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Scanning and Transmission Electron Microscopic Study of *Escherichia coli* O15 (RDEC-1) Enteric Infection in Rabbits

AKIO TAKEUCHI,[1]* LINDSEY R. INMAN,[1] PETER D. O'HANLEY,[2] J. ROBERT CANTEY,[2] AND WILLIAM B. LUSHBAUGH[2]

Department of Experimental Pathology, Walter Reed Army Institute of Research, Washington, D.C. 21102;[1] and Veterans Administration Hospital and Departments of Anatomy and Medicine, Medical University of South Carolina, Charleston, South Carolina 29403[2]

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RDEC-1 is a piliated strain of *Escherichia coli* that was isolated from and produces diarrhea in rabbits without invading the mucosa or synthesizing one of the classical enterotoxins. Previous histological and fluorescent-antibody studies of RDEC-1 diarrhea revealed an acute inflammatory response and large numbers of RDEC-1 associated with (adhering to) the mucosal surface of the ileum, cecum, and colon. The purpose of the present investigation was to further elucidate the histopathology by scanning (SEM) and transmission (TEM) electron microscopy. SEM revealed aggregates of bacteria on the surface of the gut; their distribution was patchy in the ileum and diffuse in the cecum and colon. Bacteria were in contact with each other and appeared to be closely associated with the epithelial surface. TEM showed that the brush border region of the epithelial cells was found to be in varying stages of degeneration, and the bacteria could not be seen adhering to the mucosal cells unless the brush border was absent. Bacteria were in close contact only with epithelial cells that had lost their brush border. The space between the bacteria and the epithelial cells was 11 nm, and it appeared to be filled, in most cases, with densely stained material. This *E. coli* rarely penetrated epithelial cells, but when it did; it was found in the supranuclear region and never reached the lamina propria. From previous and present studies, it seems probable that RDEC-1 produces diarrhea in rabbits by a mechanism that may be cytotoxic and differs from the classic mechanisms by which *E. coli* produces diarrhea.

Escherichia coli has been shown to produce diarrhea by invasion of the intestinal mucosa or by synthesis of heat-labile or heat-stable enterotoxins (8, 22, 26, 27). Cantey and Blake have recently described a piliated *E. coli* (RDEC-1) that reliably produces diarrhea in rabbits without benefit of any of the above mechanisms (1). O'Brien et al. have reported that RDEC-1 produces a small amount of *Shigella dysenteriae*-like enterotoxin (unpublished observations; A. D. O'Brien, M. R. Thompson, J. R. Cantey, and S. B. Formal, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B103, p. 32), but whether the toxin is important to the ability of RDEC-1 to produce diarrhea is unknown. Routine light microscopy (LM) and fluorescent-antibody (FA) techniques revealed large numbers of RDEC-1 lining the mucosal epithelium of the distal small intestine, cecum, and colon. The purpose of the present study was to further investigate the histopathology of RDEC-1 infection by means of scanning (SEM) and transmission (TEM) electron microscopy.

MATERIALS AND METHODS

Bacterial culture and infection of rabbits. *E. coli* RDEC-1 was grown in Penassay broth at 37°C for 18 h. New Zealand white rabbits weighing 0.7 to 1.0 kg were fasted overnight and given RDEC-1 by the orogastric route with 10 ml of 10% $NaHCO_3$ as previously described (1). They were examined for the presence of diarrhea, and rectal swab cultures were inoculated onto MacConkey agar. Lactose-positive colonies were confirmed as RDEC-1 by slide agglutination using specific antisera. The diarrhea reached its peak about 10 days after inoculation, at which time the rabbits were sacrificed by intravenous injection of sodium pentobarbital.

Sampling and preparation of tissues. Tissues from both infected and normal animals were collected immediately after sacrifice. Specimens of ileum, cecum, and colon were excised and processed for FA, LM, SEM, and TEM as described below.

FA technique. The first pieces were frozen in isopentane in dry ice, sectioned, stained with specifically labeled FA to RDEC-1, and counterstained with rhodamine-conjugated bovine serum albumin (1).

LM. Specimens were fixed in chilled 10% buffered Formalin and embedded in paraffin. Individual sec-

tions of paraffin-embedded material were stained with either hematoxylin-eosin or Giemsa.

