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Ultrastructural Study of Adherence to and Penetration of Cultured Cells by Two Invasive *Escherichia coli* Strains Isolated from Infants with Enteritis

STUART KNUTTON,[1] PETER H. WILLIAMS,[2]* DAVID R. LLOYD,[1] DAVID C. A. CANDY,[1] AND ALEXANDER S. McNEISH[1]

Institute of Child Health, University of Birmingham, Birmingham B16 8ET,[1] and Department of Genetics, University of Leicester, Leicester LE1 7RH,[2] England

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The adherence of invasive *Escherichia coli* strains 444-3 and 469-3 to human erythrocytes and to cultured HeLa and HEp-2 cells has been examined by electron microscopy. Bacteria elaborating type 1 fimbriae, glycocalyces, and nonfimbrial mannose-resistant hemagglutinins specific for human erythrocytes were identified in cultures of both strains, and each of these different bacterial surface components appeared to be involved in attachment of 444-3 and 469-3 to cultured epithelial cells or human erythrocytes (or to both). Both strains, which were isolated from infants with dysentery-like illness, penetrated cultured epithelial cells and existed within membrane-bounded intracellular vesicles. Mutants of 444-3 and 469-3 selected for deficiency in mannose-resistant hemagglutination did not adhere to or penetrate cultured cells. These ultrastructural studies demonstrate the complexity of the bacterial surface and show that *E. coli* strains 444-3 and 469-3 can elaborate several different adhesins, each of which could function to promote attachment to host intestinal epithelial cells. Mucosal invasion may also be an important virulence property of these strains.

Strains of *Escherichia coli* causing diarrheal disease in humans have been classified into three groups: enterotoxigenic, enteropathogenic, and enteroinvasive *E. coli*. Enterotoxigenic *E. coli* produce enterotoxins that cause cholera-like symptoms. Enteropathogenic *E. coli*, which are also noninvasive, have recently been shown to produce a *Shigella dysenteriae* type 1 (Shiga)-like toxin that may play a role in their pathogenesis. Enteroinvasive *E. coli* invade the intestinal mucosa and cause a dysentery-like illness (2).

For noninvasive organisms the ability to adhere to the mucosal surface of the intestine is now recognized as an important early event in colonization and the development of diarrheal disease (2, 10). Mucosal adherence must also precede invasion; hence in the initial stages of invasive diarrhea the ability of organisms to adhere is likely to be an important determinant of pathogenicity. Enterotoxigenic *E. coli* adhesion is mediated by filamentous bacterial structures termed fimbriae or pili, attachment being achieved by the interaction of the tips of many of these rodlike structures with specific mucosal receptors (12b). Enterocyte adhesion has recently been shown to be a common feature of intestinal colonization by human enteropathogenic *E. coli* strains (22), and ultrastructural studies show bacteria attached to and partially surrounded by cuplike projections of the apical plasma membrane with <10 nm separating the bacteria and the host membrane. At regions of attachment microvilli are lost, and the underlying cytoskeleton is disrupted or destroyed (16). Mucosal attachment of enteroinvasive *E. coli* has not been described.

In a previous study (14) in our laboratory it was shown that *E. coli* strains 444-3 and 469-3, isolated from infants with dysentery-like diarrhea (17), elaborated mannose-resistant (MR) hemagglutinins specific for human erythrocytes and that both strains adhered to HeLa and HEp-2 cells grown in culture. We have now examined the adhesion of these strains to human erythrocytes and to cultured epithelial cells by electron microscopy, and in this paper we present ultrastructural observations that indicate that type 1 fimbriae and the bacterial glycocalyx, in addition to MR hemagglutinins, are involved in mediating attachment of 444-3 and 469-3 to one or both cell types. The penetration of cultured cells with subsequent recovery of viable bacteria has also been demonstrated.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains 444-3 (O?:H4) and 469-3 (O21:H$^-$) have been described previously (14). Strains LG1412 and LG1505 are hemagglutination-deficient mutants of 444-3 and 469-3, respectively (23). Strains were grown aerobically either on nutrient agar or in Mueller-Hinton broth for 18 h at 37°C.

Antisera. Antisera against cell surface-associated MR hemagglutinins were prepared by the procedure of Evans et al. (9). Antisera raised in rabbits against Formalin-treated 444-3 and 469-3 were absorbed with live cells of the hemagglutination-deficient mutants LG1412 and LG1505, respectively, until no agglutination was detected. Sera were stored at −70°C until used.

Hemagglutination. Hemagglutination was tested at room temperature on rocked glass slides using equal volumes of a bacterial suspension (10^9/ml) and a washed 3% suspension of human group A or guinea pig erythrocytes. To test for mannose-sensitive hemagglutination erythrocytes were suspended in phosphate-buffered saline containing 1% D-mannose. Hemagglutination was graded from 0 to +4 depending on the strength and rapidity of the reaction.

Epithelial cell adhesion. Washed suspensions of bacteria in Eagle basal medium containing 10% fetal calf serum were added to subconfluent monolayer cultures of HeLa and HEp-2 cells and incubated for 3 h at 37°C. In some experi-

* Corresponding author.

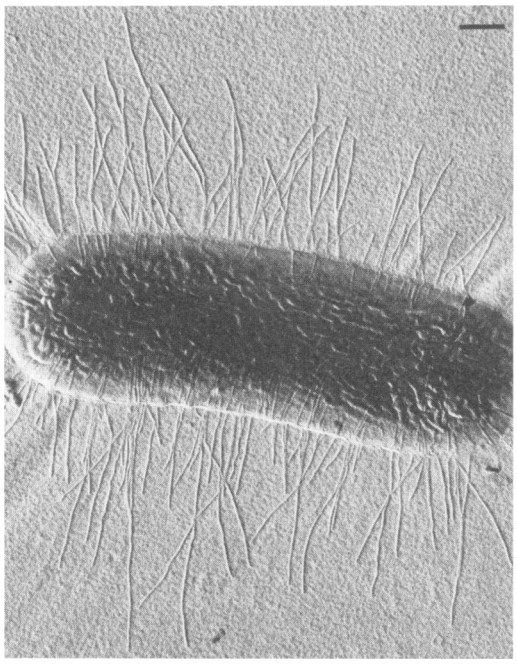

FIG. 1. Surface replica of strain 469-3. Numerous ~7-nm-diameter fimbriae project from the bacterial surface. Bar, 0.2 μm.

ments cells and bacteria were incubated at 37°C for 1 h, nonadhering bacteria were removed by washing, and the cells were incubated for up to 18 h in fresh medium. At the end of the incubation period cells were thoroughly washed with several changes of buffer before being processed for microscopy.

