

Ultrastructural Study on the Epithelial Responses against Attachment of Indigenous Bacteria to Epithelial Membranes in Peyer's Patches of Rat Small Intestine

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ABSTRACT. The ultrastructure of epithelial responses against the membrane adhesion of indigenous bacteria was investigated in the follicle-associated epithelium (FAE) of rat small intestine. The most frequent adherence of the various morphological types of bacteria to the epithelial membranes was found at the apex of the FAE. The attachment sites were deeply invaginated, and their bottoms were deformed into a sharp cone shape. Four layers with different electron densities were formed just beneath the apical membranes by microfilaments which surrounded the invaginations. The electron density of each layer was gradually decreased as being apart from the invaginations. The extremities of some bacteria in the invaginations were deformed into sharpened shapes. The cell walls of the extremities of the bacteria were occasionally dissolved in the invaginations, and their cytoplasmic matrices were slightly swollen with low electron densities. In some invaginations, the attached bacteria were eliminated to leave their fragments such as filamentous debris and a part of cell walls. Finally these remnants disappeared completely. When the bacterial colonies existed in the middle region of the FAE, the attachment of bacteria resulted in the engulfment of bacteria by M cells. The degenerated bacteria whose cytoplasmic matrices were separated into high electron dense materials and cleared materials were occasionally engulfed by ordinary microvillous columnar epithelial cells or goblet cells throughout the FAE. These findings suggest that the epithelial cells reject the attachment of live indigenous bacteria and that the M cells absorb indigenous bacteria in rat Peyer's patches.

KEY WORDS: epithelial apoptosis, indigenous bacteria, M cell, Peyer's patch, ultrastructure.

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The mucous membrane of the alimentary tract always fronts the external environment. Antigenic substances such as pathogenic microorganisms are eliminated by non-specific and specific defense mechanisms mediated by gut-associated lymphatic tissue (GALT) [11, 34]. The follicle-associated epithelium (FAE) of Peyer's patches contains M cells which play special functions that transcytose various antigens and microorganisms from the intestinal lumen to the dome area [23, 27]. Some antigens are further delivered to the antigen-presenting cells to induce the mucosal immune responses [14, 25]. However, M cells are distributed in the basal to middle regions rather than the apical regions of the FAEs in many animal species [rabbit, 15; mouse, 31; rat, 3; humans, 22, 24; chickens, 16]. Thus, M cells are away from the stream of luminal contents and are located in sites which are unsuitable for constant monitoring of luminal antigenic information under physiological conditions in animals [3, 38, 39]. Recently, a detailed study demonstrated that the colonization of indigenous bacteria on the FAE correlates with the M cell differentiation in the FAE of rat Peyer's patch. Moreover, the ultrastructural finding of the uptake of indigenous bacteria by M cells indicates that the M cells might deliver information on the expansion of the colony of indigenous bacteria to the intestinal host defense mechanism, thereby allowing the regulation of bacterial colonization [3].

However, the epithelial response to indigenous bacteria other than M cells has not been clarified ultrastructurally in the FAE.

In general, both physical and chemical barriers constitute a first defense line against microbial invaders in the intestine [4]. The physical barrier is provided by the epithelial cells themselves and by the motility of the intestinal walls. The chemical defenses are performed by digestive enzymes, bile and bactericidal substances, and so on [19, 26, 28]. The normal intestinal microflora also provide protection against infections caused by pathogenic microorganisms [2, 29], but few studies have examined the colonization mechanisms of indigenous bacteria *in vivo* [5, 9, 29]. It has been believed that the indigenous bacteria colonize on the epithelial lining, but it is still unclear whether the colonization is an acceptable phenomenon or not for epithelial cells for an animal host.

Detailed ultrastructural observations have clarified the adherence mechanisms of some pathogenic bacteria in intestines [*Vibrio cholerae*, 20; *Yersinia enterocolitica*, 10; *Escherichia coli* O103, 18; *Shigella flexneri*, 30]. A few ultrastructural observations on the adherence of indigenous bacteria have also been carried out [5, 7, 17, 37], but the adherent mechanisms and epithelial responses of indigenous bacteria have not been elucidated. In the present study, we observed and clarified the detailed morphology of epithelial responses against the colonization of indigenous bacteria on the FAE under a transmission electron microscope, and herein discuss the colonization mechanisms of indigenous

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bacteria.

