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ABSTRACT The RDEC-1 strain *Escherichia coli* is an enteroadherent bacterium that produces diarrhea in the rabbit. A histopathologically similar disease has been described in humans. The RDEC-1 bacterium adheres to the epithelium of lymphoid follicles in rabbit ileal Peyer's patches by 4 h postinoculation, 3–4 d before its adherence to absorptive epithelium. The purpose of this study was to determine whether the RDEC-1 bacterium adheres to a specific cell type in the lymphoid follicle epithelium. RDEC-1 bacteria were given in a dose of 2×10^6 by the orogastric route to postweanling rabbits. The distal ileal Peyer's patch, taken from 5 control rabbits and 43 rabbits at intervals in the first 24 h postinoculation, was examined by routine and high-voltage electron microscopy. The RDEC-1 bacterium adhered specifically to M (membranous) rather than absorptive epithelial cells of the lymphoid follicle epithelium. Further understanding of how the bacterium attaches to M cells, which transport antigens to intraepithelial lymphocytes, could be useful in designing vaccines to protect mucosal surfaces.

INTRODUCTION

The RDEC-1 strain is a serogroup 015 *Escherichia coli* (1) that has been shown to express pili under appropriate cultural conditions (2). It also possesses a prominent negatively charged surface polysaccharide (3). The bacterium is not invasive and does not synthesize any of the classical enterotoxins (1). It adheres to the lymphoid follicle epithelium of ileal Peyer's patches of postweanling rabbits by 4 h postinoculation and by 24 h microcolonies are present on the follicle tips (4). The microcolonies, which can be quite large, are the probable source of RDEC-1 bacteria adherent to the ileal, cecal, and colonic mucosa beginning 3 d postinoculation. 6–10 d postinoculation, large numbers of bacteria can be seen closely adherent to mucosal epithelial cells and diarrhea occurs (1, 3–6). Close adherence of bacteria is associated with the loss of epithelial cell microvilli. Both pili and surface polysaccharides are thought to play a role in the adherence process (3, 7, 8).

This novel type of *E. coli* diarrhea was originally described in (1) and is specific (9) for rabbits. Human cases of diarrhea due to enteropathogenic serogroup *E. coli*, in which the gut histopathology is similar to that of strain RDEC-1 diarrhea in the rabbit, have now been reported (10–13). The rabbit provides an excellent animal model for the study of the human disease.

We used ultrastructural techniques to determine whether the RDEC-1 bacterium adheres to a specific cell in the lymphoid follicle epithelium, which includes absorptive epithelial cells, and M[1] (membranous cells). M cells transport antigen to underlying intraepithelial lymphocytes (14). We conclude that the RDEC-1 bacterium adheres specifically to the M cells of the lymphoid follicle epithelium.

METHODS

Bacterial preparation

Stock cultures of the RDEC-1 bacterium were stored in tryptic soy broth (Difco Laboratories, Detroit, MI) in 50%

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[1] *Abbreviation used in this paper*: M cells, membranous cells.

glycerol at −70°C. Bacteria for the inoculation of rabbits were inoculated into static tryptic soy broth and incubated for 18 h at 37°C. The bacteria were harvested by centrifugation and resuspended in a volume of phosphate-buffered saline (PBS; pH 7.4) to give a concentration of 10^6 bacteria/ml.

Antiserum preparation

Antiserum used in this study was prepared by intravenous injection of live RDEC-1 bacteria into rabbits (1). This antiserum has been shown to have antibody to RDEC-1 surface polysaccharides but has no detectable activity against RDEC-1 pili (3).

Rabbit inoculation

New Zealand White rabbits weighing 0.7–1.1 kg were checked for gut colonization with *E. coli* by swabbing the rectum and streaking the swab onto a MacConkey agar plate (Difco Laboratories). Lactose-positive colonies were checked by slide agglutination with anti-RDEC-1 serum to be certain that the rabbits were not colonized with the RDEC-1 bacterium. Rabbits without diarrhea or gut colonization with RDEC-1 bacteria were fasted overnight, and then challenged via an orogastric tube with 2 ml of the bacterial suspension (2×10^6 bacteria) (1) followed by 10 ml of 10% $NaHCO_3$. The rabbits were then allowed food ad lib. until killing.

Tissue preparation

Conventional electron microscopy. Rabbits were killed by intracardiac injection of a lethal dose of pentabarbital. The abdomen was opened immediately and the most distal ileal Peyer's patch was excised *en toto*. The Peyer's patch, cecum, and rectum were checked for the presence of RDEC-1 bacteria by swabbing the tissues, streaking the swabs onto MacConkey's agar, and confirming lactose-positive colonies as RDEC-1 strain by slide agglutination with specific antiserum.