Epithelial cell penetration. Nalidixic acid-resistant mutants of 444-3 and 469-3 were used. Washed cover slip monolayer cultures of HeLa cells were incubated with bacteria as previously described (14). At intervals cover slips were removed, washed, and incubated in the same conditions with and without inhibitory concentrations of streptomycin (100 μg/ml) plus kanamycin (25 μg/ml) at 37°C for 1 h. Cultures were again washed thoroughly and trypsinized to suspend the cells. The cells were homogenized, serially diluted, and plated for viable counts on nutrient agar containing nalidixic acid. Colonies from the antibiotic-treated samples were checked for contaminants by drug resistance spectrum, plasmid content, and reaction with specific antiserum.

Immunofluorescence. Suspensions of bacteria of HEp-2 cell monolayers with adherent bacteria were incubated with suitable dilutions of the absorbed 444-3 or 469-3 antiserum for 30 min at room temperature. After three 5-min washes with phosphate-buffered saline, the cells were stained for 20 min with fluorescein isothiocyanate-protein A (Pharmacia) and washed a further three times with phosphate-buffered saline. Preparations stained with heterologous antiserum and fluorescein isothiocyanate-protein A or fluorescein isothiocyanate-protein A alone were used as controls. Bacteria or cover slip monolayer cell cultures were mounted on microscope slides and immediately examined under incident light fluorescence. Micrographs were recorded with a Leitz Dialux microscope equipped with Orthomat camera; phase-contrast micrographs of the same field were also recorded.

Electron microscopy. For negative staining 10 μl of a washed suspension of bacteria was mixed with equal volumes of bacitracin (150 μg/ml) and ammonium molybdate (2%, pH 7.0). A 10-μl sample was applied to carbon-coated grids for 1 min, and the excess liquid was removed with filter paper. Surface replicas were prepared by applying 10 μl of a washed suspension of bacteria to carbon-coated grids for 1 min, removing the excess liquid, and shadowing the air-dried specimens with platinum and carbon.

For thin sections, bacteria, agglutinated erythrocytes, or cell monolayers with adherent bacteria were fixed in 3% phosphate-buffered (0.1 M, pH 7.4) glutaraldehyde for 1 h at room temperature, washed, and postfixed for 2 h in 1% osmium tetroxide. Negatively charged cell suface components were labeled with either ruthenium red (13) or cationized ferritin (7). For ruthenium red staining phosphate buffer was replaced with cacodylate buffer (0.1 M, pH 7.2), and ruthenium red was present in both fixative and wash solutions at a final concentration of 0.075%. Labeling with cationized ferritin (2 mg/ml) for 30 min at room temperature took place before the primary glutaraldehyde fixation. For immunoferritin labeling cells were incubated with suitable dilutions of the absorbed antiserum for 30 min at 4°C, washed three times with phosphate-buffered saline, incubated with goat anti-rabbit serum conjugated to ferritin (Miles-Yeda) for 30 min at 4°C, and washed a further three times with phosphate-buffered saline before fixation. Samples incubated with heterologous antiserum and ferritin conjugate or ferritin conjugate alone were used as controls. Fixed specimens were dehydrated through a graded series of ethanol solutions and embedded in Epon. Sections were cut with a Sorvall MT-5000 ultramicrotome and stained with saturated aqueous uranyl acetate (ruthenium red-stained specimens) or with uranyl acetate and lead salts (18). Specimens were examined in a Philips EM301 electron microscope operated at 80 kV.

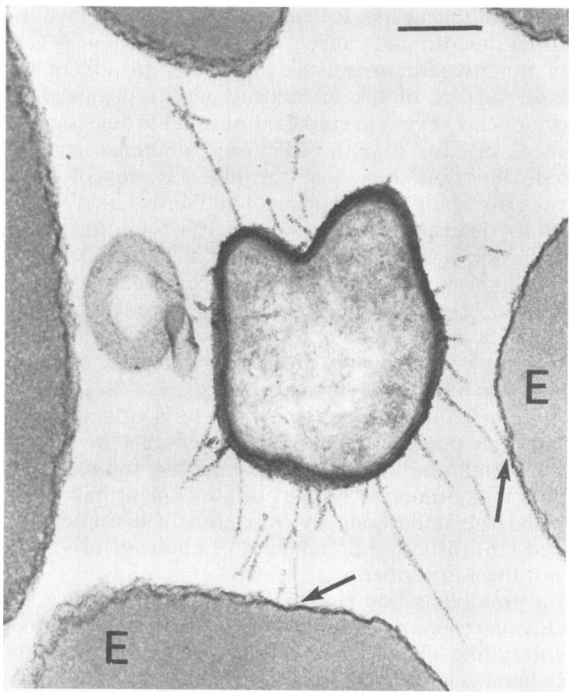

FIG. 2. Electron micrograph showing human erythrocytes (E) agglutinated by 444-3. The tips of fimbriae stained by ruthenium red can be seen to make contact with the erythrocyte membrane (arrows). Bar, 0.2 μm.