MATERIALS AND METHODS

Animals: Eight male Wistar rats aged 7 weeks (Japan SLC Inc., Japan) were maintained under conventional laboratory housing conditions. They were permitted free access to water and food (Lab MR Stock, Nosan Corp., Japan). 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 and 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 the Kobe University Animal Experimentation Regulations.

Tissue preparation: The animals were intravascularly perfused with 2.5% glutaraldehyde –2.0% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) after anesthesia with peritoneal injection of pentobarbital sodium (Dainippon Sumitomo Pharma., Japan). After perfusion, tissue blocks of the Peyer's patches were extracted and immersed in the same fixative for 24 hr at 4°C . After postfixation with 1.0% OsO_4 in PB for 2 hr at room temperature, the small specimens were dehydrated and embedded in a Quetol 812-mixture.

Transmission electron microscopy: Ultrathin sections were cut using an ultramicrotome (Sorvall MT-1, U.S.A.). The sections contrasted with both uranyl acetate and lead citrate were observed under a transmission electron microscope (Hitachi H-7100, Japan) at an accelerating voltage of 75 kV.

Quantitative histology: On the electron micrographs, the following histological factors were measured: the maximum thickness of each microfilamentous layer around 5 invaginations, the thickness of 5 cell membranes in the bacteria attaching sites and the diameter of 5 minute vesicles beneath the invaginations. Data were presented as mean number \pm standard deviation.

RESULTS

Bacteria with the same morphology were settled on each FAE of the lymphatic follicle (Fig. 1). The manner of bacterial attachment in the apical region was different from that in the middle region of FAE.

Epithelial response to bacterial attachment in the apical region of the FAE: Eight morphological kinds of bacteria adhered closely to the membranes of microvillous columnar epithelial cells in the apex of the FAE. The epithelial response against the adhesion of bacteria was almost the same. That is, at the initial stage of attachment, the microvilli that adhered to bacteria locally disappeared (Fig. 2a, b). Then the bacteria were attached closely to the cell membrane of epithelial cells (Fig. 2c). The cell membrane was deeply invaginated where four layers (I-IV layers) with different electron densities, consisting of microfilaments,

were formed just beneath the cell membrane (Fig. 3). The electron density of each layer was gradually decreased as being apart from the invagination. In some invaginations, the bottoms were deformed into a cone-shape and were bent to one side (Fig. 3). The I layer, which directly attached to the membrane of invagination, was the thinnest layer with the highest electron density. The thickness of the I layer was almost constant (45.1 ± 2.7 nm). The epithelial membrane with the I layer which surrounded the end portion of the bacterium slightly protruded, unlike neighboring portions of membrane, to attach closely against the bacterial surface (Fig. 3). The II layer microfilaments were wrapped around the extremity of the bacterium, and thus wrapped around the deep of the I layer (Fig. 3). The depth of the thickest portion was 415.7 ± 95.1 nm. The III layer wrapped almost all membranes of invagination, completely covering both the I and II layers (Fig. 3). The orientation of the microfilaments was various. The depth of the thickest site was 696.2 ± 197.6 nm. The IV layer wrapped portions of the I - III layers. The IV layer, which had variously oriented microfilaments, had the lowest electron density. The peripheral boundary of the IV layer was dim (Fig. 3).

In all filamentous layers, there were no organelles except for a few minute vesicles with diameters of 74.6 ± 11.9 nm. The vesicles which contained low electron dense substances occasionally fused the membrane in the lumen of invagination (Fig. 4). No bacterium was found in the lysosomes or other organelles of the epithelial cells. The existence or phagocytosis of bacteria was also never found in the lamina propria just beneath the epithelium at bacterial attachment.

