The Role of the *eae* Gene of Enterohemorrhagic *Escherichia coli* in Intimate Attachment In Vitro and in a Porcine Model

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Abstract

The *eaeA* gene of enteropathogenic *Escherichia coli* (EPEC) is necessary for intimate attachment to epithelial cells in vitro. Enterohemorrhagic *E. coli* (EHEC) strains also possess an *eae* gene and are capable of intimate attachment and microvillus effacement in vitro and in animal models. To assess the role of the EHEC *eae* gene in intimate attachment, we constructed an *eae* deletion/insertion mutation in wild-type EHEC O157:H7 strain 86-24 by using linear electroporation of a recombinant allele. The mutant obtained was deficient in inducing f-actin accumulation in HEp-2 cells and was incapable of attaching intimately to colonic epithelial cells in a newborn piglet model of infection. Intimate attachment in vivo was restored when the EHEC *eae* gene or the *eaeA* gene of EPEC was introduced into the mutant on a plasmid. These results indicate that the *eae* gene is necessary for intimate attachment of EHEC in vivo. In addition, the complementation achieved by the EPEC locus indicates that the *eae* gene of EHEC and the *eaeA* gene of EPEC are functionally homologous. (*J. Clin. Invest.* 1993. 92:1418–1424.) Key words: bacterial adhesion • disease models • genetics • microbial • mutations • pathogenicity

Introduction

Enterohemorrhagic *Escherichia coli* (EHEC)[1] strains cause outbreaks and sporadic cases of hemorrhagic colitis and are associated with the hemolytic uremic syndrome and thrombotic thrombocytopenic purpura (1). EHEC strains elaborate Shiga-like toxins and often belong to serotype O157:H7, but may include O26:H11 and other types as well (2–4). In addition to these properties, EHEC are capable of attaching intimately to epithelial cells and effacing microvilli in the large intestine of newborn piglets (5, 6). This attaching and effacing effect is similar to that induced by enteropathogenic *Escherichia coli* (EPEC), which in contrast occurs predominantly in the small intestine of piglets (6, 7). While attaching and effacing lesions have been reported in patients with EPEC infection (8, 9), for EHEC this effect has thus far been observed only in tissue culture and animal models (1).

Jerse and Kaper (10) reported the identification of a locus in EPEC necessary for intimate attachment to epithelial cells in vitro. This locus, now referred to as *eaeA* (11), is part of a gene cluster on the EPEC chromosome necessary for intimate attachment (12). The product of the *eaeA* locus is intimin, a 94-kD outer membrane protein recognized by sera from volunteers convalescing from experimental EPEC infection (13). A gene probe derived from sequences internal to *eaeA* recognizes sequences from the chromosome of EHEC strains (10, 14). Recently two groups have reported the cloning and nucleotide sequence of the EHEC *eae* locus (15, 16). The predicted amino acid sequences of the EPEC and EHEC loci are 83% identical and share significant sequence similarities with the invasin proteins of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* (15). The invasin protein of *Y. pseudotuberculosis* binds with high affinity to the β_1 class of integrin molecules to allow efficient invasion of epithelial cells (17).

The role of the *eaeA* gene of EPEC in intimate attachment to epithelial cells has been confirmed with the construction of an isogenic deletion mutant (18). Furthermore, a volunteer study comparing this mutant to the wild-type strain from which it was derived has confirmed that intimin is required for full virulence (19). However, the role of the EHEC *eae* homologue in intimate attachment had not heretofore been demonstrated. We now report the construction of an *eae* mutant of EHEC and demonstrate for the first time the role of an *eae* gene in intimate attachment in vivo.

Methods

Strains and plasmids. EHEC strain 86-24, an O157:H7 Shiga-like toxin II producing isolate from an outbreak of hemorrhagic colitis in Walla Walla, WA (20), was kindly provided by Phil Tarr. EDL933cu-rev is an O157:H7 laboratory derivative cured of its large plasmid (21). Prototypic EPEC O127:H6 strain E2348/69 has been described (22). BL21 (hsdS gal) (23) served as a control in piglet experiments. DH5α (supE44 ΔlacU169 (ϕ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1) was used as a host strain for plasmid transformations and as a control in adherence assays. GM48 (dam3, dcm6, gal, ara, lac, thr, leu, thi1, tonA, tsx) (24), kindly provided by Steve Moseley, was used to isolate DNA that could be restricted by BclI. Plasmids pCVD444, containing the *eae* gene from EHEC strain EDL933 (15) and pCVD438 containing the *eaeA* gene from EPEC strain E2348/69 (18) have been described. Plasmid pCVD607 contains the tetracycline gene from Tn10 (25). Bacterial strains were stored at −70°C in 50% glycerol/50% Luria-Bertani (LB) broth and grown on LB plates or in LB media. Ampicillin (200 μg/ml), tetracycline (12.5 μg/ml), and chloramphenicol (20 μg/ml) were added when necessary.