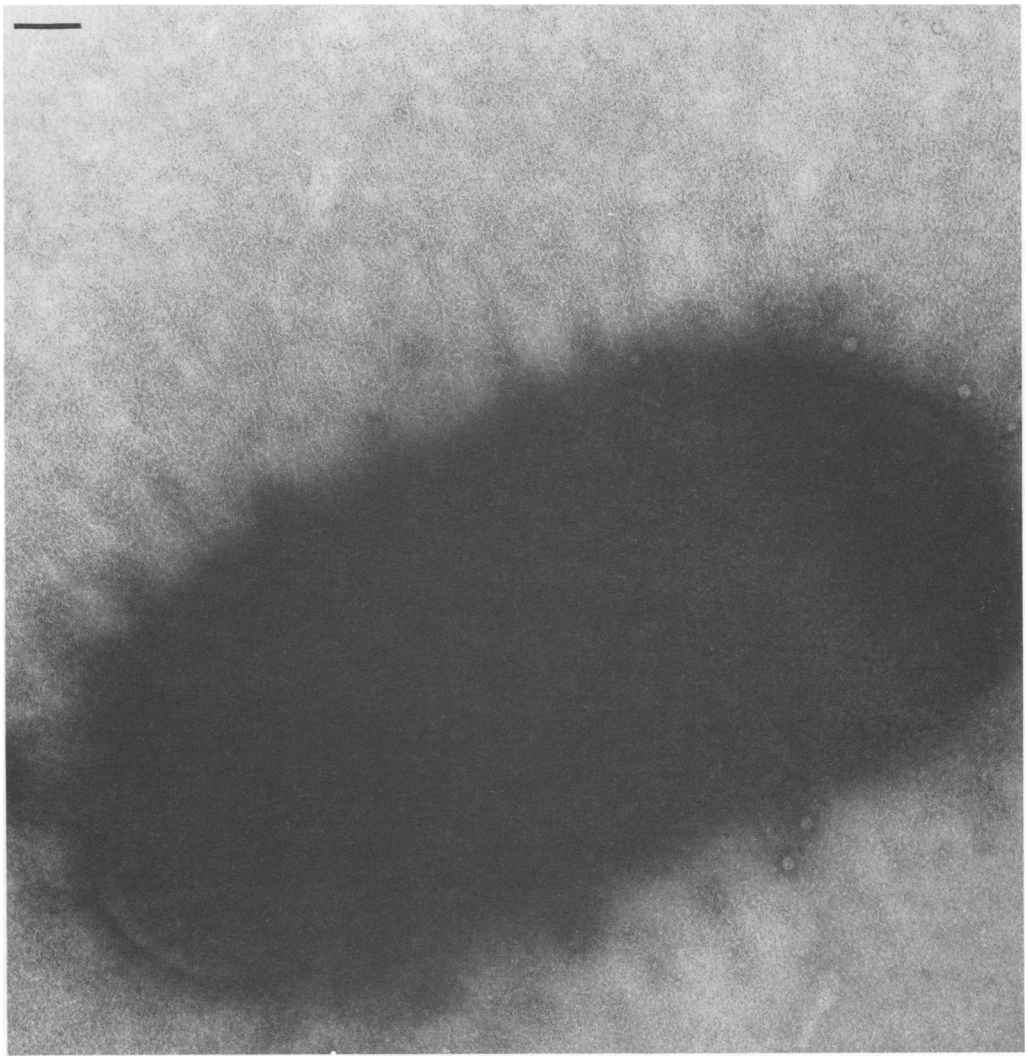

FIG. 3. Negative stain preparation of strain 444-3. The bacterial glycocalyx appears as a dense mosaic of ~2-nm-diameter flexible fibrils up to 1 μm in length surrounding the bacterium. Bar, 0.1 μm.

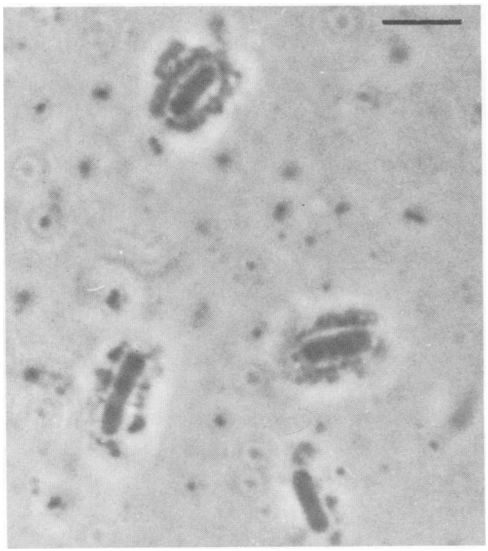

FIG. 4. Phase-contrast micrograph showing 444-3 suspended in India ink. A transparent halo surrounds bacteria possessing a glycocalyx. Bar, 5 μm.

RESULTS

Bacterial ultrastructure. Examination of bacteria by negative staining or in surface replicas showed that the expression of MR hemagglutination of human erythrocytes by strains 444-3 and 469-3 did not correlate with the presence of fimbriae (pili). The majority of bacteria were devoid of surface structures and appeared smooth, although a small percentage of fimbriate bacteria were observed in cultures of all strains examined, including the hemagglutination-deficient mutants (Fig. 1). Fimbriate organisms were more numerous in static broth cultures, and the proportion of bacteria producing fimbriae was significantly increased when bacteria were serially passaged in broth in association with the appearance of mannose-sensitive hemagglutination of guinea pig erythrocytes. In negative-stain, surface replica (Fig. 1), and thin-section preparations (Fig. 2) fimbriae appeared as ~7-nm-diameter, rodlike structures up to 1.5 μm in length.

A dense mosaic of thin ~2-nm-diameter flexible fibrils extending up to 1 μm from the bacterial surface was a feature of a further small percentage of bacteria in cultures of 444-3 and 469-3 examined by negative staining (Fig. 3). Examination of bacteria suspended in India ink confirmed the pres-

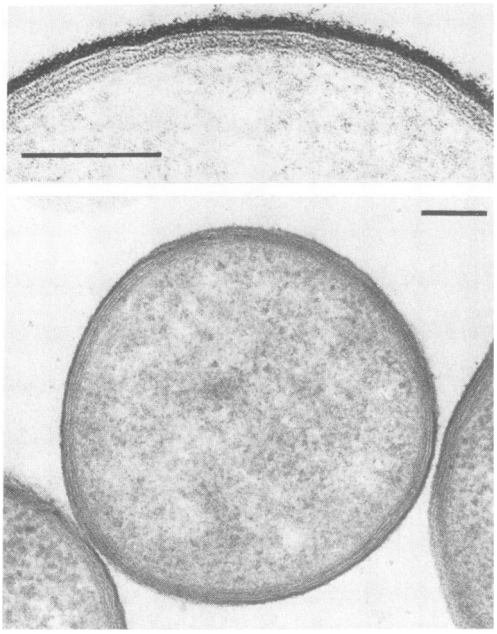

FIG. 5. Ruthenium red-stained preparation of strain 469-3 illustrating the smooth surface morphology of the majority of bacteria. A ~10-nm-thick layer at the surface of all bacteria was heavily stained by ruthenium red. Bars, 0.1 μm.

ence in cultures of both strains of bacteria producing glycocalyces (capsules). A transparent halo surrounding bacteria producing glycocalyces was seen owing to the impermeability of the glycocalyx to the carbon particles of the ink (Fig. 4). The bacterial glycocalyx is not visualized by conventional electron microscopy procedures, but can be detected with polyanion-specific stains like ruthenium red (6). Ruthenium red stained a ~10-nm-thick layer at the surface of all bacteria (Fig. 5), but large irregular-shaped particles were an additional feature of those bacteria possessing a glycocalyx (Fig. 6), because, in the absence of stabilizing agents, the glycocalyx polysaccharides collapse onto the bacterial surface during preparation for electron microscopy (1, 6). We attempted to stabilize the glycocalyx and prevent such dehydration artifacts by using the larger and more dense anion-specific molecule, cationized ferritin. With this reagent linear aggregates of ferritin molecules radiating from the bacterial surface were frequently seen (Fig. 7); in other cases, a layer of closely packed ferritin molecules surrounded the entire bacterium (Fig. 8). Bacteria elaborating glycocalyces were more numerous in cultures of 444-3 and were not seen in cultures of the hemagglutination-deficient mutants.

