regulation of epithelial cell proliferation [19, 25, 26], whereas specific host defense is induced *via* gut-associated lymphatic tissues (GALT) equipped throughout the alimentary tract [29]. The Peyer's patch which exists in the small intestine is a kind of aggregated lymphatic tissue which is the most extensively investigated among GALT. Peyer's patches mainly consist of four components, the follicle-associated epithelium (FAE), the dome area, the follicular area and the parafollicular area [4, 6]. Immune responses *via* Peyer's patch are initiated by the sampling of luminal antigens by M cells, which are specialized epithelial cells in the FAE [24]. Because of their importance, the process by which cells differentiate into M cells has been well investigated in various animals, such as chickens [16], pigs [21], mice [9] and rats [23]. M cells are generated by the follicle-associated intestinal crypts (FAIC) [9]; villous columnar epithelial cells of the follicle-associated intestinal villi (FAIV) are also generated by the FAIC. However, the peculiarity of the epithelium in the FAIC has not been fully clarified.

In the epithelium of the animal small intestine, cellular differentiation occurs from undifferentiated columnar epithelial cells in the ordinary intestinal crypts (IC). The following mature epithelial cells are mainly produced: villous columnar epithelial cells, goblet cells, Paneth cells and endocrine cells [5]. The importance of goblet cells and Paneth cells in the host defense against the indigenous bacteria has been well established. That is, goblet cells are abundantly present in the IC and secrete the mucus that forms the mucus layer which functions as a physical barrier against bacteria [17, 20]. Paneth cells secrete the various bactericidal

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peptides, such as lysozyme, soluble phospholipase A2 (sPLA2) and α-defensin [1, 13, 14, 31, 32]. However, the contribution to the host defense of the FAIC has been never clarified. Therefore, this study aimed to clarify the cell composition and morphological characteristics of the FAIC and to discuss the role of the FAIC in host defense against indigenous bacteria.

MATERIALS AND METHODS

Animals: Thirteen male Wistar rats aged 7 weeks (Japan SLC Inc., Hamamatsu, Japan) were maintained under conventional laboratory housing conditions. They were permitted free access to water and food (Lab MR Stock, Nosan Corp., Yokohama, Japan). The animal facility was maintained under conditions of a 12 hr light/dark cycle at 23 ± 1°C and 50–60% humidity. Clinical and pathological examinations in all animals confirmed no signs of disorder. This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 19-05-07 and 22-05-01) and carried out according to the Kobe University Animal Experimentation Regulations.

Tissue preparation: After euthanasia with an intraperitoneal injection of 200 mg/kg of pentobarbital sodium (Kyoritsu Seiyaku Corp., Tokyo, Japan), small tissue blocks of Peyer's patches were removed from the ileum. All tissue blocks were immersion-fixed in 4.0% paraformaldehyde fixative in 0.1 M phosphate buffer (PB, pH 7.4) for 24 hr at 4°C and then were snap-frozen in liquid nitrogen with reference to the embedding method described by Barthel and Raymond (1990) [2]. Four-μm-thick sections 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) and stored at −20°C until use.

Polysaccharide staining: Ileal samples from 5 rats were used for polysaccharide staining. For the detection of neutral mucin, the periodic acid Schiff (PAS) reaction was conducted by the routine method. For the detection of acidic mucin, Alcian blue (AB) stainings at pH 1.0 and 2.5 were also conducted by routine methods.

Immunohistochemistry: Detection of antigens was conducted using the indirect method of enzyme immunohistochemistry with ileal samples from 5 rats. Briefly, after rinsing with 0.05% Tween-added 0.01 M phosphate buffered saline (TPBS; pH 7.4), the sections were autoclaved for 20 min at 121°C for antigen retrieval. Then, all sections were immersed in absolute methanol and 0.5% H_2O_2 for 30 min, respectively. Three times TPBS rinse was performed after all preparation steps to remove any reagent residues. Following blocking with Blocking One Histo (Nacalai Tesque Inc., Kyoto, Japan) for 1 hr at r.t., the sections were reacted with anti lysozyme (C-19, diluted at 1:800) or anti sPLA2 (M-18, diluted at 1:200) goat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) for 18 hr at 6°C. The antibody specificity for rat lysozyme and sPLA2 is described in the manufacturer's specification form (lysozyme, sc-27958; sPLA2, sc-14472), respectively. Then, the sections were incubated with horseradish peroxidase-conjugated anti goat IgG mouse IgG (AP186P) (diluted at 1:200; Chemicon International Inc., Billerica, MA, U.S.A.) for 1 hr at r.t. Finally, the sections were incubated with 3,3′-diaminobenzidine (Dojindo Lab., Mashiki, Japan) containing 0.03% H_2O_2 and were counterstained with hematoxylin. Control sections were incubated with TPBS or non-immunized goat IgG instead of the primary antibody.