Tissues were placed immediately into chilled 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4, 403 mosmol/kg) for 15 min. The tissue was removed from the fixative and sliced into 1–2-mm wide by 1–2-cm long strips that ran the full width and depth of the Peyer's patch. 6–10 strips were obtained from each patch. The tissue was then returned to the fixative overnight at 4°C, rinsed three times with 0.1 M cacodylate buffer, and postfixed with 2% OsO_4 in 0.1 M cacodylate buffer at room temperature for 3 h. The prolonged period of fixation in glutaraldehyde was necessary because of the greater mucosal-serosal thickness of the lymphoid follicles as compared with absorptive epithelium and the long length (1 cm) of the Peyer's patch strips. The strips were washed three times with buffer and trimmed to 1-mm wide by 4–5-mm long strips for embedment in a capsule mold. They were dehydrated through a graded ethanol series to 100% ethanol, followed by propylene oxide. The tissue was then infiltrated 3–4 h under vacuum in a 1:1 mixture of propylene oxide and Epon 812, placed in Epon 812 overnight under vacuum and embedded in a capsule mold. The tissue strips were oriented at the time of embedment so as to present the lateral or cut surface to the knife edge.

10–15 capsule molds from each animal were thick sectioned using a glass knife, stained with toluidine blue and examined under a light microscope to locate follicles with optimum orientation. Portions of strips in capsule molds, in which lymphoid follicles were well oriented (sagittally sectioned through the thickest point), from uninfected rabbits and rabbits postinoculation with the RDEC-1 bacterium were thin sectioned with a diamond knife. An additional selection criteria for rabbits postinoculation was the presence of adherent bacteria. The thin sections were stained on the grid with uranyl acetate followed by lead citrate. Three to five grids were prepared from the selected area of each mold. At least three grids from each mold were examined with an Hitachi HU 12-A transmission electron microscope (Hitachi, Tokyo, Japan) at an accelerating voltage of 0.075 MeV (3).

Treatment of tissues with antiserum to OK antigens

Some tissues were treated with antiserum to RDEC-1 OK antigens to provide positive identification of RDEC-1 bacteria and to stabilize the surface polysaccharides for electron microscopy (3, 15). Uninfected animals as well as animals at intervals postinoculation with the RDEC-1 bacterium were anesthetized by intramuscular injection of 0.2 ml of a ketamine-HCl (Vetalar, Parke-Davis, Division of Warner-Lambert Company, Morris Plains, NJ), xylazine-HCl (Rompun, Cutter Laboratories, Shawnee, KS) mixture in a 3:2 ratio. A laparotomy was then performed and the most distal ileal Peyer's patch located. The ileum was ligated 1–2-cm distal to the patch. 1–2-cm proximal to the patch, a 22-gauge needle was inserted into the ileal loop and secured with a loose ligature. The ligatures were placed so as to completely avoid the blood supply to the patch and adjacent ileum. Approximately 0.5–0.75 ml of warm, complement-inactivated antiserum or, in controls, complement-inactivated nonimmune sera or PBS, was injected into the ileal lumen containing the Peyer's patch, and the ligature tightened about the needle. The needle was removed and the ligature was tightened. The loop was distended only slightly by the serum or PBS. The intestines were then returned to the abdominal cavity for 20 min. The rabbit was then killed by intracardiac injection of pentabarbital and the ligated ileal loop containing the Peyer's patch was excised, opened, and rinsed with PBS. The patch, cecum, and rectum were cultured as previously described. The Peyer's patch was excised from the tissue and prepared for routine or high-voltage electron microscopy.

Ruthenium red-treated tissues

Tissues to be stained with ruthenium red to emphasize negatively charged surface polysaccharides (16) were processed routinely except that the glutaraldehyde and osmium fixatives and buffer washes contained 0.33% ruthenium red (EM Sciences, Fort Washington, PA).