SEM. Ileal and proximal colonic tissues were cut into 5- by 10-mm pieces, fixed in 1.75% glutaraldehyde in 0.1 M sodium cacodylate buffer for 1 to 2 weeks, rinsed in 0.1 M cacodylate-HCl buffer, pH 7.2, and postfixed overnight in cacodylate-buffered 1% osmium tetroxide. The tissues were further stabilized in 1% thiocarbohydrazine (17), rinsed, and put back in the 1% osmium tetroxide. The tissues were then dehydrated in ethyl alcohol, critical-point dried with carbon dioxide, mounted on aluminum stubs with silver paint, vacuum coated with gold palladium alloy, and examined in a Coates & Welter scanning electron microscope at the Medical University of South Carolina.

TEM. Specimens of ileum and colon were cut into 1-mm cubes and processed for standard TEM by glutaraldehyde fixation with osmium tetroxide postfixation. Specimens of cecum were sectioned and examined more extensively than the ileum and colon specimens and were also stained with alcian blue-lanthanum and ruthenium red.

Standard TEM techniques consisted of fixing the tissues in 2.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.2 for 4 h at 4°C, washing the tissues in three changes of 0.2 M cacodylate buffer, postfixation in 1% osmium tetroxide in 0.2 M cacodylate buffer for 2 h at room temperature, and finally, two buffer rinses. Alcian blue-lanthanum staining was performed according to the method of Shea (24). The tissue was fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 containing 0.5% alcian blue for 2 h at room temperature, washed three times in 0.1 M cacodylate buffer, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h, rinsed, stained with lanthanum nitrate in 0.1 M *S*-collidine buffer, and washed two times. The procedure for ruthenium red staining was that of Luft (18) with some modification. The ruthenium red stock solution was prepared at 300 μg/ml in distilled water. Primary fixation was at 4°C for 1 h, and all buffers used were calcium-free. The fixed tissues were dehydrated in ethyl alcohol and embedded in Epon (32). Ultrathin sections were cut, stained with uranyl acetate followed by lead citrate, and examined with Hitachi HU-11C and 12A electron microscopes.

RESULTS

FA. The FA technique revealed fluorescent bacteria at the epithelial surface of both ileum and cecum. Bacteria were present only focally in the ileum, but were diffuse in the cecum. Fluorescent bacteria were not seen in the lamina propria.

LM. Histological findings in the small and large intestine of infected animals derived from paraffin sections that have previously been described (1) were confirmed by the present study and will not be repeated here. LM could not resolve the details of the association of bacteria with the mucosal epithelial cells.

SEM. The surface of the ileal mucosa of control rabbits was similar to that of normal rats (16), while the mucosal surface of the control cecum and colon bore a close resemblance to that of normal monkeys (34).

At magnification up to 300 times, the mucosal surface of the ileum, cecum, and colon infected with RDEC-1 was indistinguishable from that of the control rabbits.

At magnification ranging from 300 to 1,000 times, aggregates of bacteria were found on the mucosal surface of infected ileum and large intestine; their distribution was in a patchy fashion in the ileum and colon and was generally diffuse in the cecum. However, the mucosal surface between bacterial aggregates was not remarkable. In contrast, the control intestines were free of bacterial aggregates on the mucosal surface.

At magnification ranging from 1,000 to 5,000 times, individual bacilli were clearly discernible in the areas of bacterial aggregates and appeared to contact each other and attach closely to the microvillous border (MVB; Fig. 1 and 2). The exact interface between bacteria and MVB could not be resolved with certainty. In the proximity of the bacterial attachment, microvilli were elongated and projected into the lumen (Fig. 1 and 2), whereas the surface distal to the bacterial attachment showed the individual tips of unaltered microvilli (Fig. 2).

TEM. Ultrastructural observations confirmed LM and SEM findings and resolved further details of the histopathology. The bacterial infection of the epithelial cells at the MVB region was heavier and more diffuse in the cecum than in the ileum. *E. coli*-epithelial cell interactions were essentially the same in both the ileum and the cecum. For these reasons, the following TEM observations were made on the cecum. The glycocalyx of normal cecal samples were seen as an electron-opaque fine material that radiated from the tip of occasional microvilli. Ruthenum-stained glycocalyx, in contrast, was easily identified as a layer of dense material up to 500 nm wide and evenly distributed. Lanthanum-stained glycocalyx appeared to be moderately dense and coarsely granular and was sparsely distributed over and around microvilli in approximately 30-nm-thick layers.