Histoplanimetry: For the following histoplanimetry, sections containing ileal Peyer's patches were stained with hematoxylin-eosin.

The numbers of FAE, FAIV and IV with or without bacterial adherence were counted in 20 FAE, 40 FAIV and 200 IV randomly chosen from ileal samples of 12 rats. The relative frequencies of FAE, FAIV or IV with bacterial adherence were calculated.

The relative frequencies of goblet cells and Paneth cells were counted in the epithelia of both the follicular and anti-follicular sides of 5 FAIC from 5 rats. The means were calculated, and the data are presented as means ± standard deviations (SDs).

The relative frequencies of goblet cells and Paneth cells were calculated in the three portions, apical, middle or basal portions, of both the follicular and anti-follicular sides of 25 FAIC randomly chosen from 5 rats. The means were calculated, and the data are presented as means ± SDs.

The numbers of goblet cells per 50-μm-long epithelial segments from each region (apical, middle and basal) were counted in 10 FAE and 10 IV randomly chosen from ileal samples of 5 rats. The means were calculated, and the data are presented as means ± SDs.

Statistical analysis: Pearson's Chi-square test was performed for the comparison among FAE, FAIV and IV with bacterial adherence. The Mann-Whitney *U* test was performed for the comparison of the relative frequency of goblet cells or Paneth cells between the follicular and anti-follicular sides in FAIC. For the comparison of the relative frequency of goblet cells or Paneth cells among the apical, middle and basal portions of follicular and anti-follicular sides in FAIC, the Kruskall-Wallis test was performed first; then, the Mann-Whitney *U* test, and finally the Bonferroni correction were performed. For the comparison of goblet cell numbers between FAE and IV, the normality of distribution was assessed by the Shapiro-Wilk test, and statistical analysis was performed with Student's *t* test for parametric variables and the Mann-Whitney *U* test for non-parametric variables. When necessary, the *t* test was modified to the unequal variance with Welch's *t* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

General histology: In Peyer's patches, FAE were covered with FAIV. FAIC were situated between the FAE and FAIV (Fig. 1). FAIC were on average of 84.2 ± 11.8 μm in depth and mainly consisted of columnar epithelial cells, goblet cells and Paneth cells. M cells were located at the FAE adjacent to the crypt orifices, but were not found in the epithelium of

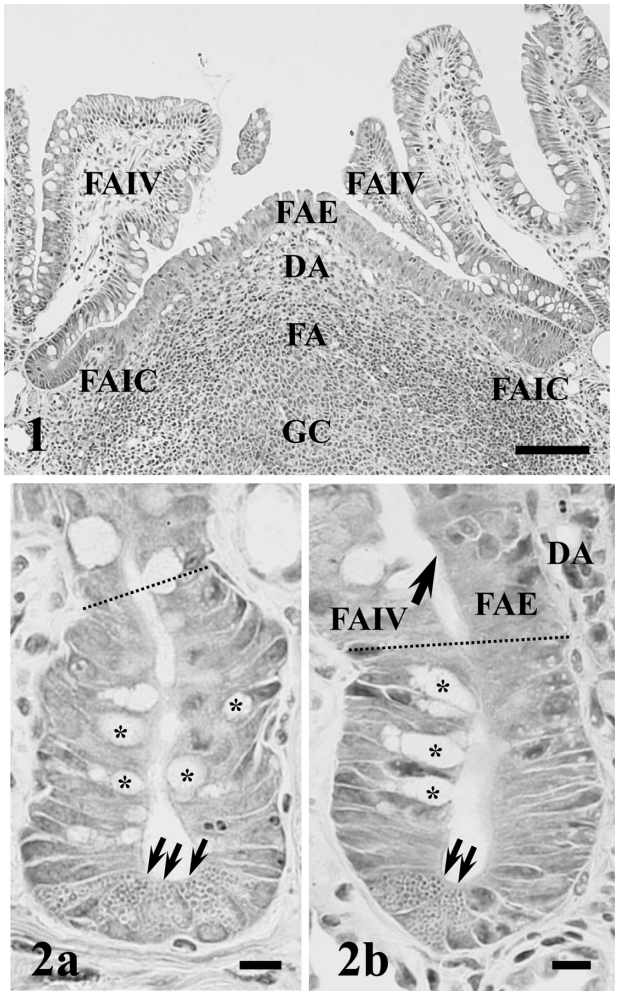

Fig. 1. Structure of Peyer's patch in the rat ileum. The follicle-associated epithelium (FAE) is incompletely covered by the FAIV. Follicle-associated intestinal crypts (FAIC) are situated between the FAE and the FAE-associated intestinal villi (FAIV). DA, dome area; FA, follicular area; GC, germinal center. Bar=100 μm.