High-voltage transmission electron microscopy

Tissues for high-voltage transmission electron microscopy were taken exclusively from the 12-h postinoculation group. We determined in preliminary studies that lymphoid follicles of tissues taken at 12-h postinoculation were most likely to have adherent RDEC-1 bacteria and show a spectrum of adherence from loose association with microvilli to close association with epithelial cells lacking microvilli. The tissues were obtained and processed as described for conventional

electron microscopy except that they were en bloc stained with aqueous 2% uranyl acetate before the dehydration steps (3). Each capsule mold was sectioned for and examined by light microscopy. Portions of capsule molds judged suitable on the basis of orientation and, in rabbits inoculated with bacteria, adherent bacteria, were thin sectioned at 0.05 μm and thick sectioned at 0.5 μm. Thin sections were stained on the grid as described for conventional electron microscopy. Thin sections were examined before cutting thick sections to be certain the section was desirable in terms of orientation and adherent bacteria. Thick sections were stained on the grid with 4% aqueous uranyl magnesium acetate and 2.5% lead citrate (3). The grids were carbon coated on both sides before viewing by high-voltage electron microscopy. The sections were observed simultaneously by both authors and photographed as stereo pairs at an accelerating voltage of 1 MeV with a high-voltage transmission electron microscope (No. 1000; Japanese Electron Optics Laboratory, Tokyo, Japan). A minimum of two grids per capsule mold were examined.

RESULTS

Peyer's patch tissues were available from 65 rabbits. 6 of the 65 rabbits were control animals and 59 were postinoculation with 2×10^6 RDEC-1 bacteria. Peyer's patches of 43 of the 59 rabbits inoculated with the RDEC-1 strain were examined by electron microscopy (Table I). Thick sections of four of the Peyer's patches taken from rabbits 12-h postinoculation, one stained with ruthenium red, two treated with antiserum against OK antigens and one patch treated with nonimmune serum (control loop), and one of the patches from an uninfected rabbit, were examined with the high-voltage electron microscope. We took 859 conventional electron micrographs and 110 high-voltage electron micrographs in the course of our studies.

Routinely prepared tissue from infected rabbits. As in our previous studies of absorptive epithelium (3, 5), several stages of bacterial approach and adherence to the epithelium were visualized in each of the 8-, 12-, and 24-h postinoculation tissue groups. Bacteria were found free in the lumen, among the microvilli, and closely adherent to apical cell membranes. They were rarely found within the epithelium or be-

TABLE I

Peyer's Patch Tissues Examined vs. Type of Treatment for Electron Microscopy. Infected Tissues Are from Rabbits Postinoculation with 2×10^6 RDEC-1 Bacteria

	Tissue treatment		
Tissue groups	Routine	Ruthenium red	Anti-OK antigen serum
Uninfected tissues	2/2*	1/2	
Ileal loops‡	2/2		
Infected tissues			
Hours postinoculation			
8	4/5	4/8	
12	3/3	7/10	
24	3/5	2/5	
Rabbits with diarrhea	2/3		
Infected tissues from ileal loops			
Hours postinoculation			
8			2/4
12	4/4§		9/12

* Number examined by electron microscopy/number prepared for electron microscopy.

‡ 1–2-cm long ileal loops were injected with PBS, nonimmune serum or immune serum and incubated in vivo in the abdomen for 20 min before killing of the animal and excision of the tissues.

§ Two of the four loops injected with nonimmune rabbit serum and two with PBS.

