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Scanning and Transmission Electron Microscopic Study of Adherence of *Escherichia coli* O103 Enteropathogenic and/or Enterohemorrhagic Strain GV in Enteric Infection in Rabbits

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The GV strain (serotype O103:H2:K−), originally isolated from a diarrheic rabbit, is an enteropathogenic *Escherichia coli* strain that produces diarrhea without synthesizing the classical enterotoxins and that is not invasive. This strain is characterized by a 117-kb plasmid (pREC-1). Histological study of the gut by scanning electron microscopy and transmission electron microscopy was performed on the GV strain, on a derivative strain cured of pREC-1, and on transconjugants obtained by transfer of pREC-1 to nonpathogenic strains *E. coli* K-12 and 6100, not belonging to the O103 serogroup. The GV strain adhered to the epithelial cells of the ileum and large intestine, whereas the cured GV strain did not. Transfer of plasmid pREC-1 to *E. coli* K-12 or 6100 allowed the bacteria to attach to the intestinal mucosa in the same manner as that of the wild-type GV strain. Thus, pREC-1 seems to play an important role in attachment to and colonization of the intestinal tract of rabbits by *E. coli* serogroup O103. Scanning electron microscopy showed numerous bacteria attached together and closely associated with intestinal villi. Transmission electron microscopy revealed effacing lesions characteristic of enteropathogenic *E. coli* strains: effacing of microvilli and cuplike projections (pedestal formations) associated with an acute inflammatory and hemorrhagic response. In contrast with the results reported for rabbit pathogenic O15 strains, it appeared that the Peyer's patches were not involved in the early stages of infection with the O103 GV strain. This strain may represent a model for the study of the virulence and pathogenic effects of enteropathogenic and enterohemorrhagic *E. coli* strains.

Cantey and Blake (6) were the first authors to describe a piliated *Escherichia coli* O15:H− strain (RDEC-1) that provokes diarrhea in rabbits without producing the classical heat-labile and heat-stable enterotoxins or invading the intestinal mucosa. Pathogenic *E. coli* O15 strains were also isolated from diarrheic weaned rabbits by Peeters et al. (25) in Belgium. These strains, which intimately adhere to the gut mucosa and provoke the loss of microvilli, are similar to human enteropathogenic *E. coli* (EPEC) strains. The results of several studies in infant and adult animals have suggested that such adherence of bacteria to the intestinal mucosa is an important factor in the pathogenesis of diarrhea caused by infection with EPEC strains (15, 19, 28).

In France, EPEC strains belonging to serogroup O15 rarely have been found up to now (4), but *E. coli* strains belonging to serogroup O103 have been isolated in 30 to 40% of diarrheic rabbits (5, 11a). It has been demonstrated previously that some strains of *E. coli* O103 are highly virulent for rabbits (2, 17, 26). One of these strains (GV), isolated from an outbreak of diarrhea and harboring an R plasmid of 117 kb (pREC-1), was studied by Reynaud et al. (27). This strain produces diarrhea without invading the mucosa and does not synthesize the classical heat-labile and heat-stable enterotoxins. It has been shown, in controlled experimental conditions, that pathogenicity for and colonization of the intestinal tract are related to the presence of this R plasmid (27).

The purpose of the present study was to investigate the histopathology of GV strain infection by means of scanning electron microscopy (SEM) and transmission electron microscopy (TEM) and to assess the role of pREC-1 in the intestinal adherence of this strain.

MATERIALS AND METHODS

Bacterial strains. The characteristics of the strains used have been previously described (27). In brief, starting from the wild-type pathogenic strain of *E. coli* O103:H2:K− harboring plasmid pREC-1 (GV strain) and two nonpathogenic strains devoid of the plasmid (*E. coli* K-12 [BM21] and an *E. coli* O?:H:K− strain [6100, not belonging to the O103 serogroup]), the following derivative strains were obtained: strain GVc, corresponding to wild-type strain GV cured of plasmid pREC-1, and transconjugants K-12/pREC-1, 6100/pREC-1, and GVc/pREC-1. Bacterial strains were stored at −75°C in 15% (vol/vol) glycerol broth until used. Before inoculation, bacteria were grown overnight in 10 ml of nutrient broth (brain heart infusion; Difco).