Fig. 2. High-magnification image of the ordinary intestinal crypt (IC) (a) and the follicle-associated intestinal crypts (FAIC) (b). a) Paneth cells (arrows) and goblet cells (asterisks) are visible on both sides of the IC. b) Paneth cells (small arrows) and goblet cells (asterisks) are visible on the anti-follicular sides, but not on the follicular sides of the FAIC. M cell (large arrow) is visible in the follicle-associated epithelium (FAE), but not in the FAIC. DA, dome area; Dotted lines, orifices of intestinal crypts; FAIV, FAE-associated intestinal villus. Bar=10 μm.

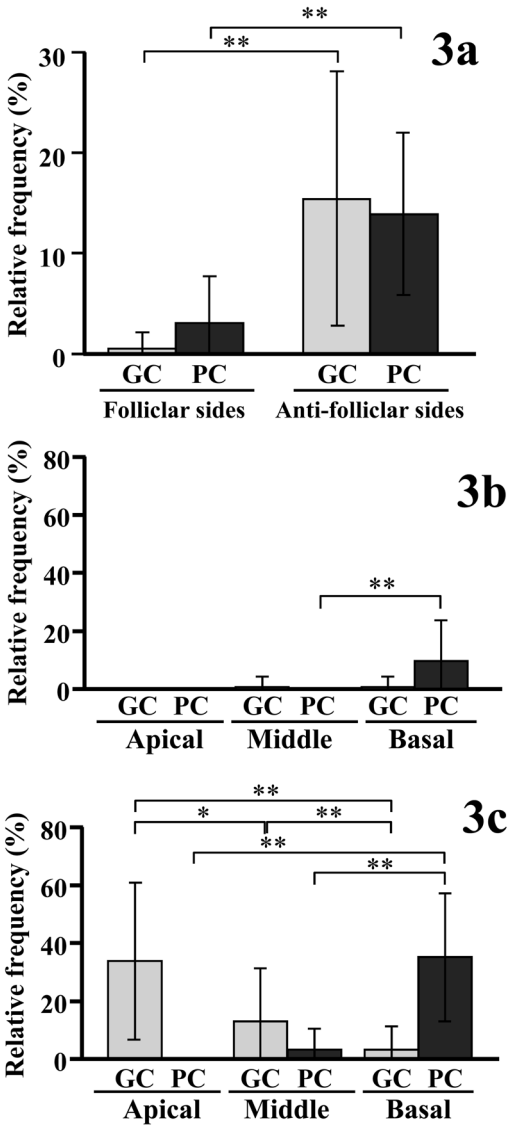

Fig. 3. a) Relative frequency of goblet cells (GC, light gray columns) or Paneth cells (PC, dark gray columns) on the follicular and the anti-follicular sides of the follicle-associated intestinal crypts (FAIC). b, c) Relative frequency of goblet cells (GC, light gray columns) or Paneth cells (PC, dark gray columns) in the apical, middle or basal portions of the follicular (b) and anti-follicular sides (c) in the FAIC. Asterisks, $P<0.05$. Double asterisks, $P<0.01$.

FAIC. M cells were never found in the FAIV, IV or IC.

Both goblet cells and Paneth cells were equally localized on both sides of IC, but markedly less frequent on the follicular sides than on the anti-follicular side of the FAIC (Figs. 2a, 2b and 3a). In the epithelium of the anti-follicular sides of FAIC, goblet cells were rare in the basal portions and significantly increased from the base toward the crypt orifices. Paneth cells were almost restricted to the crypt base (Fig. 3b and 3c). The morphological characteristics of goblet cells, Paneth cells and columnar epithelial cells in the FAIC were the same as those in the IC (Fig. 2a and 2b). The size of secretory granules in the goblet cells increased toward the crypt orifices of both the FAIC and IC. Goblet cells were significantly less frequent in the apical, middle and basal portions of the FAE than in those of the IV, respectively (Fig. 4).