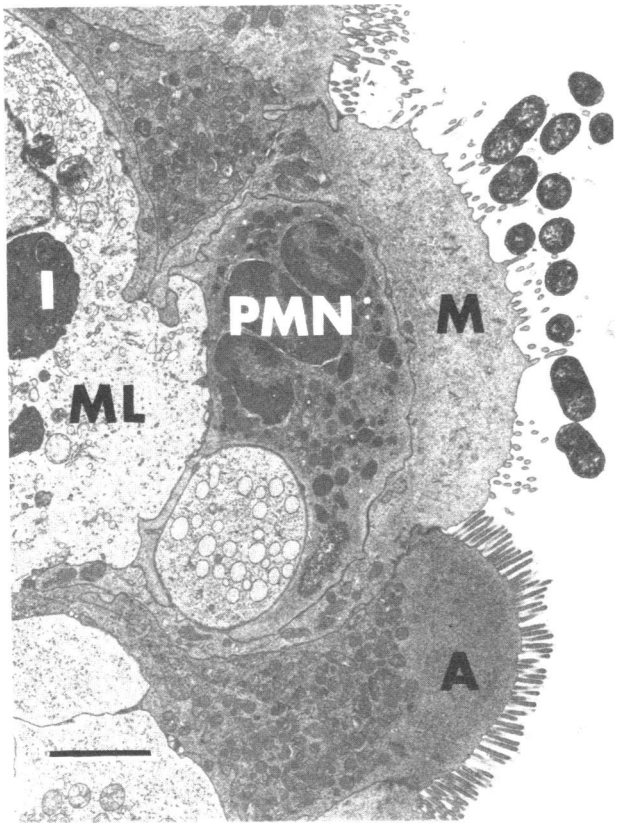

FIGURE 1 Routinely prepared tissue from a rabbit 12-h postinoculation with 2×10^6 RDEC-1 strain *E. coli*. Bacteria are in the lumen adjacent to and among the serpentine, loosely packed microvilli of an M cell (M). No bacteria are associated with the straight, tightly packed microvilli of an adjacent absorptive epithelial cell (A). A polymorphonuclear leukocyte (PMN) is on the lumenal side of the basement membrane in a position normally occupied by lymphocytes. The PMN is also intimately associated with a mononuclear leukocyte (ML) containing electron dense inclusions (I). Bar = 2 μm.

neath the basement membrane. Bacteria that were free in the lumen were distributed evenly above the different cell types of the follicular epithelium. Lumenal bacteria were rarely seen above the mucosal epithelium of the adjacent villi.

Bacteria were closely associated only with M cell microvilli or cell surfaces (Fig. 1), usually near the apex or upper one-third of the follicle. There was a striking lack of association with absorptive epithelial cells immediately adjacent to the M cells in the lymphoid follicle epithelium, until 24-h postinoculation. At that time, adherent bacteria formed large patches that included absorptive epithelial cells as well as M cells. Bacteria closely associated with intact M cell microvilli remained ~30 nm from the outer leaflet of the microvillar cell membrane (Fig. 2). In the routinely prepared tissue this area contained no stained material. Vesicles that lacked the internal structure of microvilli were present in the lumen at this stage of adherence. Bacteria closely associated with the apical surfaces of M cells that had lost their microvilli remained ~11 nm distant from the host cell membrane (Fig. 3). This adherence was ultrastructurally indistinguishable from the adherence of RDEC-1 to cecal and ileal absorptive epithelium (3, 5).

At 8-h postinoculation the majority of bacteria were in the lumen or among the serpentine M cell microvilli over the lymph follicles. 12-h postinoculation increased numbers and 24-h postinoculation large numbers of bacteria were closely adherent to M cell surfaces and microcolonies were beginning to form.

As early as 12-h postinoculation, polymorphonuclear leukocytes were seen within the lymphoid follicle epithelium beneath adherent bacteria (Fig. 1). 24-h postinoculation they were present in large numbers and occasionally could be seen in the lumen adjacent to the follicle. When present in the lumen they frequently contained phagocytized bacteria. The migration of polymorphonuclear leukocytes into the follicular epithelium was even more striking in rabbits with

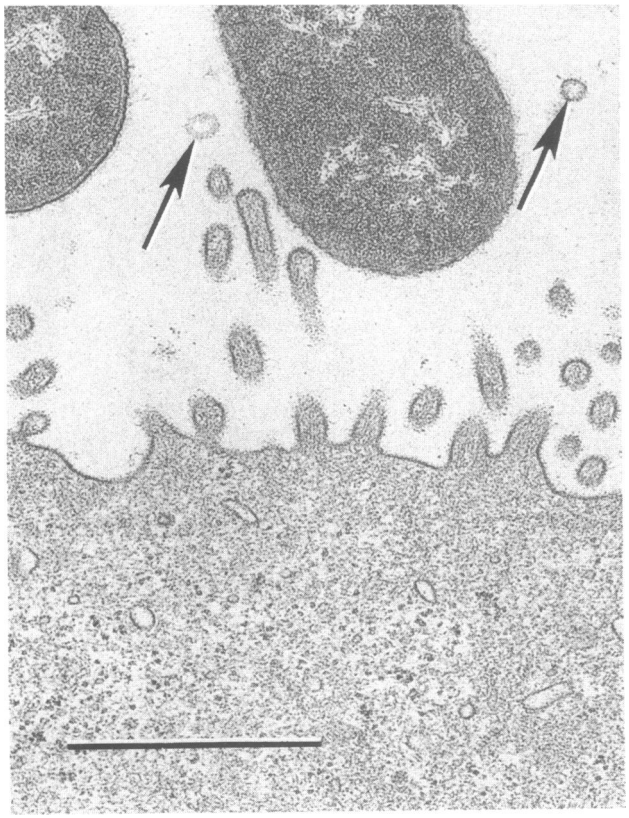

FIGURE 2 Routinely prepared tissue from a rabbit 8-h postinoculation. No material is visible in the space between bacteria closely associated with microvilli. Two membrane-enclosed vesicles (arrows) are present in the lumen. Bar = 1 μm.