Animals. A total of 91 5-week old New Zealand White rabbits, free of *E. coli* O103 (10), were used; 56 (8 per inoculated strain) were used as controls for the course of the experimental disease, and 35 were used for histological examinations. Each animal was inoculated at 32 days of age with 10^7 bacteria via an orogastric tube. They were given

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food and water ad libitum. Criteria for determining pathogenicity (diarrhea, mortality, and weight gain), enumeration, and identification of *E. coli* by means of different tests (agglutination, fermentation of rhamnose, and antibiogram) were as described previously (27).

Tissue preparation and histological procedures. Two rabbits challenged with the different bacterial strains were sacrificed at 4 and 8 h postinoculation by intravenous injection of pentobarbital. Immediately after death, the abdomen was opened and the Peyer's patch closest to the ileocecal junction was excised and fixed in a bath of 3% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.4) for 1 h at 4°C. Three other rabbits per bacterial strain were sacrificed on day 6 postinoculation, which corresponds to the peak of gut colonization by the strains harboring plasmid pREC-1 (27). Segments about 1 cm long from the duodenum, ileum, cecum, and colon were taken, opened longitudinally, and affixed mucosa side outside to Bristol sheets (1 by 2 cm). After fixation, all the specimens were washed three times with 0.2 M cacodylate buffer.

For SEM processing, pieces of 5 mm^2 were cut and postfixed for 1 h at 4°C in cacodylate-buffered 1% osmium tetroxide. Tissues were rinsed, dehydrated in ethyl alcohol, dried to the critical point with carbon dioxide, coated with gold, and observed in a Philips model 505 SEM.

For light microscopy (LM) and TEM processing, small blocks of 1 mm^3 were cut, postfixed in 1% osmium tetroxide for 1 h at 4°C, rinsed, dehydrated in a graded ethanol series, and embedded in TAAB embedding resin. Semithin sections (1 μm) obtained from the resin blocks on an E. Reichert ultramicrotome were stained with toluidine blue and examined by LM (Olympus BH2 microscope). Sections selected by LM were processed for TEM by standard methods: 70-nm sections were stained with uranyl acetate and lead citrate and examined with a Philips model EM 400 TEM at 80 kV.

RESULTS

Pathogenicity, enumeration, and identification of fecal *E. coli* strains. The course of the experimental disease in inoculated animals was as previously observed (27). No mortality was observed in animals inoculated with plasmidless strains BM21, 6100, and GVc. Death rates 87.5, 62, 50, and 25% of those associated with diarrhea, generally of the hemorrhagic type, were obtained in animals inoculated with strains GV, GVc/pREC-1, K-12/pREC-1, and 6100/pREC-1, respectively.

Numbers of fecal *E. coli* cells were also similar to those previously reported (27). The identification tests confirmed that the *E. coli* cells recovered corresponded to the inoculated strains.

Examination of Peyer's patches. No layered bacteria were seen on the Peyer's patches in animals inoculated either with nonpathogenic *E. coli* K-12, 6100, and GVc or with pathogenic *E. coli* GV and transconjugants K-12/pREC-1, 6100/pREC-1, and GVc/pREC-1 at 4 and 8 h postinoculation.

Examination of the different segments of the intestinal tract. (i) Experimental infection with *E. coli* K-12, 6100, and GVc. No bacteria were seen 6 days postinfection in rabbits inoculated with *E. coli* K-12, 6100, or GVc, which does not contain plasmid pREC-1, in any segment studied by LM, SEM, and TEM. An SEM view of the ileum of a rabbit inoculated with *E. coli* 6100 is presented in Fig. 1. Numerous transverse furrows characterize the finger-shaped or tongue-shaped villi, which are identical to those of a healthy rabbit.