MR hemagglutinins elaborated by 444-3 and 469-3 did not appear to exist as morphologically identifiable entities, and so immune labeling was used to characterize these molecules. Less than 25% of bacteria in cultures of 444-3 and 469-3 were observed to fluoresce strongly when stained with the appropriate absorbed antiserum (Fig. 9); by immunoelectron microscopy two distinct patterns of ferritin labeling were observed. Most bacteria displayed a fairly uniform labeling of the bacterial surface (Fig. 10), although, in the case of bacteria possessing glycocalyces, ferritin labeling of the glycocalyx was observed (Fig. 11). Bacteria elaborating glycocalyces were not present in cultures of the hemagglutination-deficient mutants, which would explain why glycocalyx antibodies were present in the absorbed antisera in addition to antibodies to MR hemagglutinins.

Adhesion to cultured cells. When cell monolayers were incubated for 3 h at 37°C with dilute (10^4 to 10^5/ml) suspensions of bacteria, small randomly distributed colonies of adherent bacteria were observed; with more concentrated suspensions (10^8 to 10^9/ml), adherent bacteria covered the entire apical cell surface.

Electron microscopy of cell monolayers revealed bacteria attached to both cell surface microvilli and to nonmicrovillous membrane (Fig. 12). The majority of adherent bacteria displayed a smooth surface morphology, although in some experiments up to 20% of bacteria possessed a glycocalyx; fimbrially mediated attachment of 444-3 and 469-3 to HeLa and HEp-2 cells was not observed. In the case of smooth-surfaced bacteria a small (<10-nm) gap generally separated the closely apposed bacterial and epithelial cell surfaces (Fig. 12, inset), but in ruthenium red-stained preparations intimate contacts between the two surfaces were revealed (Fig. 13). Ruthenium red appeared to be staining components of the epithelial cell glycocalyx external to the unit membrane with which bacteria were in contact.

Close apposition of bacterial and epithelial cell surfaces was also a feature of the attachment of bacteria possessing a glycocalyx when demonstrated by ruthenium red staining (Fig. 7a). In contrast, when cationized ferrin was used to visualize the glycocalyx, bacteria were separated from the epithelial cell surface by up to 1 μm, but were connected to it by glycocalyx fibrils labeled by ferritin molecules (Fig. 7b).

All bacteria adhering to HeLa and HEp-2 cells exhibited a strong immunofluorescence (Fig. 14), but when immunoelectron microscopy was used to examine attached bacteria the two patterns of ferritin labeling reported earlier were observed (Fig. 11).

Erythrocyte adhesion. When human erythrocytes agglutinated by 444-3 and 469-3 were examined by electron microscopy, morphological observations similar to those described for bacterial attachment to epithelial cells were obtained, i.e., there was either very close apposition of bacterial and erythrocyte surfaces or of the bacterial glycocalyx and

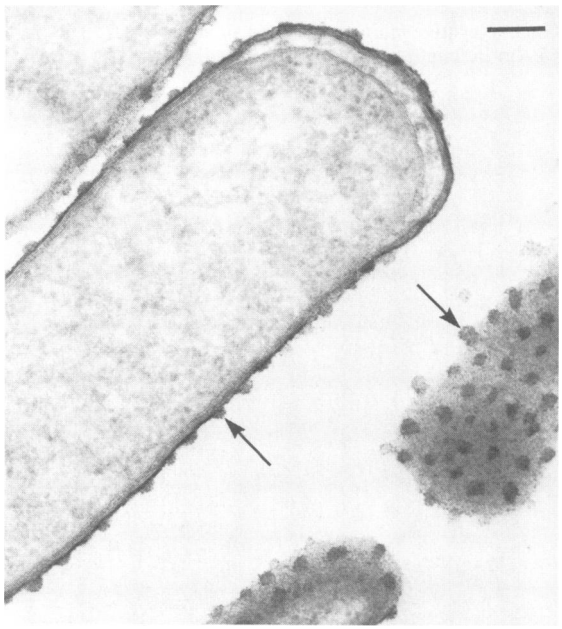

FIG. 6. Ruthenium red-stained preparation of strain 444-3. Large globular particles are a surface feature of bacteria producing a glycocalyx. Bar, 0.1 μm.