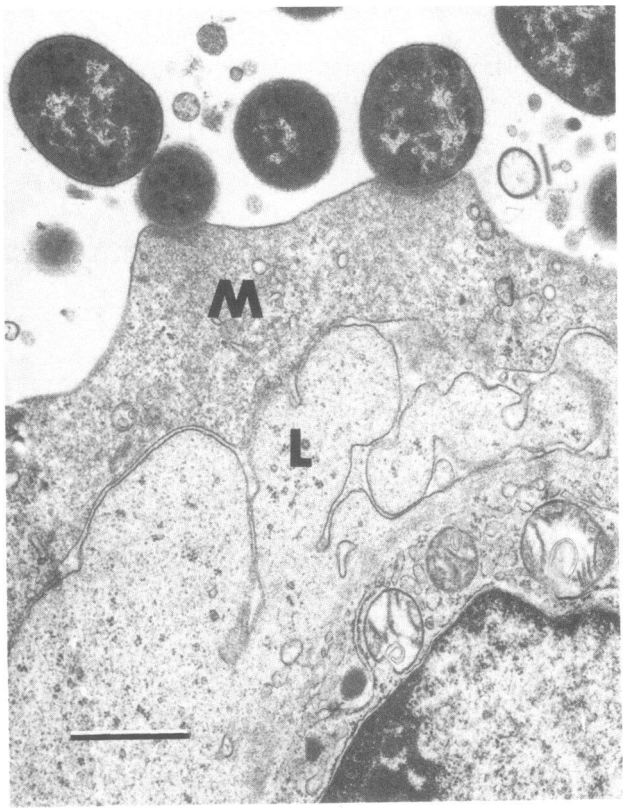

FIGURE 3 Routinely prepared tissue from a rabbit 24-h postinoculation. The bacteria are closely adherent to the surface of an M cell (M) that has lost its microvilli. The space between the bacterial cell wall and the plasma membrane of the M cell is ~11 nm. The cytoplasm of an intraepithelial lymphocyte (L) can be seen beneath the M cell. Bar = 1 μm.

diarrhea. Polymorphonuclear leukocytes were not seen in lymphoid follicles that lacked adherent bacteria.

High-voltage electron stereopair micrographs of infected tissue demonstrated previously unrecognized alterations in the ultrastructure of the microvilli (Fig. 4). Microvilli closely associated with bacteria became electron lucent, beginning at their tips, and often displayed multiple notches along their lengths. Vesicles were present in the lumen adjacent to damaged microvilli (Figs. 2 and 4).

Ruthenium red-stained infected tissues. Thin sections of ruthenium red-treated infected tissues demonstrated staining of the negatively charged bacterial surface polysaccharides, microvillar glycocalyx and amorphous material, the latter presumably microvillar glycocalyx plus bacterial surface polysaccharides, in the area between the bacteria and the M cell microvilli (Fig. 5). High-voltage electron micrographs demonstrated strands of tenuous ruthenium red-stained material extending between bacteria, M cell, and adjacent absorptive epithelial cell glycocalyx (Fig. 6).

Antiserum-treated tissues. We examined controls in which we injected PBS or nonimmune complement-inactivated rabbit serum into loops in noninfected animals and animals 12-h postinoculation with the RDEC-1 bacterium. The loops did not appear different from the routinely prepared tissues of control and infected animals when examined by conventional and high-voltage electron microscopy.

Lymphoid follicles from the antiserum-injected loops of rabbits 8- and 12-h postinoculation were examined. Where bacteria were closely associated with M cell microvilli anti-OK antigen serum-coated material was seen to span the distance between the bacterial cell wall and the M cell plasma membrane (Fig. 7). Recall that this space contained no stained material in the routinely prepared tissues (Figs. 1, 2, 4) and ruthenium red-stained material in ruthenium-red stained tissues (Fig. 5). This material was more easily visualized in high-voltage electron micrographs (Fig. 7).