The end portions of attached bacteria were deformed into cone-shapes in the invagination, whereas those of intact bacteria were generally round in the intestinal lumen (Fig. 3). In addition, some end portions of the bacteria apparently swelled, having low electron densities. The cell walls of the extremities of the bacteria also swelled and dissolved in the invaginations (Fig. 3). In several invaginations, only remnants of cell walls and filamentous debris from bacteria remained or were completely eliminated (Fig. 5a, b).

In the apical region of the FAE, some degenerated bacteria which were characterized by the separation of high electron dense materials and cleared materials were occasionally engulfed by microvillous columnar epithelial cells and goblet cells (Fig. 6a, b). During the engulfment process, high electron dense microfilaments never accumulated beneath the cell membrane of the epithelial cells. The encapsulated bacteria in small vesicles were transcytosed toward the basal lamina, passing by the nucleus. No engulfed bacteria were in the lysosomes of epithelial cells.

The exfoliating microvillous columnar epithelial cells which had been adhered by bacteria were accompanied completely or incompletely with the microfilamentous layers described above. However, in the exfoliated epithelial cells which were swollen and attached with numerous bacteria, no such microfilamentous layers were

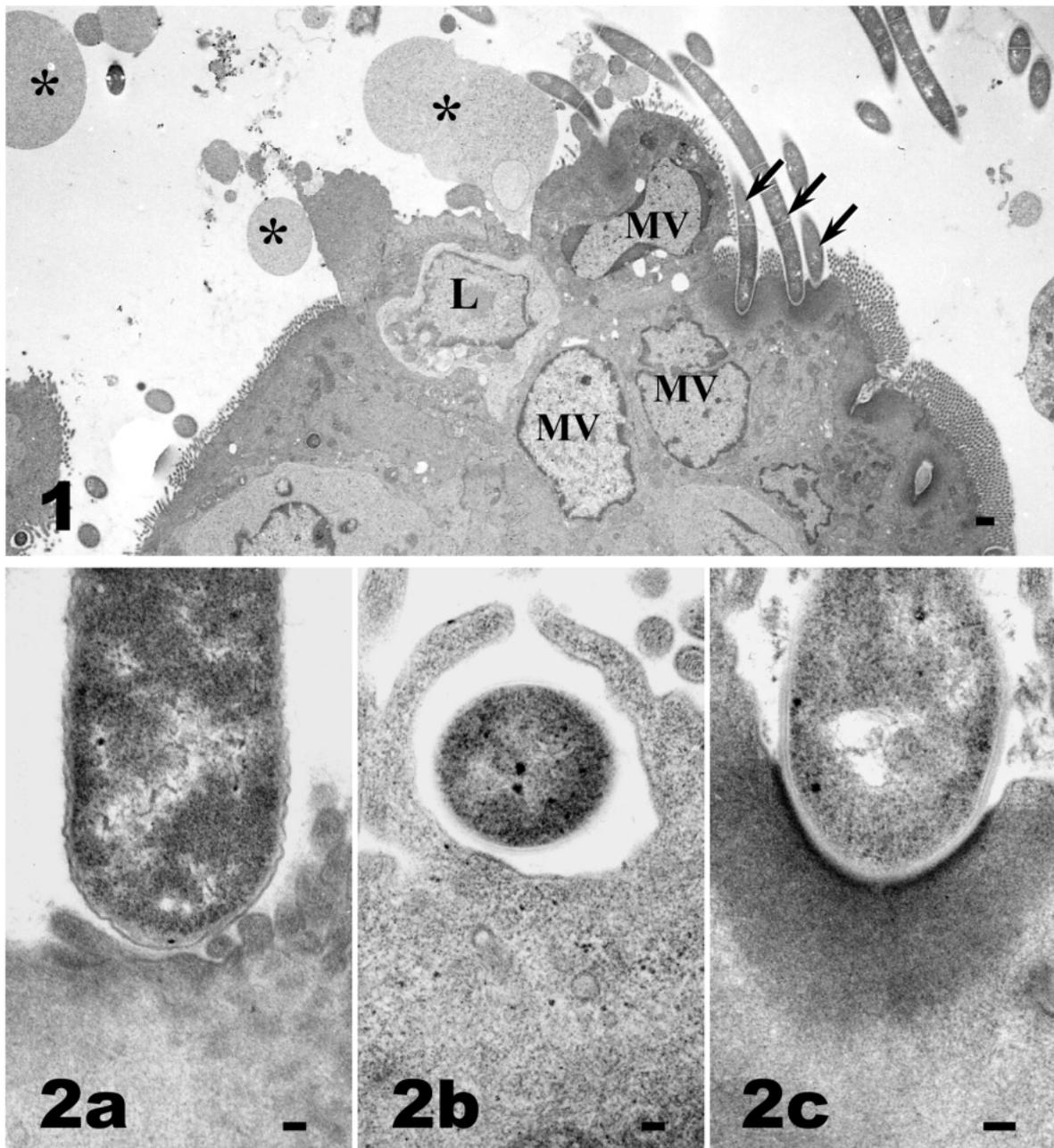


Fig. 1. On the apex of the FAE of rat Peyer's patch, numerous bacteria with a filamentous form (arrows) attach on the cell membrane of microvillous columnar epithelial cells (MV). L: Lymphocyte; *:Exfoliated apoptotic bodies of epithelial cells. Bar = 1 μ m.