Construction of an eae mutant. Plasmid pCVD444 was transformed into GM48 by using a rapid dimethylsulfoxide method (26). Plasmid

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1. *Abbreviations used in this paper:* EHEC, enterohemorrhagic *Escherichia coli*; EPEC, enteropathogenic *Escherichia coli*.

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DNA was isolated by alkaline lysis (27), cut with BclI, and religated to create pMSD100, which has an internal deletion of nucleotides 835–1959 of the *eae* coding sequence. The tetracycline resistance gene from transposon Tn10 was excised from pCVD607 on a BglII fragment and ligated to BclI cut pMSD100 to create pMSD101, which now has an insertion of the tetracycline resistance gene into the *eae* locus. This plasmid was then introduced into EDL933cu-rev by electrotransformation in 0.2 cm cuvettes using a Gene Pulser (BioRad Laboratories, Richmond, CA) at 2.5 kV/25 μF. This step was added to allow the DNA to be modified by native EHEC enzymes. Plasmid pMSD101 was then reisolated by $CsCl_2$ density gradient centrifugation. Modified pMSD101 was cut with BstYI and treated with the Klenow fragment of DNA polymerase in the presence of thionucleotides using a commercial kit (Pharmacia LKB Biotechnologies, Uppsala, Sweden) to confer resistance to exonuclease digestion (28). The 3.1-kb BstYI fragment was then separated from residual uncut plasmid DNA by excision from a 1.5% agarose gel followed by purification with Gene Clean (Bio101, La Jolla, CA). The purified linear fragment was then introduced into 86–24 by electroporation. Transformants selected on tetracycline plates were predicted to have undergone a double recombination event replacing the native *eae* allele with the recombinant allele that has a deletion of internal sequences and an insertion of the gene for tetracycline resistance.

Genomic DNA isolation (18) and Southern hybridization (29) of BglII digested DNA was performed as described under stringent conditions (50% formamide, 37°C, 750 mM NaCl; 65°C wash with 750 mM NaCl) to confirm the construction. Hybridization was performed with a previously described *eae* DNA probe (10) labeled along with lambda DNA with [α-^{32}P]dATP by the random primer method (30). Plasmids pCVD444 and pCVD438 were introduced into the mutant by electroporation.

In vitro adherence assays. In vitro studies were performed as described by Cravioto et al. (31) with minor alterations. Briefly, semiconfluent monolayers of HEp-2 cells on glass coverslips or in eight-well plastic Chamber Slides (Nunc, Naperville, IL) were overlaid with Earle's minimal essential medium (EMEM, Bio-Whittaker, Gaithersburg, MD) supplemented with 0.4% sodium bicarbonate and 1% mannose which contained 20 μl/ml (vol/vol) of an overnight culture of bacteria (each inoculum was $\geq 10^7$ bacteria). The infected monolayers were incubated at 37°C in a 5% CO_2 atmosphere. After 3 h the medium with the nonadherent bacteria was aspirated and the monolayers washed once with sterile 10 mM PBS. Fresh EMEM/bicarb/mannose medium was added to the cells which were then incubated for an additional 3 h. The monolayers with the adherent bacteria were washed six times with PBS. Each wash was gently removed by aspiration to avoid disturbing the monolayers. Each assay was done five times in duplicate to allow for both Giemsa and FITC-phalloidin (FAS) staining. For Giemsa staining, the HEp-2 cells and adherent bacteria were fixed with 70% (vol/vol) methanol and stained with 1:10 Giemsa (Sigma Chemical Co., St. Louis, MO) for 20 min. To assess the FAS phenotype, the FITC-phalloidin (Sigma) staining procedure of Knutton et al. (32) was used. EPEC strain E2348/69 and K-12 strain DH5α served as positive and negative controls, respectively.

Newborn piglet model of EHEC infection. E. coli strains to be tested were grown overnight in tripticase soy broth containing appropriate antibiotics and inoculated orally into pairs of colostrum-fed piglets 12–18 h after birth. Each inoculum consisted of a 2-ml suspension containing $\sim 10^9$ viable organisms. Selection for plasmids was maintained with chloramphenicol at a dosage of 62.5 mg per os twice per day (for piglets receiving the mutant with plasmid pCVD438) or ampicillin at a dosage of 250 mg per os per day (for piglets receiving the mutant with plasmid pCVD444). Antibiotics were begun at the time of inoculation and continued until the end of the experiment.