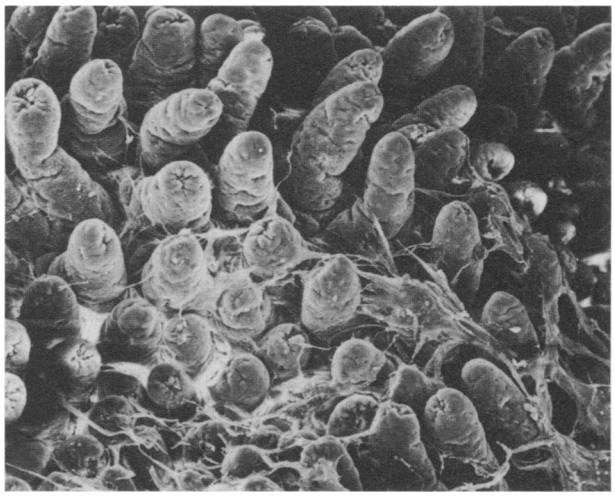

FIG. 1. SEM view of the ileum of a rabbit inoculated with *E. coli* 6100. Finger-shaped or tongue-shaped villi with multiple transverse furrows are shown. Magnification, ×100.

(ii) Experimental infection with *E. coli* GV and transconjugants K-12/pREC-1, 6100/pREC-1, and GVc/pREC-1. By LM, only a few bacteria were found lining the mucosa of the duodenum of animals inoculated with strain GV (Fig. 2) or with the transconjugants. No further examination was done on the duodenum. LM examination revealed a large number of bacteria attached to the epithelial cells of villi as well as of the crypts in the ileum, cecum, and colon. In comparison with the ileum of rabbits inoculated with *E. coli* K-12, 6100, or GVc, partial atrophy of villi could be observed in the ileum of rabbits inoculated with strain GV or with the transconjugants.

By SEM and TEM, it was not possible to detect any difference between animals inoculated with strain GV or with the different transconjugants. At a low magnification, SEM showed a patchy distribution of bacterial aggregates in the ileum and in the colon, while in the cecum the distribution seemed to be more diffuse. The bacterial aggregates

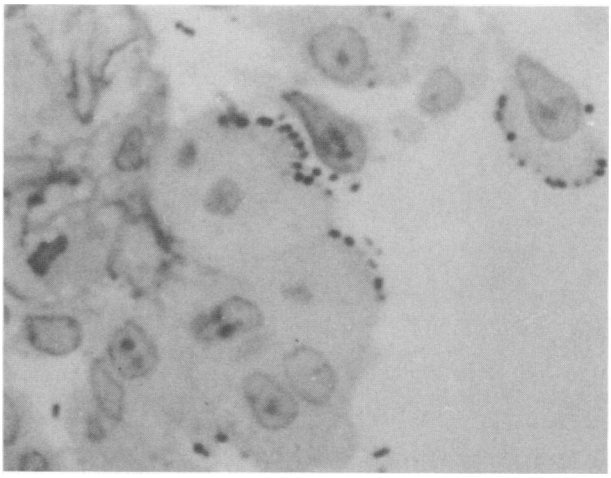

FIG. 2. LM view of the duodenum of a rabbit inoculated with *E. coli* GV showing only a few bacteria lining epithelial cells in the process of desquamation. Magnification, ×625.

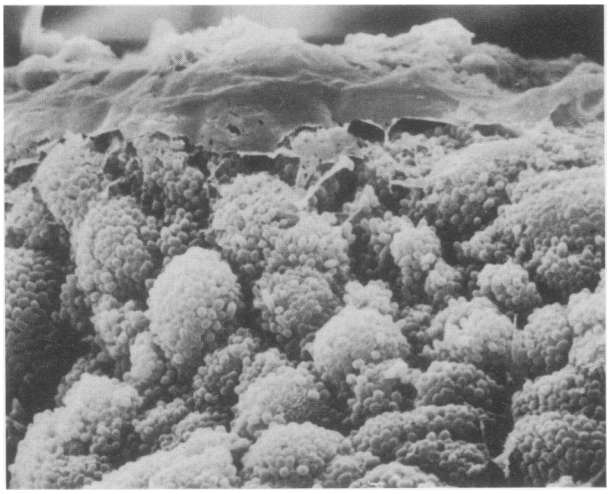

FIG. 3. SEM view of the ileum of a rabbit inoculated with *E. coli* K-12/pREC-1 showing heavily coated mucosa and bacteria covered in places with mucus. Bacteria are aggregated and piled on top of each other. Magnification, ×2,000.