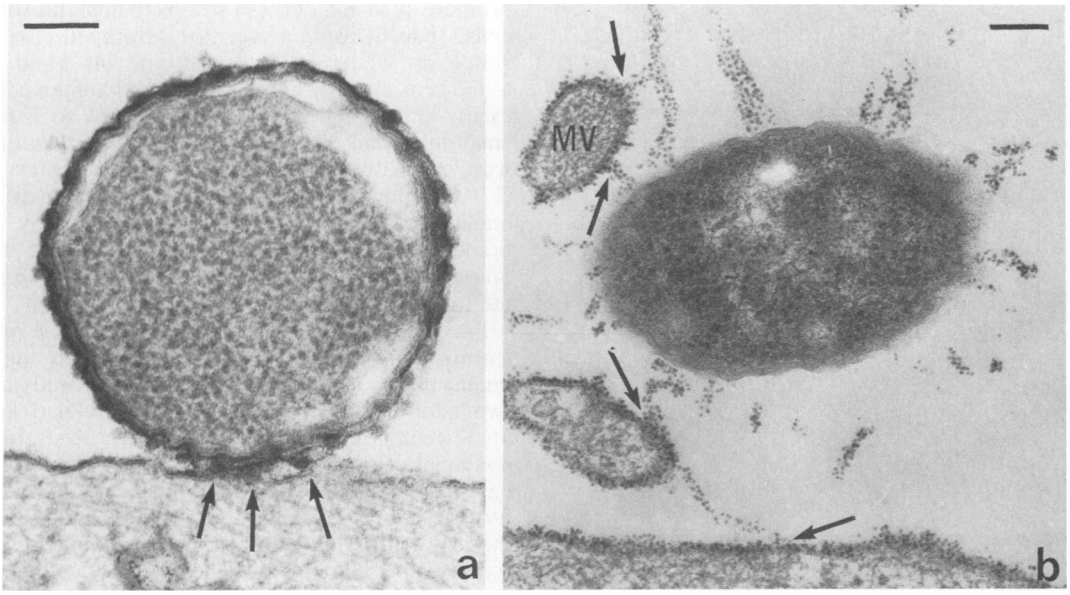

FIG. 7. Attachment of bacteria possessing a glycocalyx to HEp-2 cells. In ruthenium red-stained preparations large globular particles on the bacterial surface make contact with the HEp-2 cell membrane (a); in cationized ferritin-labeled preparations it is linear glycocalyx fibrils that are seen to make contact with the HEp-2 cell surface (b, arrows). MV, microvilli. Bar, 0.1 μm.

erythrocyte surface (Fig. 8). In addition, fimbrially mediated attachment of 444-3 and 469-3 to human and guinea pig erythrocytes was observed (Fig. 2); fimbriae are also visualized by ruthenium red staining (12b). Bacteria were separated from erythrocytes by up to 1 μm, and numerous ~7-nm-diameter fimbriae were seen to connect bacteria to the erythrocyte surface; only the tips of fimbriae appeared to make contact with the erythrocyte membrane (Fig. 2).

Epithelial cell penetration. The invasive properties of strains 444-3 and 469-3 were investigated by two independent methods. Penetration of HeLa cells was examined by a procedure that involved recovering internalized bacteria from cells after the killing of surface-associated bacteria with inhibitory concentrations of antibiotics (see above). The data from five separate experiments are shown in Fig. 15. For both 444-3 and 469-3 the fraction of bacteria protected

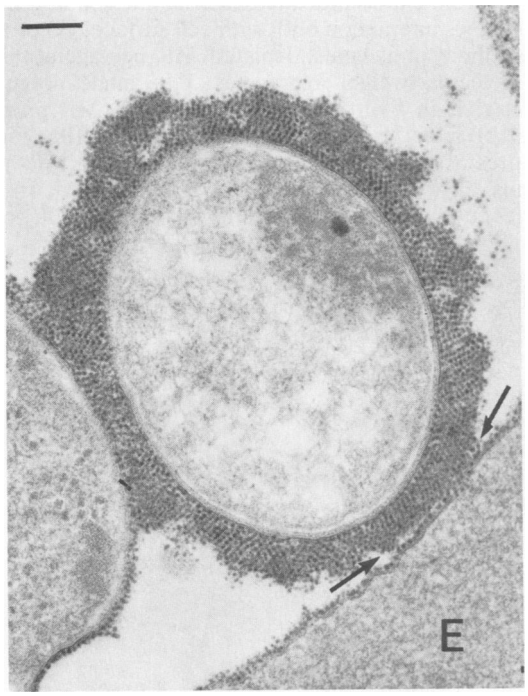

FIG. 8. Adhesion of bacteria possessing a glycocalyx to human erythrocytes. The ~0.2-μm-thick glycocalyx labeled by cationized ferritin makes contact with the erythrocyte (E) membrane (arrows). Bar, 0.1 μm.

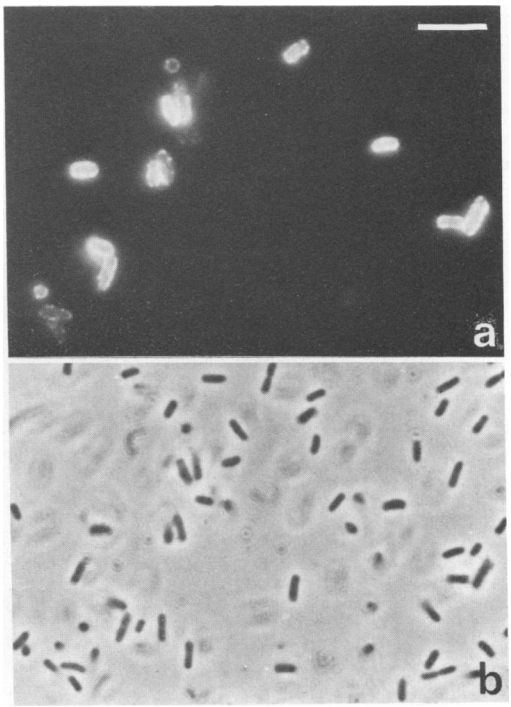

FIG. 9. Indirect immunofluorescence staining of strain 444-3 (a) and a phase-contrast micrograph of the same field (b). Less than 25% of bacteria exhibit a strong fluorescence. Bar, 10 μm.

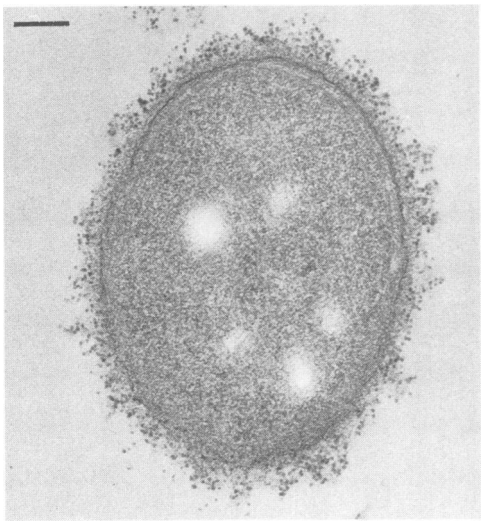

FIG. 10. Immunoferritin labeling of strain 469-3. Antigens labeled by ferritin are uniformly distributed over the entire bacterial surface. Bar, 0.1 μm.

against killing by antibiotics (viable counts after antibiotic treatment/viable counts of untreated samples) showed saturation kinetics and was maximal after approximately 8 h; this fraction is equivalent to the protection of ~1 bacterium per HeLa cell.

Examination by electron microscopy of cell monolayers which had been incubated with adherent bacteria for 8 h revealed bacteria inside most cells (Fig. 16). Bacteria were generally located close to the apical surface within membrane-bounded vesicles; within the time course of these experiments (maximum 18 h), bacteria free within the cytoplasm were not observed. The hemagglutination-deficient mutants did not adhere to or penetrate cultured HeLa or HEp-2 cells.