Piglets were housed individually in cages in an environmentally-controlled room and fed twice daily with 200 ml of milk-based diet. Sequential pairs of piglets were inoculated with different test strains over a period of 6 wk to avoid cross infection. Piglets were euthanized 48 h after inoculation and six intestinal sections (duodenum, jejunum, proximal and terminal ileum, cecum, and colon) were fixed in either formalin for light microscopy or in glutaraldehyde for electron microscopy. Colonic swabs obtained at death from each piglet were cultured to confirm the presence of the inoculated strain.

Tissue preparation and microscopy. Samples of intestinal mucosa were fixed in Trump's fixative in cacodylate buffer, pH 7.2, postfixed in 1% osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4, dehydrated through graded ethanol solutions, stained en bloc with 5% uranyl acetate and embedded in Embed-812 epoxy resin. For light microscopy 1-μm-thick sections were stained with toluidine blue. For electron microscopy, 50–70-nm-thin sections were cut, stained with uranyl acetate and lead citrate, and photographed with a Philips EM 201 electron microscope.

Results

Construction of an EHEC eae mutant. Initial attempts to construct an EHEC *eae* mutant using electroporation of linear pMSD101 DNA from DH5α were unsuccessful. Therefore, to enhance the stability of the DNA after entry into strain 86-24, two steps were added. The first was passage of the plasmid in EDL933cu-rev to allow the DNA to be modified by enzymes present in a wild-type EHEC strain and therefore to reduce possible DNA digestion by host restriction enzymes. The second was modification of the DNA with thionucleotides to protect the ends of the fragment from cellular exonucleases. After these additional steps were added, a single transformant was isolated. This strain is referred to as UMD619, and can be described as 86-24 Δ*eae*1::Tc^R.

The deletion of 1.1 kb of the *eae* gene and the addition of the 2.9-kb fragment containing the tetracycline resistance gene yielded a hybridizing fragment 1.8 kb larger than that from the wild-type strain. The results of Southern hybridization confirming these changes are shown in Fig. 1.

HEp-2 cell assays. EHEC strain 86-24 adhered in a localized manner to the HEp-2 cells after 6 h in the adherence assay and was positive in the FAS test for f-actin accumulation. In contrast, the *eae* mutant (UMD619) and the mutant transformed with recombinant plasmids containing cloned *eae* genes from EHEC (pCVD444) and EPEC (pCVD438) resem-

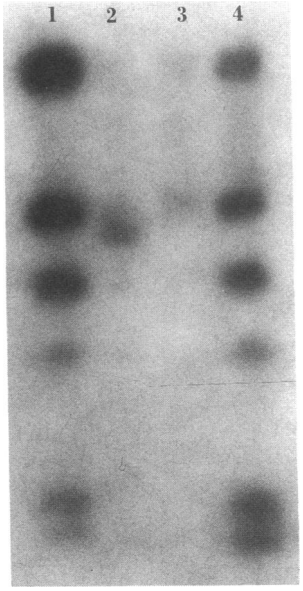

Figure 1. Southern hybridization of genomic DNA digested with BglII and probed with a radiolabeled internal fragment of the EPEC *eaeA* gene. A radiolabeled lambda DNA probe was also included to visualize molecular weight standards (lanes *1* and *4*, HindIII digested lambda DNA). Lane *2*, wild-type 86-24. Lane *3*, UMD619, the *eae* mutant. The replacement of the wild-type allele with the mutated allele results in a 1.8-kb increase in the size of the hybridizing fragment. The diminished signal seen in the mutant is due to deletion of all but 223 of the 1052 bp of DNA recognized by the probe.