Fig. 2. The microvilli adhered by bacteria are gradually shortened (a) and disappear locally (b). The cell membrane to which bacterium is adhered is slightly invaginated and accompanied with dense filamentous layers (c). Bar = 0.1 μ m.

formed (Fig. 7a, b).

Epithelial response to bacterial attachment in the middle to basal region of the FAE: The close attachment of bacteria to ordinary microvillous epithelial cells was never found, whereas the close attachment was frequently found at the apical membranes of the M cells. In the bacterial attachment sites of the M cells, the I layer was not formed,

but small microfilamentous accumulation was occasionally formed in the M cells. The engulfed bacteria were attached to the epithelial membranes, which were slightly thick (17.3 ± 0.4 nm) (Fig. 8a, b). Some bacteria were engulfed by migrating cells in the pocket of the M cells, but the engulfment of bacteria never occurred in the lamina propria. Degenerated bacteria were also engulfed by microvillous

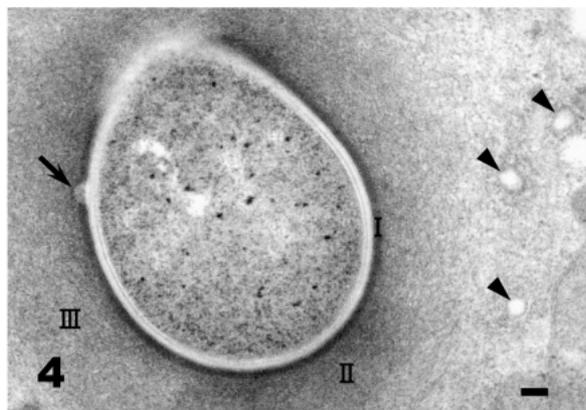
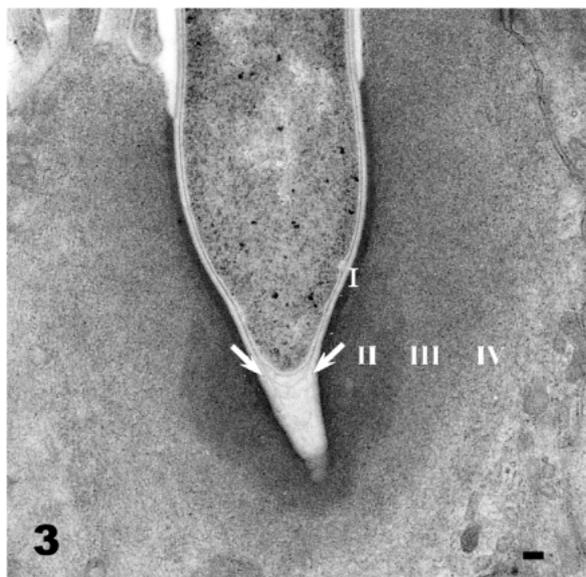


Fig. 3. An epithelial invagination with a bacterium is deformed into a cone shape and bent to one side. Four layers (I - IV) with different electron densities, which consisted of microfilaments, are formed just beneath the cell membrane. The electron density of each layer is gradually decreased as being apart from the invagination. The epithelial membrane with the I layer is slightly protruded and attaches closely to the bacterial surface. The end portion of the bacterium is deformed into a cone shape and is swelled, having slightly low electron densities. The cell walls of the extremities of the bacteria also swell and dissolve (arrows). Bar = 0.1 μ m.