DISCUSSION

The elaboration by *E. coli* strains 444-3 and 469-3 of surface components that interact with receptors at the surface of erythrocytes and cultured epithelial cells reflects the presence of adhesive structures that could function to promote adhesion of these strains to host intestinal epithelial cells. Indeed, using a recently developed enterocyte adhesion assay we have shown that both 444-3 and 469-3 adhere to the brush border of freshly isolated human colonic enterocytes, although neither strain adhered to isolated human small intestinal enterocytes (12). The fact that these strains were originally isolated from cases of dysentery-like disease (17) and the observation that they can penetrate cultured human epithelial cells and exist intracellularly within membrane-bounded compartments in the manner described for *Salmonella* sp. (11) suggest that invasion could also contribute to their virulence.

Previous studies (14, 23) demonstrated the production by strains 444-3 and 469-3 of MR hemagglutinins specific for human erythrocytes. This ultrastructural study has identified two additional surface structures that also appear to be involved in promoting attachment of these strains to cultured epithelial cells or human erythrocytes (or to both). On the basis of species specificity of hemagglutination and sensitivity to D-mannose, fimbriae elaborated by 444-3, 469-3, and the MR hemagglutination-deficient mutants are identified as type 1 fimbriae common to many *E. coli* strains (8). Bacteria possessing a glycocalyx were also present in cultures of 444-3 and 469-3. The majority of bacteria, however, did not appear to produce any of these surface components. Since both strains were originally derived from single colonies, the presence of several different surface phenotypes in bacterial cultures is perhaps surprising, although it is well known that the formation of attachment structures such as fimbriae may be subject both to spontaneous variation and to variation determined by the culture conditions (8); glycocalyx production usually ceases when bacteria are subcultured in vitro (6).

MR hemagglutinins of strains 444-3 and 469-3, unlike glycocalyx and fimbrial structures, do not appear to exist as morphologically identifiable entities, although these molecules must be exposed at the bacterial surface in a manner which allows interaction both with cell surface receptors and with antibody molecules. Isolated MR hemagglutinins are large-molecular-weight aggregates of a single polypeptide subunit rich in hydrophobic amino acids (23), properties typical of many isolated fimbrial adhesins (10). Fimbrial structures associated with bacteria elaborating MR hemagglutinins have not been detected, and although it is our experience that some fimbrial antigens are difficult to dem-

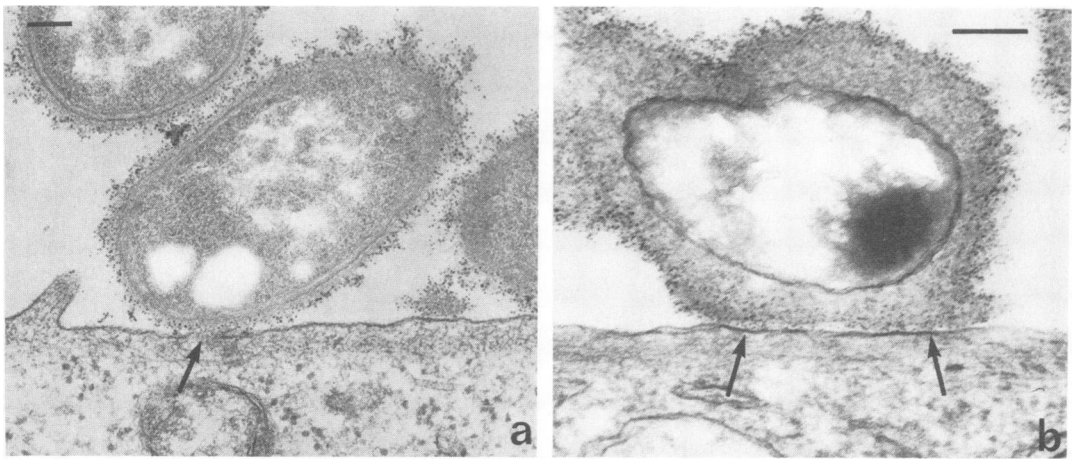

FIG. 11. Immunoferritin labeling of HEp-2 cell adherent 469-3 (a) and 444-3 bacteria (b). Ferritin-labeled bacterial surface antigens (a) or glycocalyx antigens (b) were present at or close to sites of bacterium-HEp-2 cell contact (arrows). Bar, 0.1 μm.

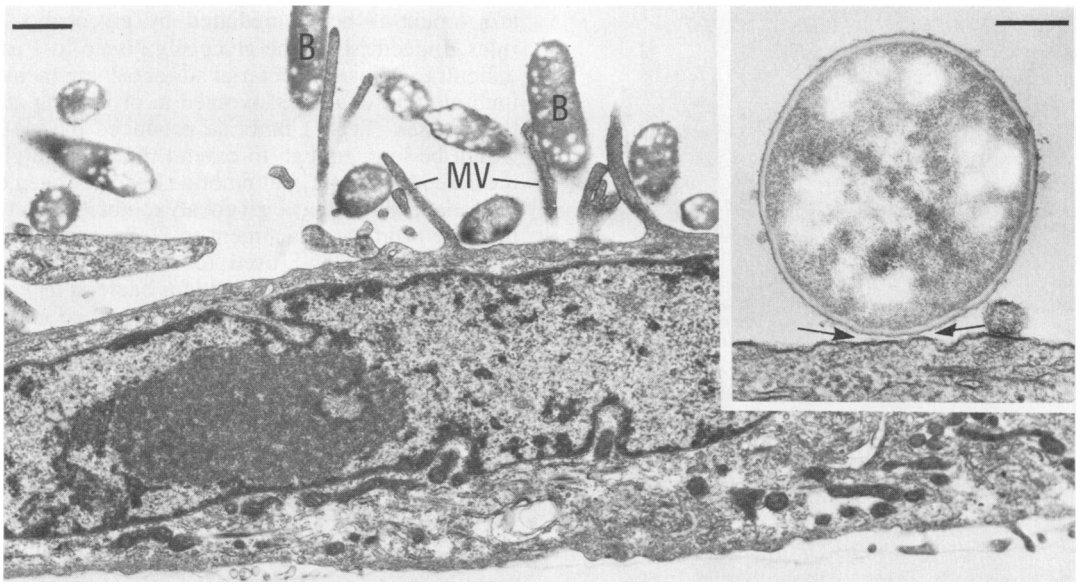

FIG. 12. Electron micrograph of an HEp-2 cell with adherent 469-3 bacteria. Bacteria (B) bind to both microvilli (MV) and to nonmicrovillous membrane. A small gap generally separated closely apposed bacterial and HEp-2 cell surfaces (inset, arrows). Bar, 0.5 μm. Inset bar, 0.2 μm.

onstrate, available ultrastructural evidence indicates that MR hemagglutinins produced by strains 444-3 and 469-3 are nonfimbrial surface components.