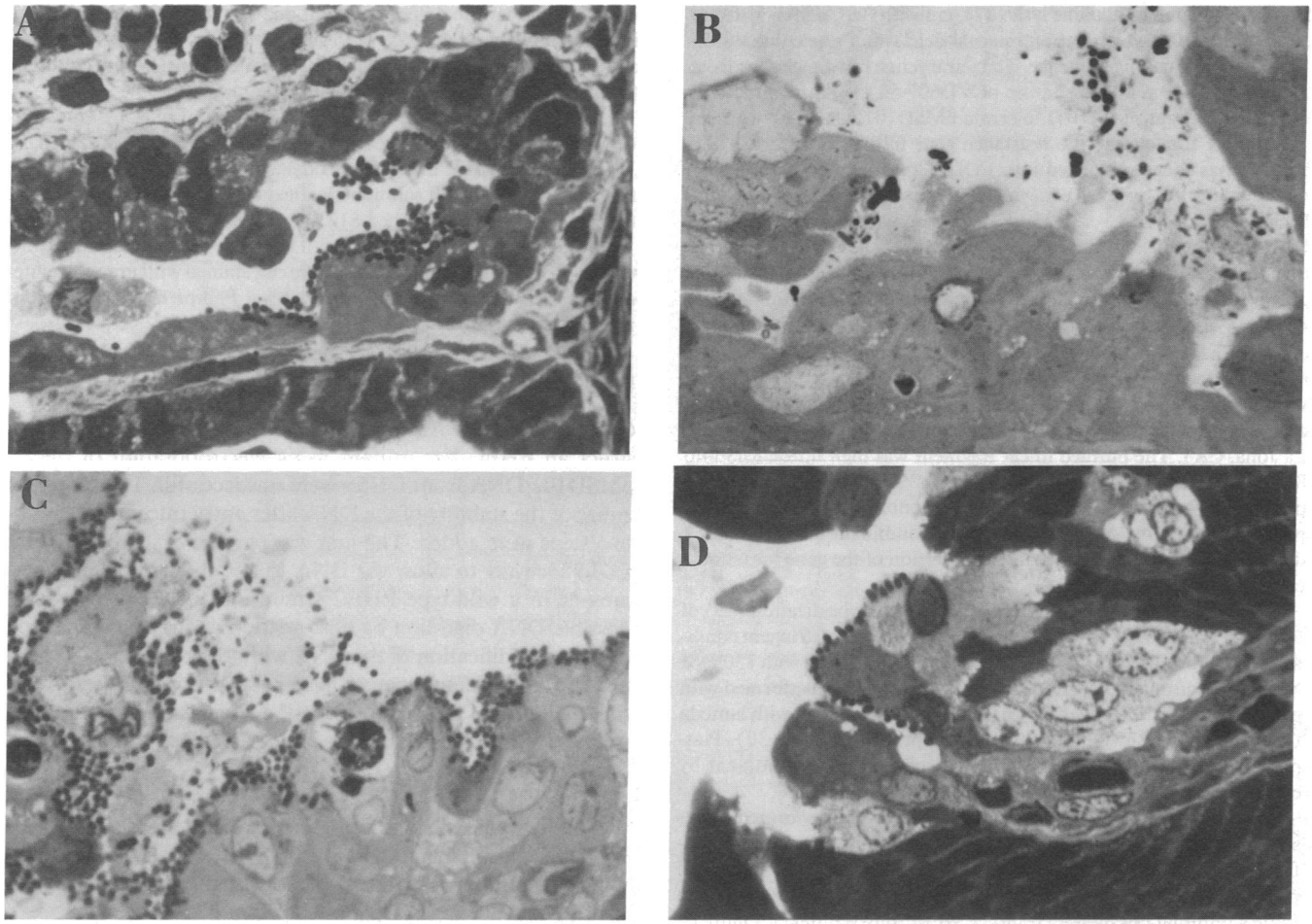

Figure 2. Light micrographs from sections of the large intestine of piglets orally inoculated with one of four *E. coli* strains stained with toluidine blue (×912). (*A*) Colonic crypt from a piglet inoculated with wild-type EHEC strain 86-24 showing bacteria attached to colonocytes. The crypt cells appear desquamated and irregular. (*B*) Colonic surface from a piglet inoculated with the EHEC *eae* mutant UMD619, showing intact cells with free bacteria and yeast in the lumen. (*C*) Cecal surface from a piglet inoculated with the EHEC *eae* mutant containing the cloned EPEC *eaeA* gene on a plasmid, showing bacteria attached to the apical surface. (*D*) Colonic surface from a piglet inoculated with the EHEC *eae* mutant containing the cloned EHEC *eae* gene on a plasmid, showing bacteria attached to the surface of cells.

bled the K12 control; no adherence or FAS staining was detected (data not shown).

Effects of the eae gene in a newborn piglet model of infection. Control *E. coli* strain BL21, EHEC strain 86-24, mutant UMD619, UMD619 containing the EHEC *eae* plasmid pCVD444, and UMD619 containing the EPEC *eaeA* plasmid pCVD438 were each fed to one pair of piglets. None of the 10 inoculated animals developed diarrhea or any other sign of illness during the observation period of 48 h. Colonic contents of all piglets contained the expected bacteria.