Fig. 4. A transverse section of invagination. There are no organelles except a few minute vesicles (arrowheads). A minute vesicle fuses the membrane of invagination (arrow). Bar = 0.1 μ m.

columnar epithelial cells and goblet cells in the same manner as described in the apical region of the FAE.

DISCUSSION

The attachment of enteropathogenic *Escherichia coli* induces the disappearance of microvilli and the

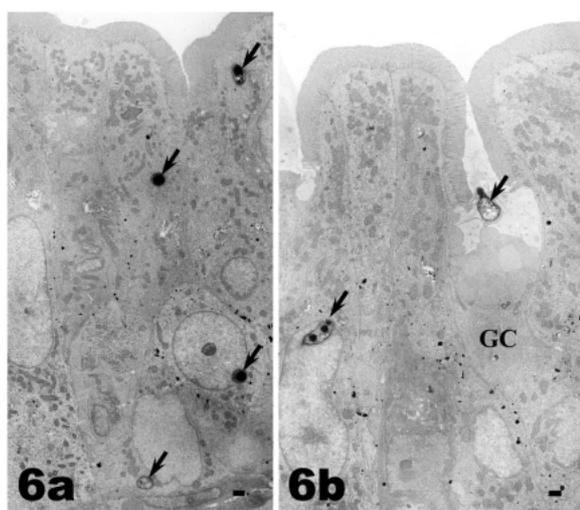
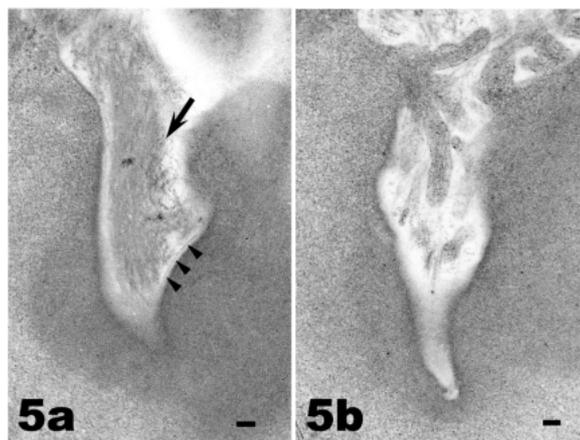


Fig. 5. a) In an invagination, only remnants of cell walls (arrowheads) and filamentous debris from bacterium (arrow) are visible. b) The invagination contains no bacterial components. Bar=0.1 μ m.

Fig. 6. In the apical region of the FAE, the degenerated bacteria (arrows), which are partially or entirely condensed, are engulfed by microvillous columnar epithelial cells and goblet cells (GC). Bar = 1 μ m.

rearrangement of actin filaments in apical cytoplasm of epithelial cells in the human large intestine [6]. After the disappearance of microvilli, pedestals which are composed of the dense accumulation of fibrillar materials are formed at the attachment sites of the enteropathogenic *Escherichia coli* in the small and large intestines of various animals [piglets, 36; calves, 36; rabbits, 12]. The attachment of pathogenic bacteria results in the internalization of bacteria into the intestinal epithelial cells by the invasin-integrin interaction and by receptor-mediated endocytosis [13]. The attachment of nonpathogenic and indigenous bacteria, segmented filamentous bacteria, also causes similar morphological changes in the chicken, in that the attachment sites are surrounded by an electron-dense layer [37]. In the present study, the microvilli disappeared at the