Infected cecum examined at low magnification revealed innumerable bacteria that were usually localized on the luminal surface of mucosal epithelial cells. MVB and a substantial portion of the apical cytoplasm of columnar cells had disappeared or were degenerating. The microvilli and terminal web were totally absent in close proximity to bacteria. Bacteria were arranged randomly and usually were embedded side by side in areas of severe infection (Fig. 3). Some were found perpendicular and others horizontal to the apical cytoplasm, where they appeared

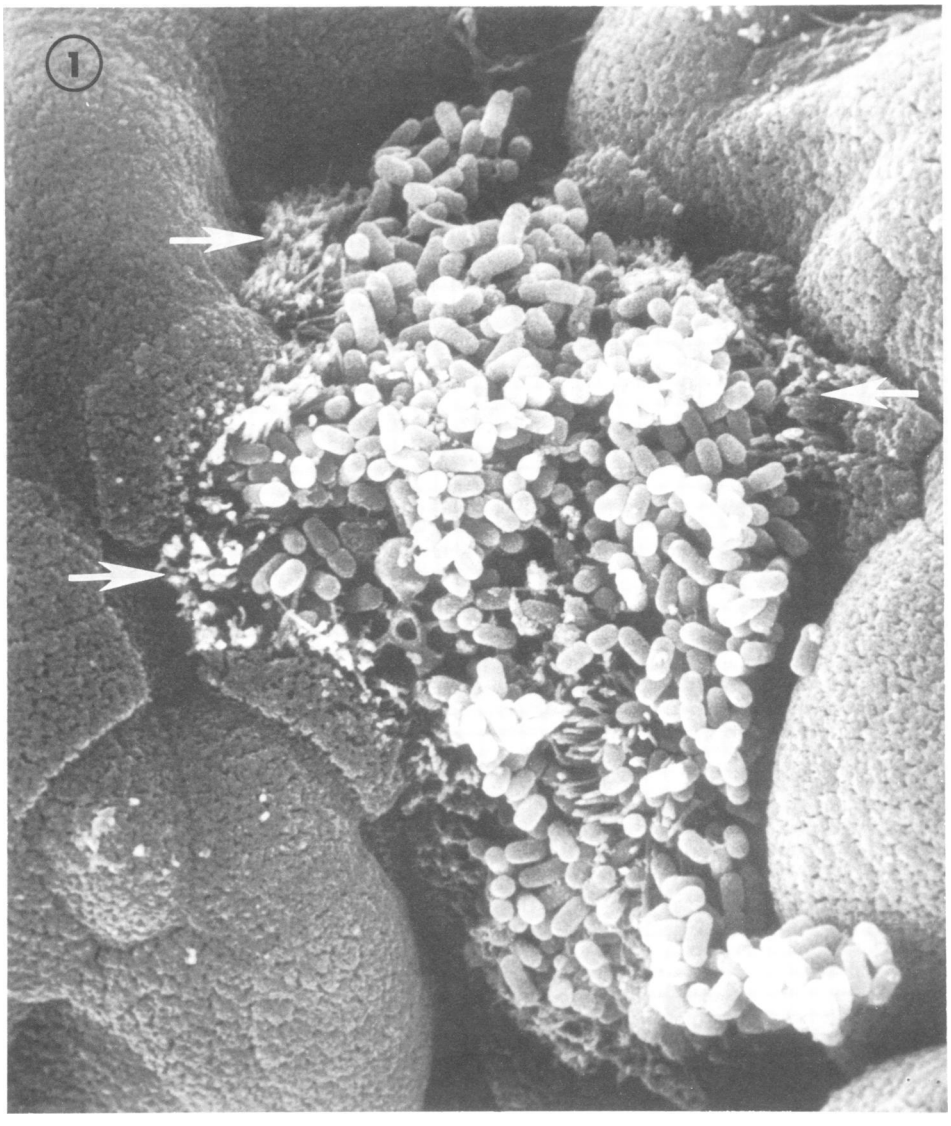

FIG. 1. *SEM view, surface of the ileal mucosa of rabbit infected with RDEC-1. Bacilli are aggregated and piled on each other. Elongated microvilli are present adjacent to the aggregates of bacteria (arrows). The surrounding epithelial surface appears unaltered.* ×6,600.

to attach to the luminal plasma membrane (Fig. 3). Both the infected cells and noninfected cells were in the process of desquamating into the gut lumen (Fig. 3).