Indigenous bacteria were occasionally found on the FAE, but never found in the lumen of the FAIC. From

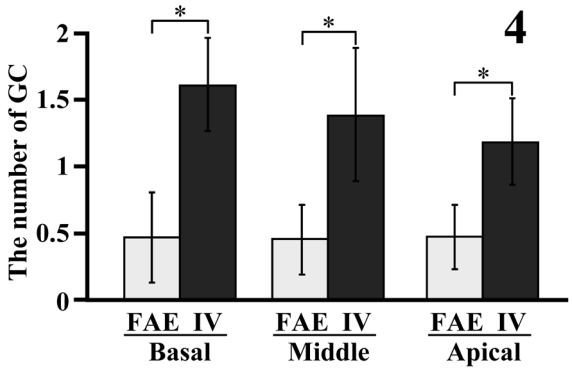

Fig. 4. The number of goblet cells (GC) per 50-μm-long epithelial segments taken from the basal, middle or apical portions of the follicle-associated epithelium (FAE, light gray columns) or the ordinary intestinal villus (IV, dark gray columns). Asterisks, $P<0.01$.

histoplanimetrical analysis, indigenous bacteria were significantly more adhered to the FAE than to the FAIV or IV (Table 1).

Table 1. The appearance frequencies of FAE, FAIV and IV with bacteria adherence

	FAE	FAIV	IV
Bacteria adherence	17	21	83
No bacteria adherence	3	19	117
Relative frequency (%)	85.0 [a, b)]	52.5[a)]	41.5[b)]

FAE, follicle-associated epithelium; FAIV, FAE-associated intestinal villus; IV, intestinal villus. a) $P<0.05$, b) $P<0.01$.

Histochemical and immunohistochemical characteristics of Paneth cells and goblet cells: In AB (pH 1.0 and 2.5) staining, the secretory granules of the goblet cells were almost positive in the FAIC as well as in IC, although they were occasionally negative for AB (pH 1.0) in the IC. The secretory granules of Paneth cells were almost negative for AB (pH 1.0 and 2.5) in the FAIC as well as IC, whereas Paneth cells with secretory granules positive for AB (pH 1.0 and 2.5) were rarely found only in the FAIC (Fig. 5a–5c). Ten AB-positive Paneth cells were found in 26 FAIC, but none were found in the 337 IC observed. In the PAS reaction, the secretory granules of Paneth cells and goblet cells of both