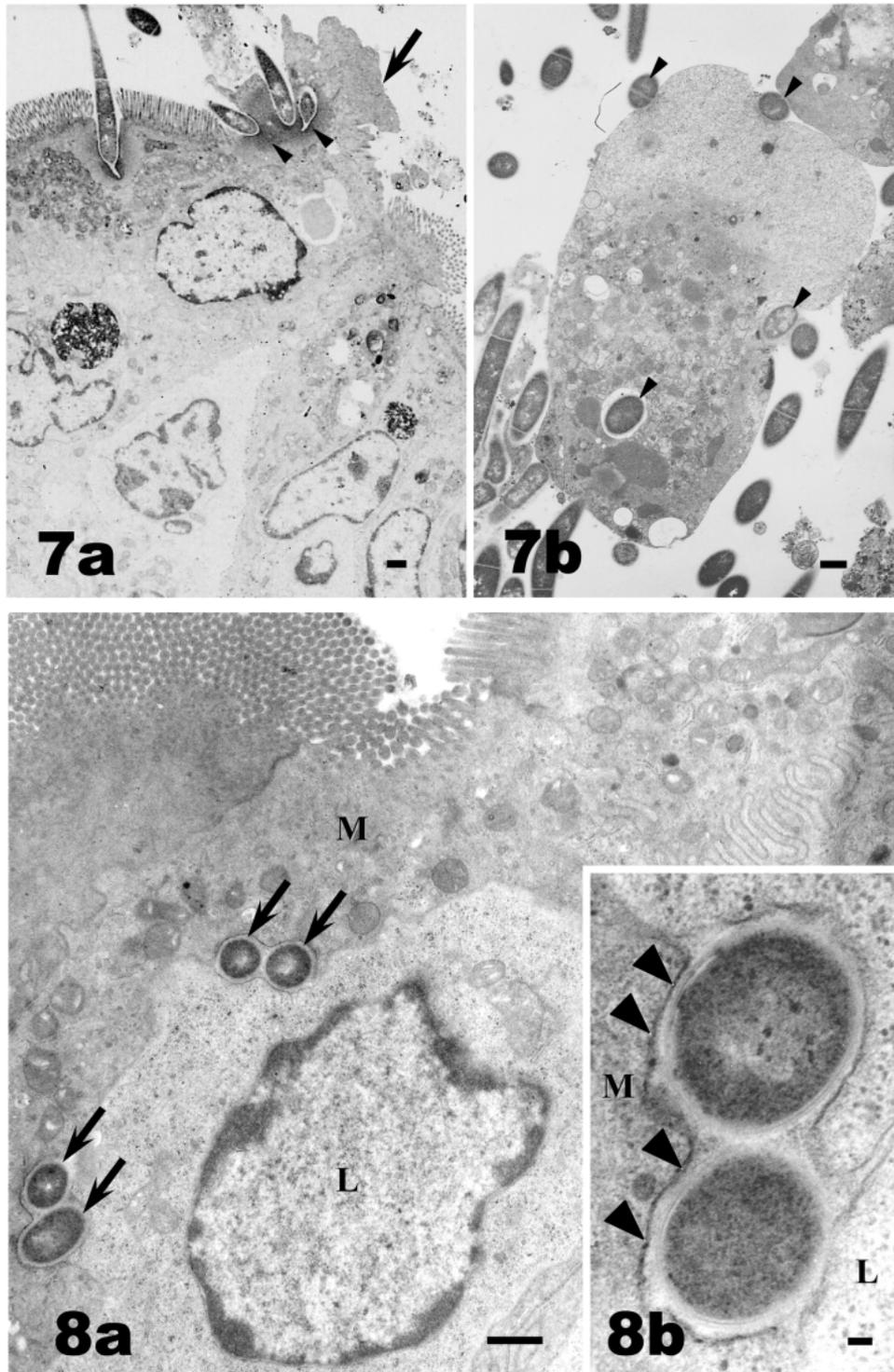


Fig. 7. a) The exfoliating microvillous columnar epithelial cells to which bacteria adhered are accompanied with microfilamentous layers. b) The exfoliated epithelial cell which is swollen and attached with numerous bacteria (arrowheads) has no filamentous layer. Bar=1 μ m.
 Fig. 8. a) In the basal portion of the FAE, a M cell (M) transcytoses bacteria into an epithelial pocket. The attachment sites of bacteria (arrows in 8a) are formed no filamentous layer. Bar=1 μ m. b) A high magnification micrograph shows the thickened membrane of bacterial attaching sites in the M cell, whereas the lymphocyte membrane to which bacteria adheres is not thickened (arrowheads). Bar = 0.1 μ m.