Individual bacilli were identified at higher magnification and measured up to 4 μm in length and 1.5 μm in diameter. They had the characteristic structure of the gram-negative bacteria (Fig. 4), with occasional bacteria showing binary fission. Distinct structures were occasionally identified radiating from the bacterial cell wall (Fig. 4). These structures could be aggregated capsular material or, perhaps more likely, pili. A few blunt microvilli remained (Fig. 5) that were still covered by glycocalyx in areas adjacent to bacteria, and the apical plasmalemma of such cells was irregularly contoured (Fig. 5 and 6). A part of the cell wall of the bacteria was closely abutted to the trilamellar membrane of host cells, separated by a space of 11 nm. The luminal membrane of the epithelial cells formed cytoplasmic invaginations, projections, and blebs (Fig. 3). Some cavities of the invaginations contained electron-dense structures that could be a

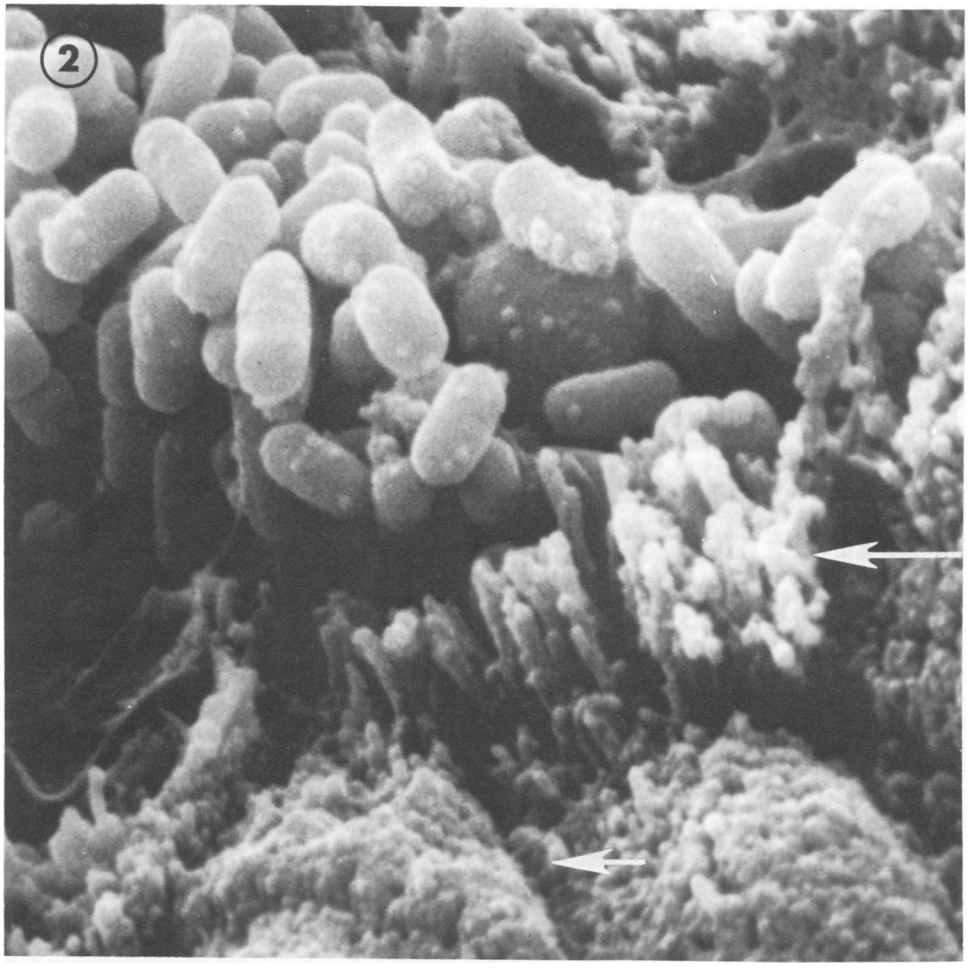

FIG. 2. *SEM view, aggregates of bacilli on the mucosal surface of the cecum. Individual bacilli are in contact with each other and are attached to the epithelial surface (long arrow). The interface between bacteria and MVB is not clear (short arrow). The tips of microvilli away from the aggregates are well discernible. ×14,700.*

demonstration of cross-sectioned pili (Fig. 4). Some of these cytoplasmic projections and blebs, together with coherent bacteria, appeared to be detached from the host cytoplasm into the lumen (Fig. 3). When bacteria were found at the intercellular tight junction, the junctional complex was displaced; however, the structural integrity of the complex remained unaltered (Fig. 6).