The predominant mode of attachment to both cultured cells and to human erythrocytes involved close apposition of bacterial and epithelial or erythrocyte surfaces. Close apposition is also a feature of the attachment of human enteropathogenic *E. coli* strains (16) and rabbit RDEC-1 *E. coli* (21) to host intestinal mucosa. These organisms also induce a very striking pathogenic lesion which involves the loss of

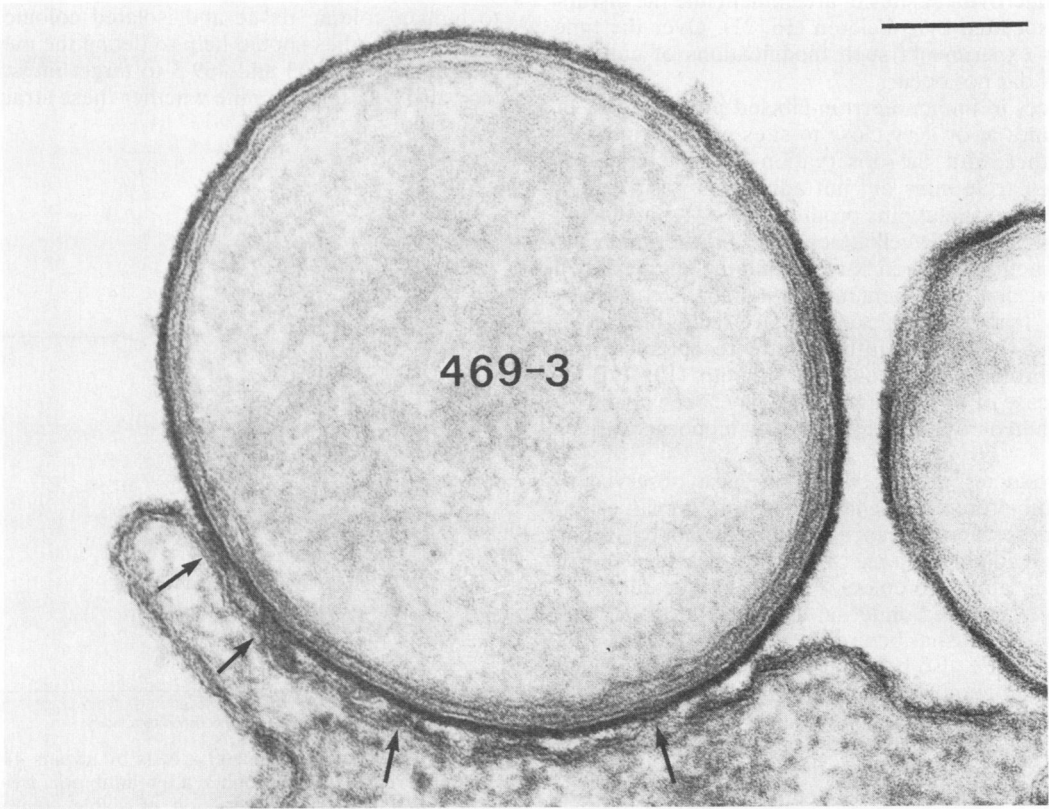

FIG. 13. Electron micrograph showing attachment of a smooth-surfaced bacterium to an HEp-2 cell. Contacts between the bacterium and epithelial cell glycocalyx components stained by ruthenium red can be seen (arrows). Bar, 0.1 μm.

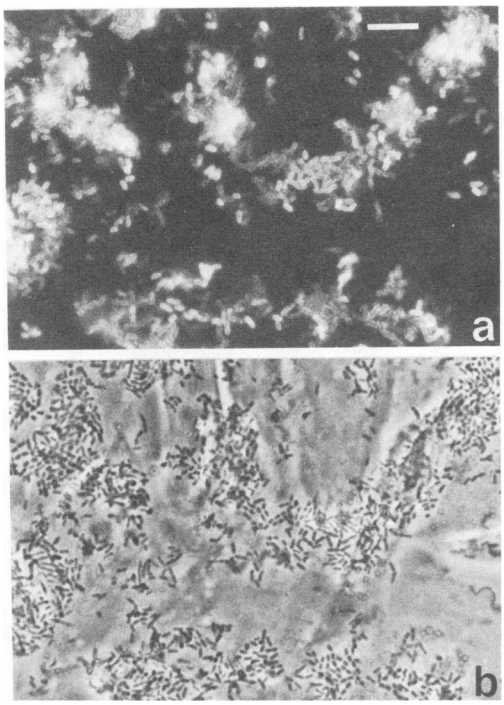

FIG. 14. Indirect immunofluorescence staining of 444-3 bacteria attached to a monolayer of HEp-2 cells (a) and a phase-contrast micrograph of the same field (b). All adherent bacteria exhibit a strong fluorescence. Bar, 10 μm.

brush border microvilli at sites of attachment and the disruption of the associated cytoskeleton (16, 21). Over the time course of our experiments such modifications of cultured epithelial cells did not occur.

The presence, in immunoferritin-labeled preparations, of ferritin molecules at or very close to sites of bacterium-cell contact, together with the observation that bacteria that lacked MR hemagglutinins did not adhere, is positive evidence that MR hemagglutinins produced by 444-3 and 469-3 are involved in promoting cell attachment. Furthermore, the observed interaction between bacteria and the epithelial cell glycocalyx revealed by ruthenium red staining is consistent with the view that bacterial adhesins are lectin-like molecules that bind to specific carbohydrate receptors of cell surface glycoproteins or glycolipids or both (10, 15), although in the case of 444-3 and 469-3 we have been unable to prevent attachment by a wide range of monosaccharides (23).