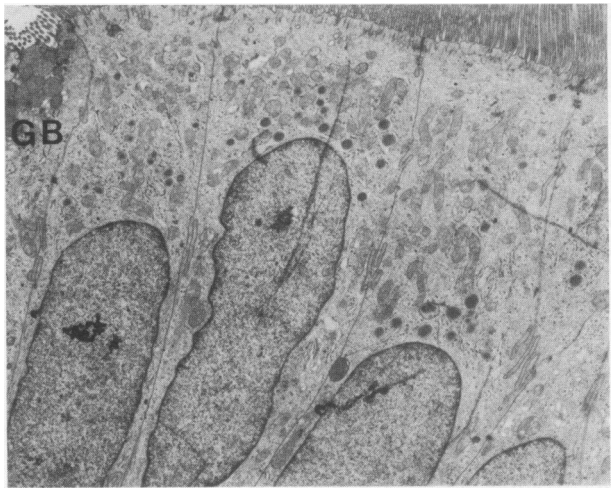

FIG. 5. TEM view of the ileum of a rabbit inoculated with *E. coli* K-12 showing typical columnar cells with elongated nuclei and unaltered microvilli. GB, goblet cell. Magnification, ×4,290.

were sometimes covered with a layer of mucus (Fig. 3). The cecum was hemorrhagic and edematous, and erythrocytes could frequently be seen in the lumen. Villi devoid of bacterial aggregates appeared normal. At higher magnifications, the mucosal surface colonized by bacteria showed a considerable number of aggregated *E. coli* cells intimately attached to the epithelium. Honeycomblike structures could sometimes be observed on infected epithelial surfaces from which the bacterial aggregates had been detached (Fig. 4), whereas on intact villous surfaces, the hexagonal lining of individual cells was hardly visible and the microvilli were intact.

More details on the attachment of and cellular changes induced by *E. coli* O103 were obtained by TEM, with similar findings in all the segments examined; only the observations made in the ileum will be reported here. Animals infected with *E. coli* harboring plasmid pREC-1 showed numerous bacteria localized at the luminal surface of the epithelial cells. The shapes of these cells were notably modified: they were rounded and had spherical nuclei, in contrast to the columnar form with elongated nuclei observed in rabbits inoculated with the plasmidless derivative (Fig. 5). A mild to pronounced inflammatory process associated with intense edema of the lamina propria infiltrated by polymorphonuclear leucocytes could be detected. The apical poles of enterocytes were strongly altered. The majority of affected cells had lost their microvilli, and their cytoplasmic membranes showed invaginations (Fig. 6) and cuplike structures (pedestals) to which bacteria were attached. A narrow space of about 10 nm separating the cell wall and epithelial cell

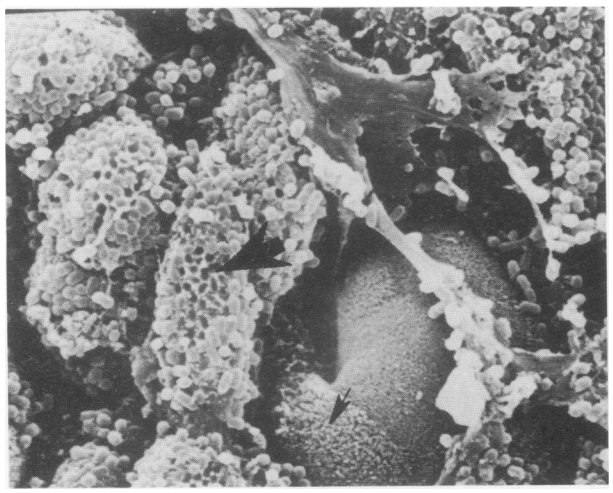

FIG. 4. SEM view of a rabbit inoculated with *E. coli* 6100/pREC-1. Honeycomb like structures of villi are visible where bacteria are detached (thick arrow). A villous surface with no associated bacteria seems to remain intact (small arrow). Magnification, ×2,500.