Cytoplasmic organelles in infected cells showed a variety of changes. Cisternae of rough and smooth endoplasmic reticulum as well as Golgi apparatus were often dilated; vesicles and vacuoles of different size were easily identified (Fig. 3). The mitochondria were swollen and showed deranged cistae with opacified matrix (Fig. 3). Membrane-bound lipid droplets were common. There was also an increased number of phagosomes containing altered host cytoplasmic components. The nuclei, however, remained unchanged.

Bacteria, in rare instances, were identified free in the cytoplasm and at the perinuclear region, where they were enclosed in membrane-bound vesicles and vacuoles. The majority of noninfected epithelial cells remained unchanged, although some of them topographically close to as well as away from the regions of bacterial adherence revealed short, blunt microvilli, occasional phagosomes, and increased numbers of vesicles.

The structure of the blood vessels in the lamina propria remained unchanged. The extravascular space was filled with finely granular ma-

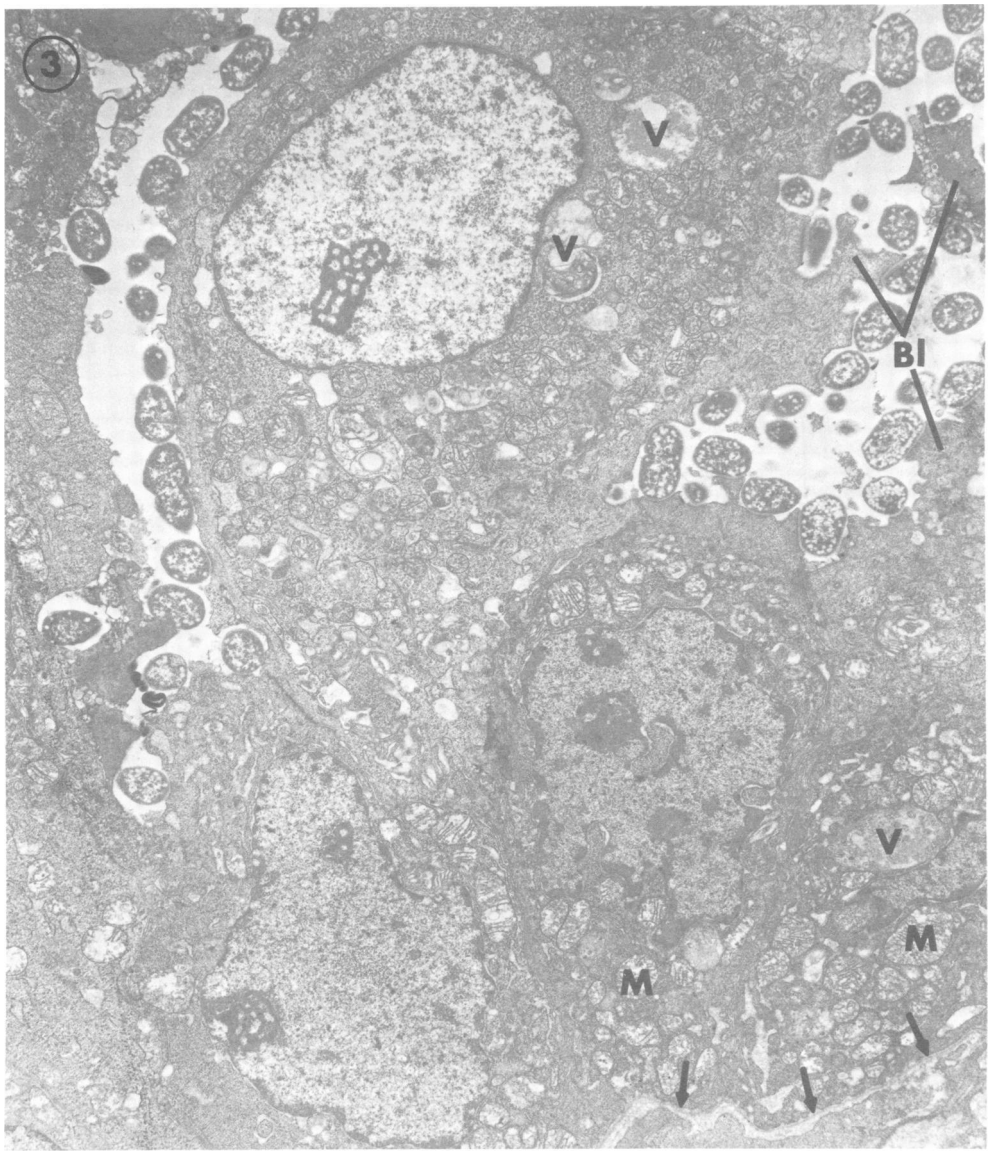

FIG. 3. *Infected cecal epithelial cells showing multiple bacteria at the cell surface. Glycocalyx and microvilli are totally absent. Infected epithelial cells are low columnar and contain increased numbers of vesicles, vacuoles (V), and swollen mitochondria (M) in their cytoplasm. Apical cytoplasm proximal to bacterial coherence shows cytoplasmic invaginations, projections, and blebs (Bl). An epithelial cell in the center projects toward the gut lumen, suggesting that it is in the process of shedding from the epithelial lining. Arrows indicate the basal lamina of the epithelium. Alcian blue-lanthanum treatment, lead citrate-uranyl acetate stain; ×6,500.*

terial. Occasional polymorphonuclear leukocytes were identified in both the gut lumen and mucosa. Leukocytes in both locations showed no evidence of phagocytized bacteria within their cytoplasm. Other mesenchymal cells remained unchanged.

DISCUSSION

The initial description of RDEC-1 diarrhea, which was based on FA and standard histological techniques for LM, revealed: (i) great numbers of RDEC-1 *E. coli* localized at and associ-