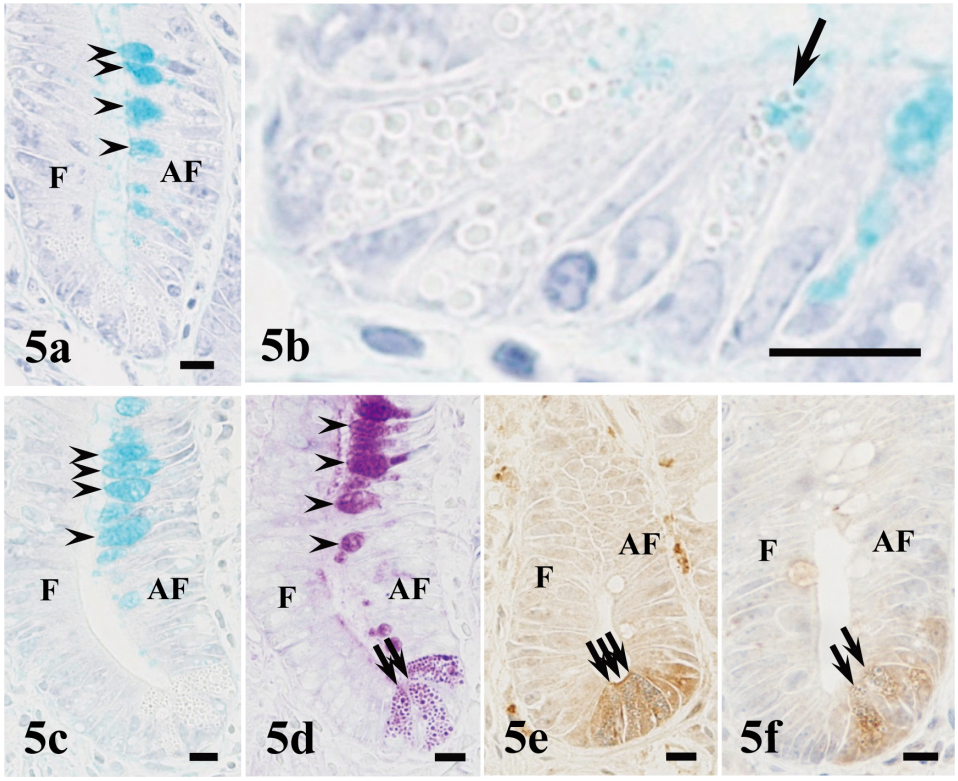

Fig. 5. Histochemical characteristics of the secretory granules of Paneth cells and goblet cells in the follicle-associated intestinal crypts (FAIC). a-c) AB pH 1.0 (a, b) and 2.5 (c)-positive goblet cells preferentially locate on the anti-follicular side (AF) of FAIC (arrowheads). b) High-magnification image of the crypt base in (a). A part of Paneth cells has AB-positive granules (arrow). d) PAS-positive goblet cells (arrowheads) and Paneth cells (arrows) preferentially locate on the AF of the FAIC. e, f) Lysozyme (e) and sPLA2 (f)-positive Paneth cells preferentially locate on the AF of the FAIC (arrows). F, follicular side. Bar=10 μm.

the FAIC and IC were positive (Fig. 5d).

In the immunohistochemistry, lysozyme and sPLA2 were detected in the secretory granules of Paneth cells in the FAIC as well as in IC (Fig. 5e and 5f). Goblet cells and columnar epithelial cells were negative for lysozyme in the FAIC as well as in IC. sPLA2 was almost negative in the goblet cells and columnar epithelial cells, although goblet cells with the secretory granules positive for sPLA2 were occasionally found in both the FAIC and IC. PAS- and AB (pH 1.0 and 2.5)-positive goblet cells and both lysozyme- and sPLA2-positive Paneth cells were preferentially found on the anti-follicular side than on the follicular sides of the FAIC.

DISCUSSION

In general, intestinal epithelial cells are generated in the IC and exfoliate from the apices of the IV or FAE in the small intestine [10, 23, 27, 28]. In the IV, epithelial cells progress through both the apoptotic process and maturation with an increase of functional proteins, such as sucrase-isomaltase and maltase-glucoamylase, during epithelial migration along villous axes in the rat small intestine [7, 22]. On the other hand, in the FAE, the cellular differentiation involved in M cells is accompanied without apoptotic process. Thus, the cellular differentiation and maturation accompanied by apoptosis are inhibited in the FAE [22]. In the present study, the relative frequencies of goblet cells and Paneth cells were significantly less in the epithelium of the follicular sides than on the anti-follicular sides of the FAIC. Furthermore, goblet cells were significantly fewer in the FAE than in the IV. On the other hand, M cells were found only in the FAE, but not in the epithelium of the FAIV or IV. From these findings, cellular differentiation into secretory cells was inhibited in the epithelium of the follicular sides of FAIC, so that the differentiation into M cells might be admitted in the FAE of rat Peyer's patches.

M cells differentiate in the FAE in response to the indigenous bacterial proliferation and transcytose the luminal antigen into subepithelial lymphatic tissue [6, 33]. The proliferation of indigenous bacteria in the intervillous spaces leads to various host responses, such as the reduction of villous height, the acceleration of epithelial cell migration [25] and the transient secretion of lysozyme and sPLA2 from Paneth cells [31, 32]. In the present study, two types of exocrine epithelial cells, namely goblet cells and Paneth cells, were markedly less frequent in the epithelium of the follicular sides than in that of the anti-follicular sides in FAIC, and the bactericidal peptides, lysozyme and sPLA2 were detected in the Paneth cells. Furthermore, indigenous bacteria more frequently adhered to the FAE than to the FAIV or IV. From these findings, host defense by the secretion of bactericidal substances against indigenous bacteria is suggested to be inhibited on the follicular sides of FAIC; this might contribute to preferential settlement of indigenous bacteria on the FAE.

From the ultrastructural observation of the mouse duodenum by Troughton and Trier (1969), intermediate cells have mucus-like granules with the dense cores which resemble the secretory granules of Paneth cells in the IC [30]. The intermediate cells are able to be light-microscopically observed as AB-positive Paneth cells at the base of the IC in the mouse small intestine [15]. From these findings, AB-positive Paneth cells found in the present study are probably intermediate cells between Paneth cells and goblet cells. Under physiological conditions, the intermediate cells are most frequently found in the duodenum and jejunum in rats aged 21 to 28 days old, but are rare in rats aged 50 days or more [18]. In the present study using 7 weeks old rats, AB-positive Paneth cells were found only in the FAIC, but not in the IC. From these findings, the intermediate cells were suggested to be preferentially differentiated in the FAIC of the Peyer's patches compared to the IC. The manner of differentiation into Paneth cells in the FAIC is probably different from that in the IC, although the nature of intermediate cells is controversial, because of their scarcity. FAIC will be the helpful subject to clarify the nature of intermediate cells.

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