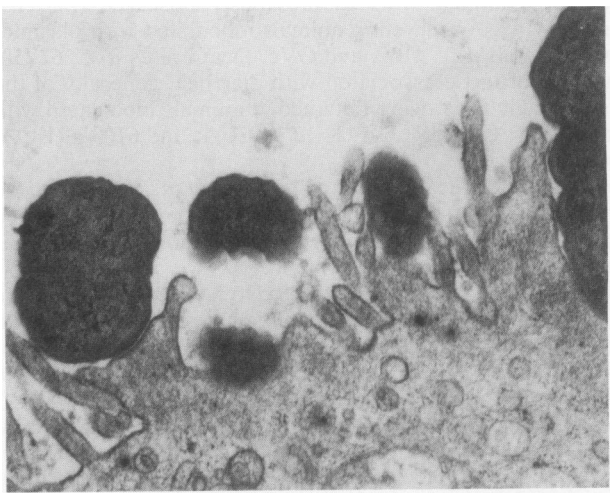

FIG. 6. TEM view of the ileum of a rabbit inoculated with strain GV. The glycocalyx is absent, and microvilli are destroyed. Note the pedestal formation with a bacterial cell intimately associated with the host epithelial cell. A narrow space of about 10 nm separates the bacterial and enterocyte membranes. The cytoskeleton is disrupted beneath attached bacteria. Magnification, ×33,000.

membranes could be distinguished. In the area immediately adjacent to the *E. coli* cell-host cell attachment, microvilli were elongated, vesiculated, or destroyed. The cytoplasm was disorganized beneath attached bacteria. The cytoskeleton associated with microvilli was disrupted, and several blebs were hardly recognizable. Only enterocytes with adherent bacteria were damaged: cells with no associated bacteria, even if closely adjacent to affected cells, remained intact, with nonaltered microvilli. No bacteria were seen in the cytoplasm of enterocytes or in intraepithelial leucocytes.

DISCUSSION

Diarrhea in weanling rabbits is the main problem of rabbit intestinal pathology. Besides coccidiosis (9), rabbit diarrhea is often associated with *E. coli* infections (5, 24). It has been shown experimentally that some strains belonging to serogroup O103 are highly virulent for rabbits (2, 17, 26). Our previous study of an *E. coli* O103 strain (GV) harboring an R plasmid of 117 kb (pREC-1) allowed us to establish the role of this plasmid in the colonization of the intestinal tract and in the pathogenicity of the strain (27). Adherence of pathogenic *E. coli* O103 to the intestinal mucosa had been suggested previously by Camguilhem et al. (3).

In the present study, histological examinations revealed a large number of bacteria attached to the luminal sides of enterocytes in rabbits inoculated with wild-type strain GV, whereas the same strain cured of its plasmid, pREC-1, was not found adhering to intestinal epithelial cells. None of the recipient strains studied (*E. coli* K-12, 6100, and GVc, which do not carry any plasmid) were able to attach to enterocytes, whereas transfer of plasmid pREC-1 to these nonadherent *E. coli* strains allowed them to adhere in the same manner as that of wild-type strain GV. This result confirms the role of plasmid pREC-1 in the colonization of the intestinal tract and subsequently in adherence and in pathogenicity and may account for the high intestinal numbers of *E. coli* GV cells observed in this study and in our previous work (27). Whether plasmid pREC-1 plays a direct role in adherence or merely in the survival of its host strain in the intestinal contents cannot be established from the present results.