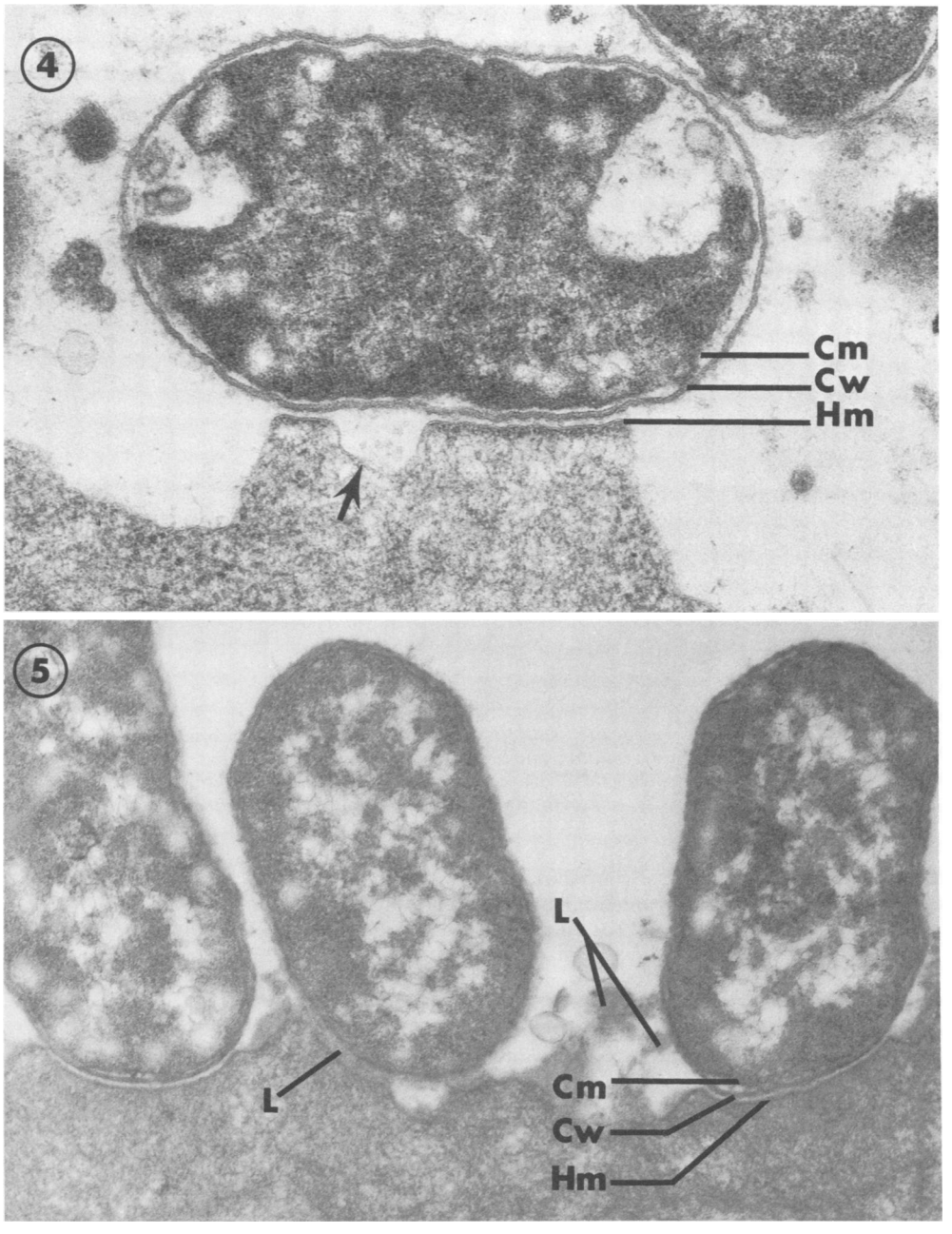

FIG. 4. *Bacterium is closely abutted to the host trilamellar membrane in a horizontal fashion. The glycocalyx and microvilli are totally absent in this routinely fixed tissue. Note that a number of slightly electron-dense and relatively well-defined structures are identified at the invagination (arrow) and radiating from bacterial cell wall. Abbreviations: Cm, Bacterial cytoplasmic membrane; Cw, cell wall; Hm, host cell membrane. Alcian blue-lanthanum treatment, lead citrate-uranyl acetate stain; ×57,160.*

FIG. 5. *Bacteria are oriented perpendicular to and approximated closely to the invaginated host epithelial cell membrane (Hm). Note that a remnant of