bacterial attachment sites of the epithelial cells at the apical region of the FAE of rat Peyer's patches, followed by the formation of four layers with the accumulation of microfilaments beneath the attachment sites. In particular, the membranes which were directly surrounded by the I layer with the highest electron density closely adhered to the bacterial surfaces and pushed against the bacteria. Consequently, the end portions of the cell invaginations and the extremities of the bacteria were deformed into cone shapes. Therefore, the regular and dense arrangement of microfilaments that formed at the bacterial attachment site suggest a physical defense structure against the attachment of indigenous bacteria by host epithelial cells.

Innate chemical defense is essentially constituted by natural antibodies and various bactericidal substances, such as defensin and lysozyme, in mucosal host defense against microbial invaders [26]. Therefore, these substances seem to play a role in the regulation of the intestinal indigenous bacteria. Lysozyme selectively disrupts bacterial cell walls and membranes [4, 26]. In this study, we found a few minute vesicles in the microfilament accumulation. Some vesicles fused the membrane of invagination to open to the lumen of invagination. Consequently, the cell wall of the extremity of the bacterium was dissolved, and the bacterial cytoplasm was swollen. Punctured bacteria and their remnants also remained in the invaginations. These findings suggest that chemical secretions, such as lysozyme, dissolve the bacterial cell wall and might prevent bacterial invasion. Therefore, the direct attachment of indigenous bacteria to the epithelial membrane might be an unacceptable event for the host cells in intestines rather than an acceptable event.

The appearance of M cells depends on the stimulation of bacterial colonization on the FAE in rat Peyer's patch. Bacteria which adhered to M cells are engulfed and transcytosed into the pockets of M cells [3]. In this study, the microfilaments slightly or never accumulated in the bacterial attachment sites of the M cells. Some bacteria were engulfed by migrating cells in the pocket of M cells. These findings indicate that the response of M cells to the adhesion of bacteria is different from that of the apoptotic epithelial cells in the apical region of the FAE. Furthermore, the membrane of vesicle which was attached by a bacterium, was thickened in this Peyer's patch. This finding suggests that the engulfment of bacteria by M cells is mediated by some membrane receptors that recognize the surface substances on indigenous bacteria.

Toll-like receptors (TLRs) function as pattern recognition receptors that recognize conserved pathogen-associated molecules (PAMPs) expressed in the cell membranes by a wide spectrum of microorganisms [8]. TLRs play a role in the detection of pathogen subtypes, including gram-positive and gram-negative bacteria, DNA and RNA viruses, fungi and protozoa [21]. The recognition of PAMPs is considered to induce the antimicrobial activity by both the innate and acquired immune system [33, 35]. M cells play an important role in the uptake of foreign antigens such as

bacteria by expressing TLRs [1, 32]. The functions of TLRs in host defense against microbial pathogens have not been fully clarified [8], but TLRs might be involved in the engulfment of indigenous bacteria by M cells in the present study.

In the apices of intestinal villi, both antigenic molecules and particulates derived from foods are persorbed from the intestinal lumen to the circulating blood by effete epithelial cells in the late apoptotic stage. However, macromolecules or particulates from ingested food are never absorbed by intact villous columnar epithelial cells [38, 39]. In this study, only the degenerated bacteria were engulfed and transcytosed by the intact columnar epithelial cells in the FAE of Peyer's patch. This phenomenon has never been reported, and might be of significance in the host defense mechanism against the indigenous bacteria. Further investigation is needed to explain the significance of this phenomenon.

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