In piglets inoculated with the wild-type EHEC strain 86-24, adherence of bacteria to the enterocytes of the cecal and colonic mucosa, and occasionally in the terminal ileum was observed by light microscopy (Fig. 2). Intimate attachment of bacteria to colonocytes and microvillus effacement was observed by electron microscopy of thin sections of colonic mucosa from piglets inoculated with the wild-type strain (Fig. 3). Similar effects were observed in light micrographs and electron micrographs from piglets inoculated with the mutant UMD619 only when it contained either the EHEC *eae* gene or the EPEC *eaeA* gene cloned on a plasmid (Figs. 2 and 5). In contrast, no evidence of attaching and effacing lesions was obtained with either the control *E. coli* BL21 or the EHEC *eae* mutant UMD619 (Figs. 2 and 4). There were no detectable differences in the distribution of the lesions among the three positive strains regardless of the origin of the *eae* locus.

Discussion

In this report we show that the *eae* gene of EHEC is necessary for intimate attachment of the bacterium to colonic epithelial cells in vivo. This result was demonstrated by constructing a specific mutation in the *eae* gene and observing a lack of intimate attachment by the mutant to the colonic mucosa in a newborn piglet model. The restoration of the ability to perform intimate attachment by reintroduction of the EHEC *eae* gene on a plasmid shows that the loss of activity was indeed due to mutation in the target gene, rather than to polar effects of the insertion on downstream loci. Furthermore, complementation by the *eaeA* gene of EPEC demonstrates that these loci are functionally homologous.