lanthanum-stained material is still present on the host cell surface (L) and also between the bacterial cell wall (Cw) and host membrane. Cm, Bacterial cell membrane. Alcian blue-lanthanum treatment, lead citrate-uranyl acetate stain; ×57,160.*

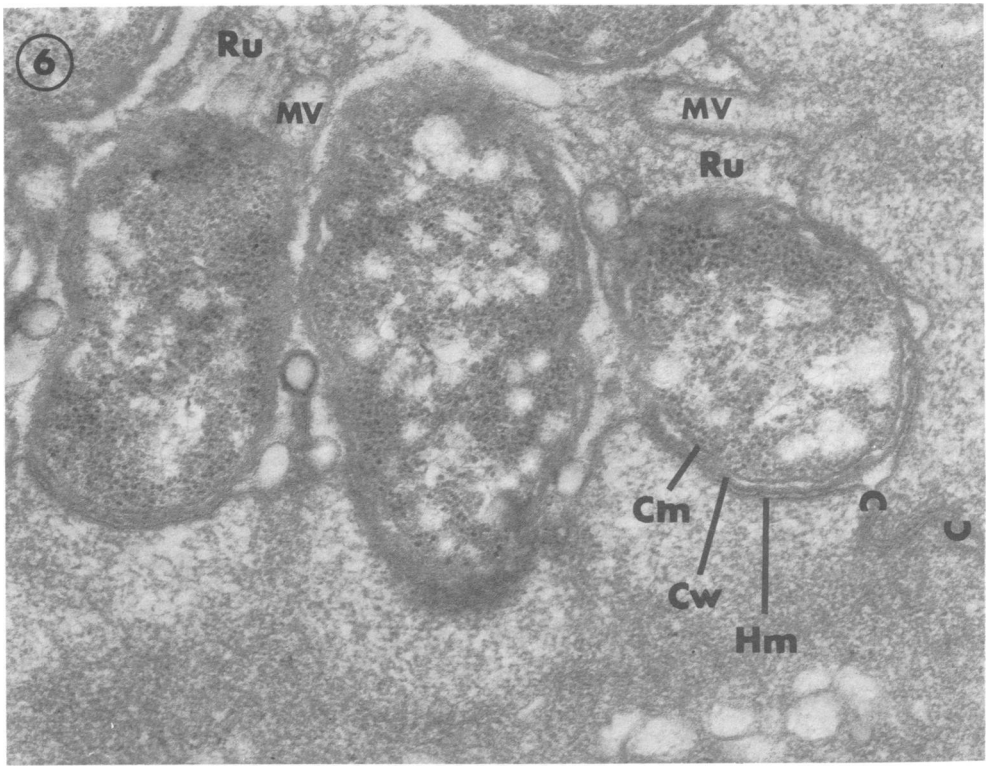

FIG. 6. *Bacteria are intimately associated with the invaginated host cell membranes (Hm) of two adjacent epithelial cells. Note that the intercellular junctional complex is displaced but structurally unaltered (parentheses). Remnants of microvilli (MV) are covered by ruthenium red-positive glycocalyx (Ru, Cm, bacterial cytoplasmic membrane; Cw, cell wall). Ruthenium red treatment, lead citrate-uranyl acetate stain; ×57,160.*