Inman and Cantey (13, 14) demonstrated that attachment of rabbit pathogenic O15 strain RDEC-1, which involves specialized M cells from the Peyer's patches of the intestine, was mediated by surface pili, named adherence factor/rabbit 1 (AF/R1) and encoded by a 130-kb plasmid. When transfer of this plasmid was carried out in a *Shigella flexneri* strain, AF/R1 pili were expressed and adherence to rabbit ileal cells in vitro occurred without cytopathic effects (attaching or effacing lesions), although attachment of bacteria to M cells appeared early in experimental infections (8, 14). This result may be explained by the fact that *S. flexneri* is not the usual intestinal host in rabbits. A study by Wolf et al. (31) confirmed that the 130-kb plasmid of RDEC-1 mediates the expression of AF/R1 pili. These authors localized the genes responsible for the expression of AF/R1 pili and constructed a mutant from RDEC-1 (M34) that does not express AF/R1 pili. When inoculated into rabbits, M34 led to characteristic effacing lesions similar to those provoked by RDEC-1, although the lesions were less frequent and did not involve the ileum. It was concluded that AF/R1 pili promote adhesion but are not essential for attaching and effacing lesions. This conclusion is in agreement with the results of Knutton et al. (15) with human EPEC strains. These authors studied a strain of *E. coli* O127:H6 cured of a 90-kb plasmid involved in intestinal colonization. The cured strain was able to colonize cultured intestinal mucosa, with much less efficiency than the parental strain, but was able to induce the typical effacing of microvilli, indicating that plasmid-encoded factors are not entirely responsible for adherence and epithelial cell-damaging properties. In another experiment, Cantey et al. (7) showed that a mutant of RDEC-1 that does not express AF/R1 pili caused diarrhea as readily as did the parental strain and adhered to absorptive epithelium but not to Peyer's patches. In our experiments, the loss of the plasmid mediating attachment resulted in a complete loss of all the characteristics of EPEC adherence, suggesting that all of these properties are encoded by plasmid pREC-1 in the O103 strain studied. We could not clearly establish whether fimbrial material is involved in attachment of strain GV, although surface pili were revealed by negative staining (27). Esslinger et al. (11) recently reported that *E. coli* O103 strains expressed pili that played a role in attachment in vitro, with a diffuse adherence pattern, and presumably in vivo. They identified a 32-kDa protein in all of the EPEC and adherent *E. coli* O103 strains studied (18). The presence of similar structures will now be investigated in our strain GV.

The microlesions of the epithelial cells due to strain GV are strikingly similar to those reported for human EPEC strains (1, 15) or for the rabbit *E. coli* O15 strains studied by Takeuchi et al. (29) or by Peeters et al. (22, 23): they show effacing of microvilli, attachment to the apical cell membrane, cuplike projections (pedestal formations), and disruption of the cytoskeleton near the attached bacteria. No bacteria were seen inside cells by TEM, confirming that strain GV does not possess invasive properties. Like *E. coli* O15 strains, strain GV preferentially adheres to the mucosa of the ileum and of the large intestine, while human EPEC strains preferentially affect the duodenum (15) or the distal part of the small intestine and large bowel as well as the proximal midintestine and *E. coli* O109 from diarrheic suckling rabbits attaches to epithelial cells from the distal part of the small intestine and large bowel as well as the proximal midintestine (22). However, the kinetics of adhesion during infection seem different, as evidenced by the fact that no bacteria were seen on the Peyer's patches before 8 h in animals inoculated with *E. coli* O103, while this time corresponds to the early stage of infection of the Peyer's patches in rabbits infected with strain RDEC-1 (13, 22). Another difference is that severe intestinal hemorrhages were regularly observed in rabbits inoculated with strain GV, whereas such lesions have never been reported for *E. coli* O15. Thus, whereas it exerts all the characteristic histopathological effects of EPEC strains, strain GV may also be related to enterohemorrhagic *E. coli*. Like the O157:H7 strains of human origin (16, 30), strain GV affects the large intestine. Hall et al. (12) recently demonstrated that an *E. coli* strain of serotype O103:H2, isolated from a child with diarrhea, hybridized with a probe derived from a plasmid found in an *E. coli* O157:H7 strain. The acute inflammatory response and the extent of the lesions might be explained by the secretion of toxins differing from the heat-labile and heat-stable enterotoxins. O'Brien et al. (20) have already provided evidence that some human EPEC strains as well as strain RDEC-1 from rabbits produce small amounts of a Shiga-like toxin, and this toxin is also produced by enterohemorrhagic *E. coli* strains (21), but how this toxin interacts with intestinal cells remains unclear. The search for this toxin in strain GV is in progress.

In conclusion, our histological findings indicate that strain GV, belonging to the O103 serogroup, possesses character-

istics in common with other EPEC strains, such as RDEC-1, but some differences need further investigation.

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