Bacteria producing glycocalyces were also observed to adhere to both cultured epithelial cells and to human erythrocytes. The presence of glycocalyx antigens among strains of *E. coli* causing diarrhea in pigs and calves has been shown to increase their ability to colonize the small intestine (19), and glycocalyx-mediated mucosal attachment of *E. coli* strains lacking fimbriae has been reported (3, 4). Glycocalyx-enclosed bacteria are also better protected from mechanical forces and host immunological defense mechanisms (6).

Unlike ruthenium red, cationized ferritin and glycocalyx antibody (present in the absorbed antisera) appeared to stabilize the glycocalyx and thus revealed a truer representation of the spatial relationship between bacteria and the cell surface. The observed contact between bacterial glycocalyces and erythrocyte or epithelial cell surfaces suggests that attachment is being mediated by glycocalyx polysaccharides. Indeed, since the glycocalyx is up to 1 μm thick it is difficult to envisage bacterial surface components other than fimbriae that could be involved in promoting attachment in these cases. Type 1 fimbriae produced by 444-3 and 469-3 would be long enough to extend through the glycocalyx to mediate attachment, but fimbriae have not been observed on bacteria elaborating a glycocalyx, nor, in fact, do type 1 fimbriae promote attachment to HeLa or HEp-2 cells.

Since the method used to select mutants deficient in hemagglutination also selected for bacteria in which glycocalyx synthesis was repressed, the absorbed antisera used in this study and thought to retain only anti-hemagglutinin antibody were also observed to retain anti-glycocalyx antibody. The method of producing antisera to specific surface antigens by raising antisera to whole bacteria and absorbing with variants which lack the antigen is frequently used (9, 20). Our observations indicate that care is required to ensure the monospecificity of such antisera.

Type 1 fimbriae enable bacteria to adhere to a wide variety of eucaryotic cells, including epithelial cells (8); although there is strong evidence for the role of fimbriae in bacterial adhesion (10), observations of ours (12a) and others (5) indicate type 1 fimbriae do not promote mucosal attachment.

Protection from killing by antibiotics is consistent with epithelial cell penetration, and internalization of 444-3 and 469-3 was confirmed by electron microscopy. Although the extent of penetration was relatively low, the observation that a noninvasive human enteropathogenic *E. coli* (O127:H6) strain did not penetrate HEp-2 cells even though bacteria adhered strongly (unpublished observations) indicates that strains 444-3 and 469-3 possess specific invasive as well as adhesive properties. We are currently studying attachment to human colonic tissue and isolated colonic enterocytes, and those studies should help to define the mechanism(s) of attachment of 444-3 and 469-3 to target intestinal cells and may also help to determine whether these strains are enteroinvasive.

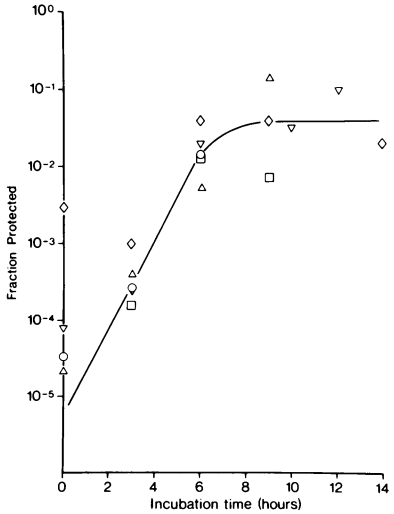

FIG. 15. Penetration of HeLa cells by strains 444-3 and 469-3. Points represent viable counts after antibiotic treatment (as described in the text) as a fraction of viable counts of untreated samples. The graph shows data from four experiments with strain 469-3 (○, △, ▽, □) and one with strain 444-3 (◇).

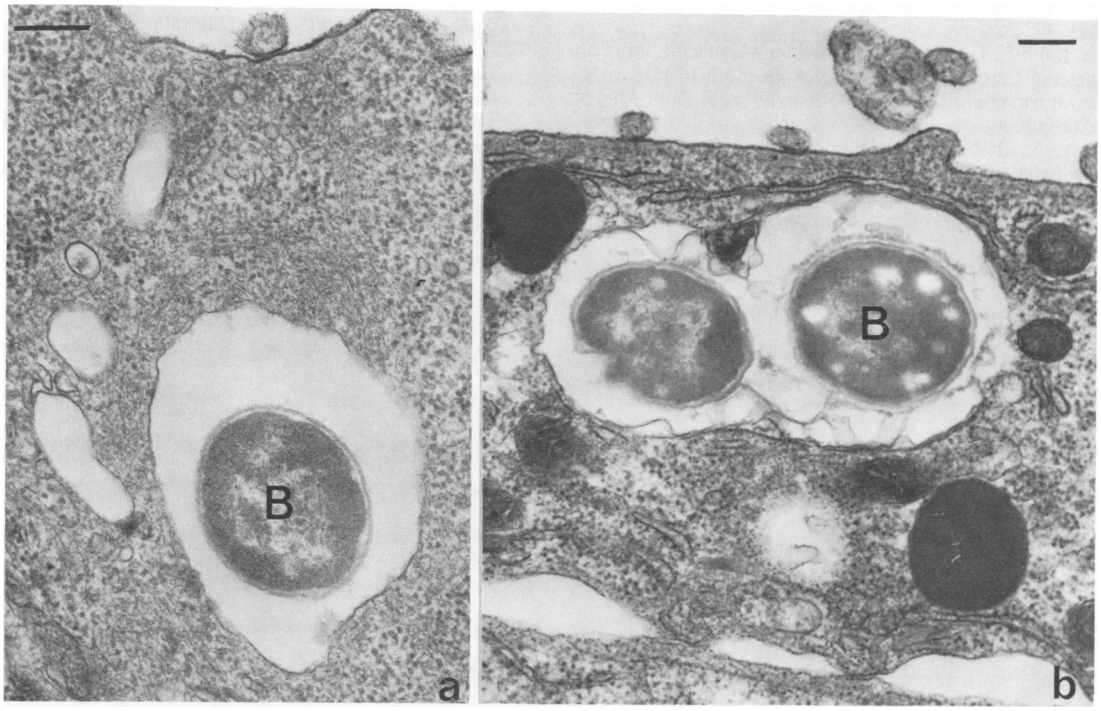

FIG. 16. Electron micrographs of HeLa (a) and HEp-2 cell monolayers (b) after incubation with 444-3 (a) and 469-3 (b) for 8 h at 37°C. Bacteria (B) located within membrane-bounded vesicles were seen in most cells. Bars, 0.2 μm.

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