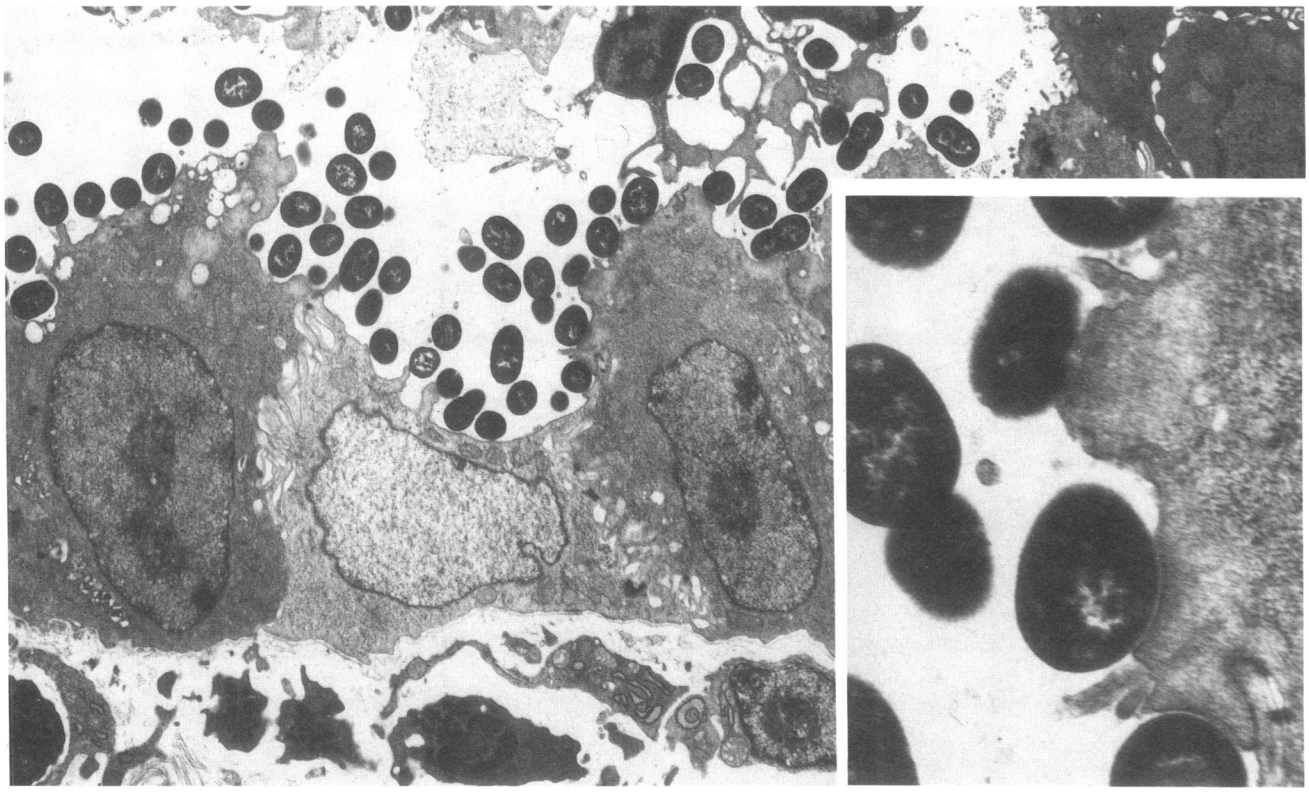

Figure 3. Electron micrograph of surface colonocytes from a piglet infected with wild-type EHEC strain 86-24 (×5,000). Colonocytes with irregular surfaces that have lost their microvilli as well as desquamated cells are coated with bacteria. Inflammatory cell infiltrates and edema are evident beneath the surface layer. *Inset:* Higher magnification reveals plasmalemmal cupping or pedestal formation around the bacteria characteristic of the attaching and effacing effect (×23,000).

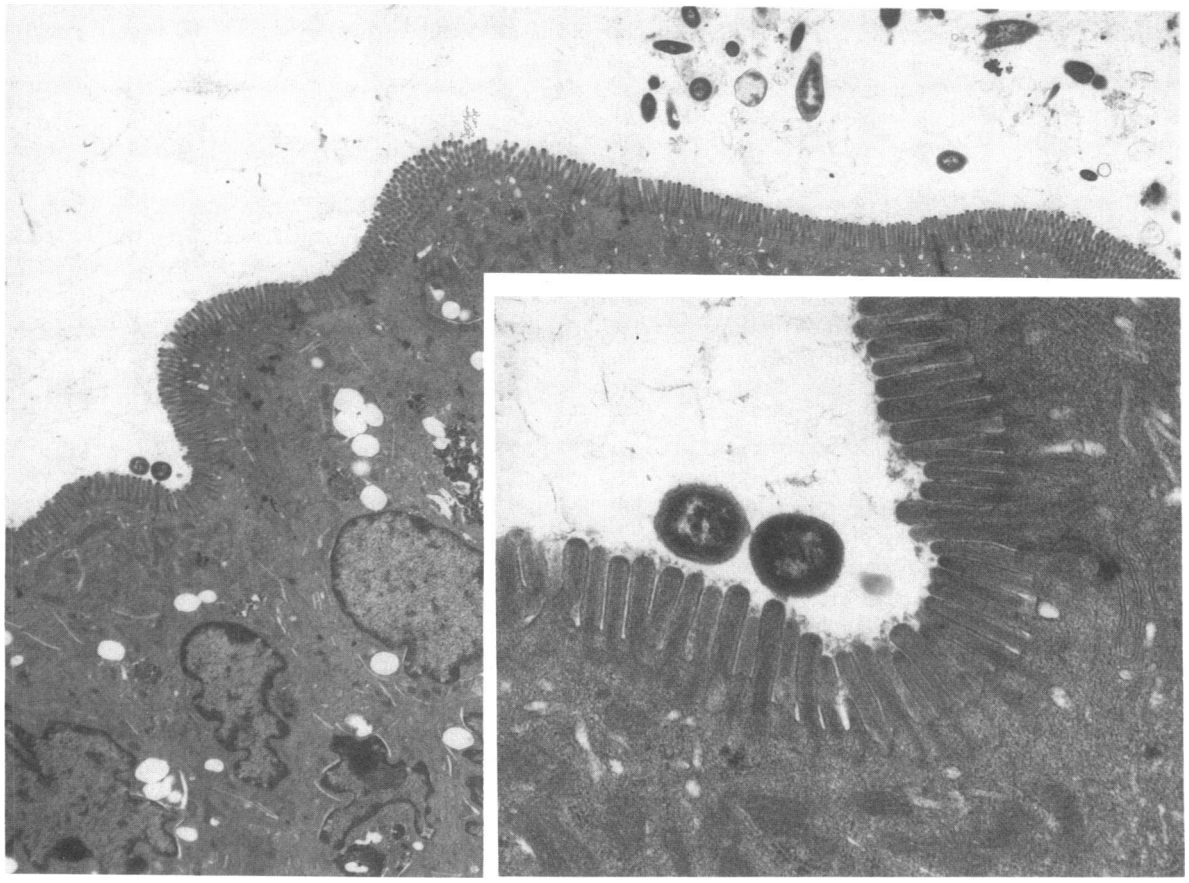

Figure 4. Electron micrograph of colonocytes from a piglet inoculated with the mutant strain UMD619, showing largely intact cells with normal microvillus border. No bacteria were observed attaching intimately to epithelial cells or effacing microvilli in any section (×5,000). *Inset:* Higher magnification illustrates the nature of association of two bacterial cells with the microvillus border (×20,000).