ated with the luminal surface of the villus and surface epithelium of the small and large bowel, and (ii) that the MVB and part of the epithelial cell cytoplasm were destroyed in the presence of the attached bacteria (1). In the present EM observations, SEM has shown how RDEC-1 *E. coli* inhabit their natural environment; three-dimensional views revealed how bacteria intimately populated the surface of the gut mucosa. TEM has clearly demonstrated that RDEC-1 *E. coli* multiply, destroy the MVB, and closely abut the luminal plasma membrane of epithelial cells.

The ability to adhere to mucosal surfaces which, in some cases, may be mediated by bacterial pili, is thought to be important for bacteria that infect such surfaces (25). Those who would apply these allied concepts to the area of bacterial diarrhea have based their opinions on data obtained in artificial systems utilizing separated gut epithelial cells, intestinal epithelial strips, isolated MVB, neonatal animals, and the rabbit ileal loop (6, 7, 9, 10, 13–16, 19, 20, 21, 28, 36, 38). All such studies have failed to consider that LM cannot resolve the fine details of the relationship between bacteria and the MVB of the epithelial cell surface or that several barriers stand between bacteria and the MVB in the intact animal, including the glycocalyx (11, 12, 16), the mucus layer, and the unstirred water layer (5). Evidence against adherence of bacteria to MVB was obtained in the present study. RDEC-1 was found adhering not to the MVB but to the surface of mucosal epithelial cells that had lost their MVB. Although we were not able to demonstrate with certainty that pili were responsible for or involved in the bacterial-host cell interaction, some sections did reveal distinct dense structures radiating from the bacterial surface and within cytoplasmic invaginations in the area of contact between the bacteria and the host cell membrane (Fig. 4). The narrow 11-nm space between the bacteria and the host cell membrane would, however, appear to mitigate against the possibility that these structures are pili. The space could easily contain RDEC-1 capsular material that stains well with ruthenium red (2).

TEM studies in enteric infections with *Salmonella typhimurium* in guinea pigs (29), *Shi-*

gella flexneri in guinea pigs (35) and monkeys (30), and invasive *E. coli* in rabbits (1a) revealed that the bacteria passed through the gut epithelial barrier, destroyed the MVB in the region of bacterial penetration, and produced a severe inflammation in the lamina propria. On the other hand, it has been shown at the fine structural level that several microbes characteristically destroy the MVB of the gut epithelial cells and attach preferentially to the luminal plasma membrane of host cells. They include spirochetes and flagellated microbes on the mucosal epithelium of the colon of monkeys and humans (31, 34, 35), segmented, filamentous microbes in murine ileum (4), and cryptosporidia in the ileum of guinea pigs (37). These microbes, however, cause no acute inflammatory response in the gut mucosa. It is evident that injury to the microvillous membrane region and the attachment of microbes to the host membrane does not necessarily cause acute inflammation. However, other studies have provided evidence that some strains of *E. coli* can penetrate the glycocalyx and contact the MVB with pilus-like appendages, but without causing damage to the MVB (H. W. Moon, personal communication). RDEC-1, in contrast, is able to: (i) destroy the microvillous membrane and the apical cytoplasm of mucosal epithelial cells, (ii) produce a mild to moderate acute inflammatory response in the lamina propria, and (iii) elicit a diffuse and intense edema in the lamina propria. The destructive and inflammatory capabilities of RDEC-1 may be due to the small amount of *S. dysenteriae*-like enterotoxin that it synthesizes (O'Brien et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B103, p. 32). The edema may be due to the release of vasoactive amines such as serotonin and histamine, both of which are abundant in the intestinal mucosa.

Detailed ultrastructural observations of RDEC-1 *E. coli* diarrhea in rabbits have thus shed new light and raised additional questions concerning the understanding of bacteria-host cell interactions and mechanisms of *E. coli* diarrhea. They did not resolve the question of whether pili and/or capsular material are responsible for bacterial adherence to epithelial cells.

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