Lymphoid follicle epithelium morphology. The

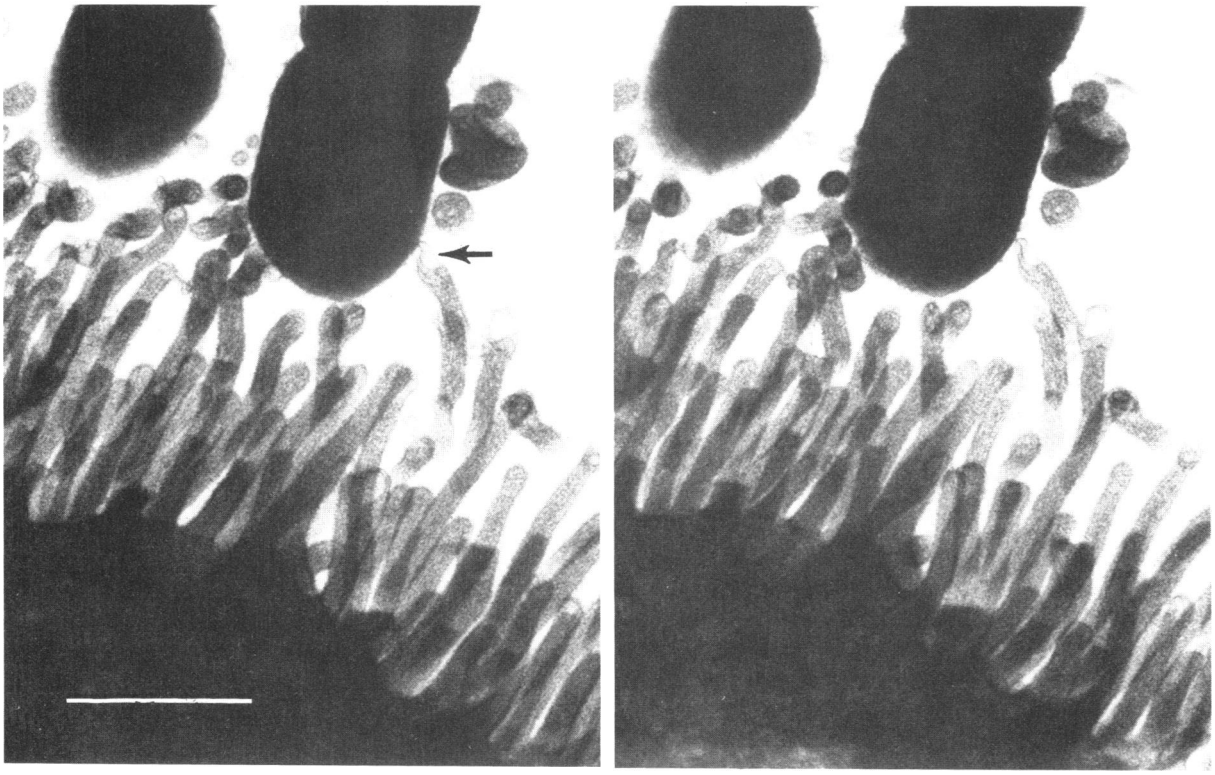

FIGURE 4 Stereopair high-voltage transmission electron micrograph of Peyer's patch tissue taken from an ileal loop injected with PBS (control loop) of a rabbit 12-h postinoculation. If no stereoviewer is available, the pair may be viewed in depth by crossing the eyes and concentrating on the middle of the three images that result. The M cell microvilli are of variable length, snake back and forth through the depth of the field, and have an irregular basal attachment. Some microvilli have formed lucent blebs at their tips (arrow). Note how several of the microvilli appear to curve towards and adhere to the bacterium. Little or no stained material can be seen in the space between the bacterium and the microvilli. Bar = 1 μm.