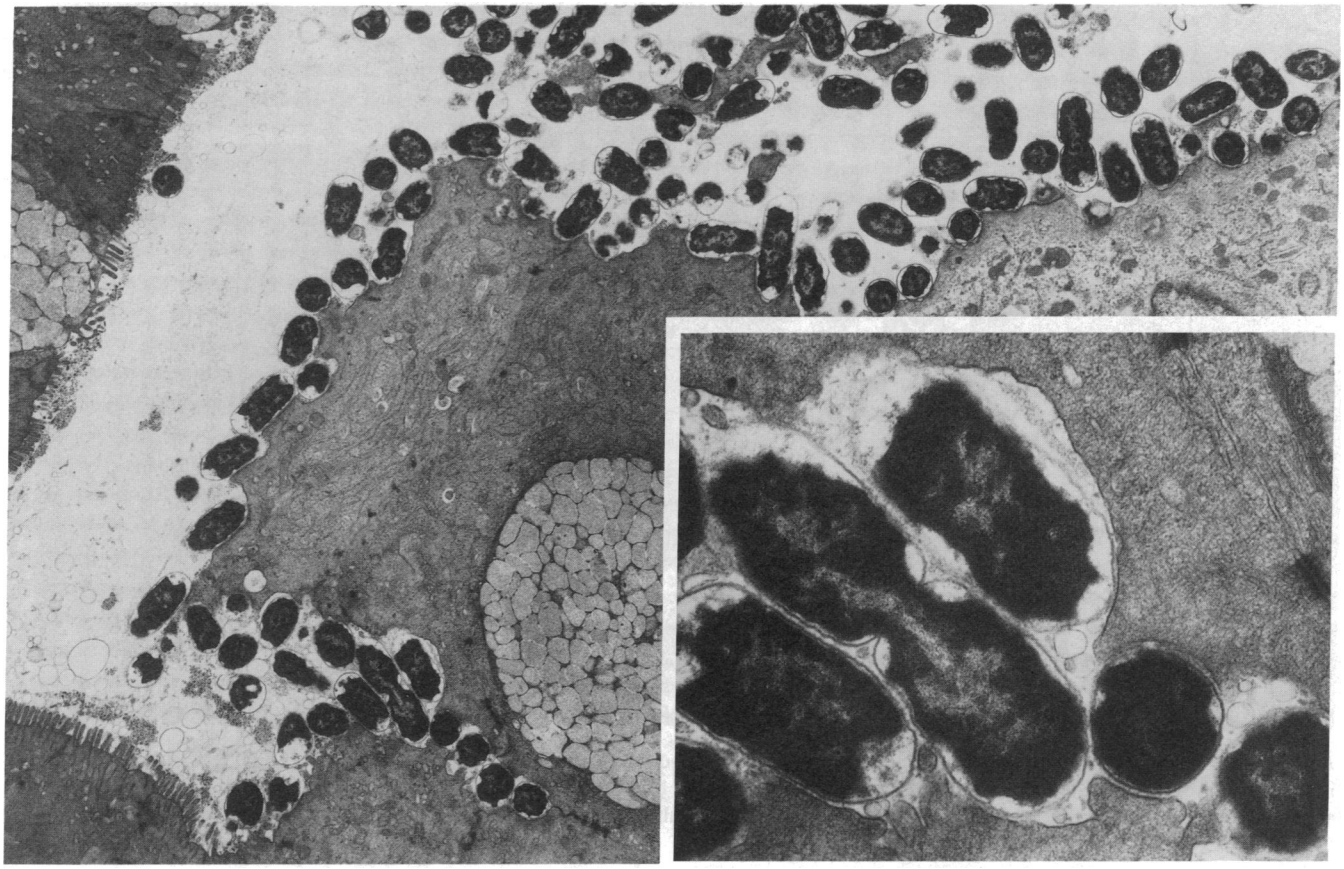

Figure 5. Electron micrograph of colonocytes including a goblet cell from a piglet inoculated with *eae* mutant UMD619 containing the cloned EPEC *eaeA* gene on plasmid pCVD438. Note the complete lack of microvilli in cells heavily colonized by bacteria on the right, as compared with cells on the left which show presence of microvilli on uninfected cells (×5,000). *Inset:* Higher magnification of a region between cells which shows intimate attachment of bacteria to the cell membrane and bacterial cell division (×23,000).

Previous studies have shown that the EPEC *eaeA* locus is required for intimate attachment in tissue culture (10, 18). In addition, a volunteer trial has demonstrated unambiguously that the *eaeA* gene is a virulence locus required for full EPEC pathogenicity (19). However, this is the first demonstration in vivo of the role of an *eae* gene in the ultrastructural damage characteristic of the attaching and effacing lesion.

The requirement for an intact chromosomal *eae* locus for attaching and effacing activity contrasts with the demonstration by Toth and colleagues that in vitro attaching and effacing activity could be conferred upon HB101 after introduction of the large plasmid from O157:H7 EHEC strain 7785 (33). It is difficult to reconcile these discordant findings. The two studies used different EHEC strains, but in view of the close clonal relationship of O157:H7 EHEC, significant strain differences are unlikely to explain this paradox (34). Furthermore, the *eae* gene has been detected only on the chromosome of EHEC strains (14). Nonetheless, it is conceivable that the plasmid elements detected by Toth et al. are either not present in strain 86-24, or not active in vivo.

Indeed, our study illustrates the fact that in vitro activity does not always correlate with in vivo activity. Although the *eae* mutant was deficient in attaching and inducing the accumulation of f-actin in tissue culture cells, we were unable to demonstrate complementation by the cloned *eae* gene of EHEC or the cloned *eaeA* gene of EPEC to restore activity in vitro. The explanation for failure of in vitro complementation by plasmids that complement in vivo is not clear. It would seem, however, that there are differences between attachment in tissue culture and in the pig intestine. Furthermore, it should be noted that the mutation we engineered has an insertion of the tetracycline resistance gene within *eae,* which has the potential for disrupting transcription of cistrons downstream of the target gene. It is therefore conceivable that a downstream gene, which is necessary for attachment or actin polymerization in vitro, is not required in vivo. Indeed, a recently constructed in-frame *eae* deletion mutant of strain 86-24, which should be free of polar effects on downstream loci, can be complemented by cloned *eae* sequences to restore the ability to induce f-actin accumulation in vitro (M. L. McKee and A. D. O'Brien, unpublished results). An alternative explanation for the discrepancy between in vivo and in vitro results deals with the variable adherence of some EHEC strains in tissue culture and the occasional difficulty identifying zones of f-actin accumulation in epithelial cells infected with EHEC strains (35). Thus, while a positive result in this assay can be taken as definitive evidence for activity, a negative result is more problematic.

The precise role of intimin, the product of the *eae(A)* loci of EHEC and EPEC, in the attaching and effacing phenomenon is unproven. Intimate attachment and the signal transduction events in epithelial cells that result in profound cytoskeletal disruption are clearly separable phenomena. While EPEC

eaeA mutants are incapable of intimate attachment, they retain the ability to induce host cell tyrosine kinases and actin accumulation (36). In contrast, other TnphoA mutants of EPEC that are deficient in cytoskeletal disruption remain capable of intimate attachment (11, 36, 37). Thus intimin is crucial only for the intimate attachment aspect of attaching and effacing. The simplest interpretation is that intimin is an adhesin that binds to a host cell receptor. The sequence similarities among intimins and invasins are consistent with this hypothesis. Moreover, Sherman and colleagues have shown that antibodies reacting with a 94-kD EHEC outer membrane protein (the same M_r as EPEC intimin) block EHEC attachment to HEp-2 cells (38). However, since the antigen responsible for inducing these blocking antibodies has not been shown to be the EHEC intimin, a direct role of intimin in adherence remains speculative. Formal proof of whether intimin is the intimate adhesin awaits binding studies performed with the purified protein.

The divergence between the predicted amino acid sequences of EPEC and EHEC intimins toward the carboxyl-terminus of the proteins (15) is of interest, especially since this is the domain of invasin responsible for binding to its receptor (39). We were interested in whether the difference in distribution of EPEC, which is found primarily in the small intestine, and EHEC, which is confined to the colon and distal ileum (6, 7), might be explained in part by the divergence in the putative receptor-binding domain of the intimins. However, we observed no differences in the distribution of attaching and effacing lesions between piglets infected with the *eae* mutant containing the cloned *eae* locus of EHEC and those infected with the mutant containing the cloned EPEC gene.

What is the role of the EHEC intimin in diarrhea? We did not observe diarrhea in these studies. We chose to sacrifice the animals at 48 h in order to maximize the probability of observing attaching and effacing lesions prior to the onset of diarrhea and complications of infection (40). Intimin is required for full virulence of EPEC, but diarrhea can still develop in its absence (19). These results are compatible with intimin's role in intimate attachment and with the fact that other EPEC genes are involved in signal transduction events necessary for diarrhea. As for the EHEC intimin, experiments are currently underway to investigate the role of *eae* in diarrhea in a gnotobiotic piglet model.

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