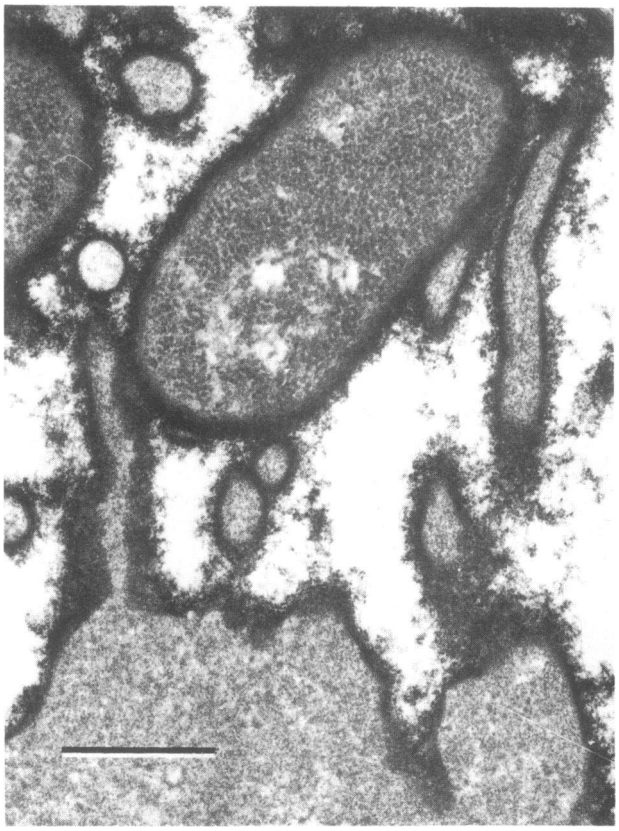

FIGURE 5 Thin section of ruthenium red-treated tissue from a rabbit 12-h postinoculation. The surfaces of the bacteria and the M cell microvilli are heavily stained with ruthenium red. The bacterium is closely associated with or adherent to several of the microvilli. Ruthenium red-staining material is visible in the space between the microvilli and the bacterium. Bar = 0.5 μm.

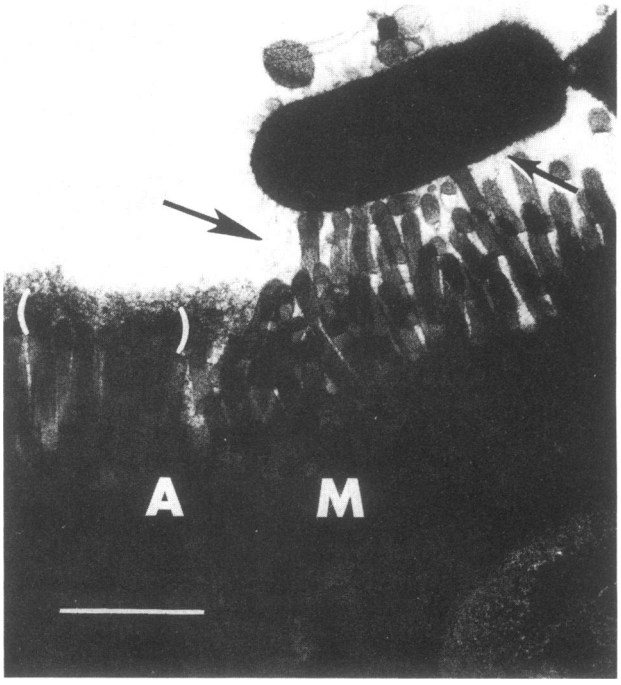

FIGURE 6 High-voltage transmission electron micrograph of ruthenium red-treated tissue from a rabbit 12-h postinoculation. The absorptive epithelial cell (A), has straight, closely packed microvilli with an abundant ruthenium red-positive glycocalyx mesh (). In contrast, the microvilli of the adjacent M cell (M), which are longer and not so closely packed, have minimal glycocalyx. The bacterium is seen closely associated with M cell microvilli that are oriented towards the bacterium. Electron lucent vesicles are in the lumen adjacent to the bacterium. Strands of ruthenium red-positive material can be seen extending between the bacterial capsule and the microvillar glycocalyx of the M (small arrow) and absorptive epithelial cell (large arrow). Bar = 1 μm.

epithelium of Peyer's patch lymphoid follicles from uninfected rabbits and the epithelium of lymphoid follicles of infected rabbits that lacked adherent RDEC-1 bacteria, conformed to descriptions of lymphoid follicle epithelium published elsewhere (14, 17–19, 20–23). The decreased glycocalyx and serpentine microvilli of the M cells were strikingly evident in the thick sections (Figs. 4, 6, and 7). Bacteria of varying morphology were sometimes present in the vicinity of, but were never found adhering to the lymphoid follicles of uninfected rabbits.

DISCUSSION

Thus, the RDEC-1 bacterium adheres specifically to the M cells of ileal lymphoid follicles very early postinoculation. Piliated RDEC-1 bacteria have been shown to agglutinate partially purified microvillus borders of rabbit ileal mucosal cells (7, 8). Pili may also mediate adherence of the RDEC-1 bacteria to M cells. Although bacterial surface polysaccharides were prominent in the space between adherent RDEC-1 bacteria and M cell plasmalemma, we could not draw any conclusions concerning their possible role in the adherence process.

Specificity of intestinal pathogens for the Peyer's patch is not novel. Reovirus particles injected into ligated loops of the gastrointestinal tract of mice gain entrance to the lymphoid follicle via the M cell (24). Salmonellae invade Peyer's patches preferentially, but whether they adhere to or invade through a specific cell in the lymphoid follicle epithelium is unknown

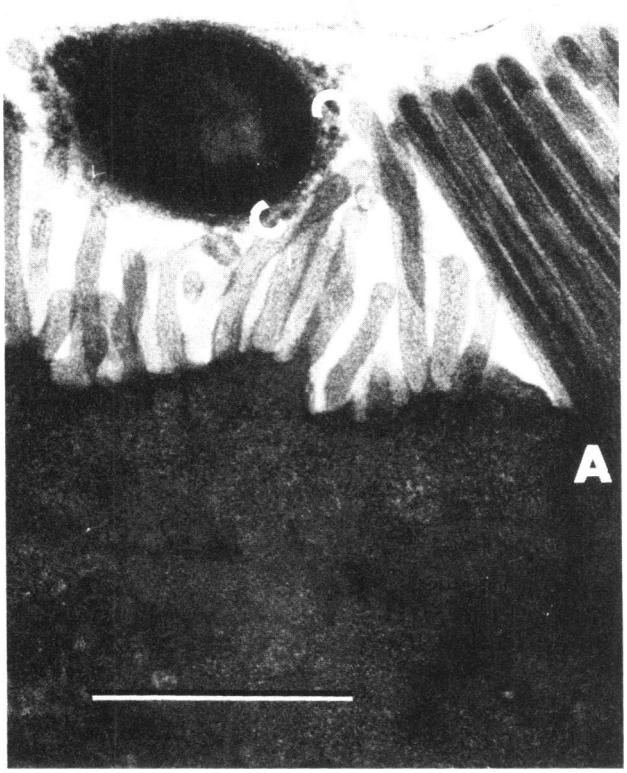

FIGURE 7 High-voltage transmission electron micrograph of tissue treated with antiserum to RDEC-1 OK antigens from a rabbit 12-h postinoculation. An antiserum-coated bacterium is closely associated with M cell (M) microvilli, some of which appear to bend towards the bacterium. The antibody that coats the bacterium and gives it a fuzzy appearance, bridges the space between the bacterium and the M cell microvilli ( ). There was no stained material in this space in Figs. 1 and 4. Ruthenium red-stained material was present in the space in Figs. 5 and 6. The M cell microvilli are of variable length and weave back and forth through the section. Microvilli of an adjacent absorptive epithelial cell (A) are straight, closely packed and normal in appearance. Bar = 1 μm.

(25, 26). *Giardia muris* is phagocytized by the lymphoid follicle (20). Whether the interaction of these organisms with the Peyer's patches is due to receptor specificity or to diminished interfering mucus and glycocalyx over the lymphoid follicle epithelium (14, 17–19), or to unknown factors, is uncertain.

Bacteria were not seen adhering to the lymphoid follicle epithelium of uninfected rabbits, consistent with the previous observations using the light microscope (4). This is surprising in view of the frequent presence of bacteria in the vicinity of the follicles and is further evidence of the specificity of the adherence of the RDEC-1 bacterium to the lymphoid follicle epithelium (4).

The blebbing and notching of the M cell microvilli closely associated with bacteria probably eventuated in the formation of the vesicles so numerous in the gut lumen in the area of adherent bacteria. The vesicles may be the M cell variant of the "round bodies," thought to be formed from degenerating microvilli of absorptive epithelial cells (27–28). Blebs and vesicles were noted in earlier studies of absorptive epithelial cells (3) and are no doubt due to microvillar damage associated with close adherence of the RDEC-1 bacteria.

In our previous study of absorptive epithelium we noted "pedestal" formation by the plasmalemma of epithelial cells with adherent bacteria. Pedestals were defined as extrusions of apical plasmalemma and cytoplasm to which bacteria were adherent and which appeared to cup the bacteria (3). They have also been described in tissues of patients infected with an enteroadherent *E. coli* (11, 13). Pedestals were very commonly seen in high-voltage electron micrographs of absorptive epithelial cells and their absence among M cells suggests a difference between the two cell types in membrane response to bacterial adherence.

The early appearance of polymorphonuclear leukocytes in the lymphoid follicle epithelium and in the lumen adjacent to the lymphoid follicle was surprising in view of the mild to moderate acute inflammation observed in the ileum, cecum, and colon of rabbits with RDEC-1 strain diarrhea (1, 5). The nonspecific local production of leukocyte chemotaxins by intraepithelial lymphocytes exposed to M cell transported RDEC-1 antigens could be an explanation for this event.

It is likely that the surface structure of the RDEC-1 bacterium necessary for specific adherence to the M cell can be determined. If human strains of enteroadherent *E. coli* also adhere to Peyer's patches and the surface structures responsible for their adherence can be ascertained and transferred to other bacteria, then the potential for enhancing the effectiveness of oral vaccines designed to protect mucosal surfaces is great.

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