INFECTION AND IMMUNITY, Sept. 1995, p. 3621–3627 Vol. 63, No. 9
0019-9567/95/$04.00+0

The Role of the *eaeA* Gene in Diarrhea and Neurological Complications in a Gnotobiotic Piglet Model of Enterohemorrhagic *Escherichia coli* Infection

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Received 13 April 1995/Returned for modification 1 June 1995/Accepted 21 June 1995

We reported previously that mutation of the chromosomal gene *eaeA* from enterohemorragic *Escherichia coli* (EHEC) serotype O157:H7 prevented bacterial attachment in vivo. Attachment was restored when the EHEC or enteropathogenic *E. coli* (EPEC) *eaeA* gene was introduced into the mutant on a plasmid. In this communication we have compared in gnotobiotic piglets the pathogenicities of wild-type O157:H7 strain 86-24 and its *eaeA* mutant UMD619 with those of the two plasmid-complemented strains expressing $Intimin_{O157}$ (EHEC) and $Intimin_{O127}$ (EPEC). 86-24 colonized the surface and glandular epithelium of the large intestine and induced diarrhea, while UMD619 did not colonize any intestinal site and induced little or no diarrhea. Surprisingly, strain UMD619 expressing $Intimin_{O127}$ behaved in pigs more like EPEC than EHEC strains; it colonized the distal half of the small intestine and the surface of the large intestine, inducing serious diarrhea. In contrast, strain UMD619 expressing $Intimin_{O157}$ colonized the colon extremely poorly, inducing little or no diarrhea. While only the two strains causing extensive attachment—86-24 and UMD619 expressing $Intimin_{O127}$—induced diarrhea, neurological symptoms attributed to Shiga-like toxin II occurred equally in all four groups of animals. The intimate bacterial attachment and mucosal damage were not a prerequisite for Shiga-like toxin II translocation from the gut lumen into the circulation. $Intimin_{O127}$ appears not only to facilitate intimate attachment to cells but also to influence the site of intestinal colonization and other characteristics of EPEC infection.

Enterohemorrhagic *Escherichia coli* (EHEC) is the category of *E. coli* organisms that is responsible for sporadic cases and outbreaks of hemorrhagic colitis and hemolytic-uremic syndrome (HUS) (8, 11). EHEC strains of O157:H7, the serotype most frequently isolated from such cases, possess several virulence factors whose precise contribution to either hemorrhagic colitis or HUS is not entirely clear. The most predominant and distinguishing feature, however, is the production of one or more Shiga-like toxins (SLT-I, SLT-II, and SLT-IIv), which closely resemble the Shiga toxin produced by *Shigella dysenteriae* type 1 strains. The role of SLT in the pathogenesis of hemorrhagic colitis remains unclear, and while SLT-II is closely linked to HUS (15), neither the mechanism of mucosal translocation of the toxin nor its precise mode of action which leads to HUS and often to neurological complications in children is understood. Intimate bacterial attachment-effacement (A-E) of the gut epithelium, also a characteristic of enteropathogenic *E. coli* (EPEC) strains (14, 16, 20, 22), can be demonstrated in tissue culture and animal models of EHEC infection (6, 19, 21) and in many EHEC strains of other serotypes. The production of bacterial A-E lesions in the colonic mucosa observed in the gnotobiotic piglet model is probably partly responsible for the manifestation of diarrhea associated with EPEC and EHEC strains (18). The *eaeA* gene of EPEC strains encodes a 94-kDa outer membrane protein known as Intimin (2, 3, 10) that is required for intimate attachment of EPEC strains to epithelial cells in vitro and in vivo. An EPEC *eaeA* deletion mutant is markedly attenuated for virulence in experimental human infection (9). The *eaeA* gene of EHEC,

TABLE 1. Summary of experimental inoculation of groups of gnotobiotic piglets[a]

Group no.	*E. coli* strain	Total no. of animals	No. of animals with clinical outcome:		A-E lesions (result)[b]	
			Diarrhea	Other[c]	SI	LI
1	86-24	6	5	6	−	+
2	UMD619	4	1	4	−	−
3	UMD619 expressing $Intimin_{O157}$	6	2	5	−	±
4	UMD619 expressing $Intimin_{O127}$	6	6	6	+	+
5	C600	2			−	−

[a] Groups of pigs inoculated with wild-type O157:H7 strain 86-24, its mutant from which the *eaeA* gene had been deleted (UMD619), two UMD619 derivative strains containing plasmids expressing $Intimin_{O157}$ and $Intimin_{O127}$, and *E. coli* K-12 (C600) were compared.

[b] −, no bacterial A-E lesions were observed in any of the animals in this group; +, extensive lesions were observed in the large intestine and in the distal half of the small intestine in all of them; ±, minimal (only one or two foci) lesions were observed in the colon in all animals. SI, small intestine; LI, large intestine.

[c] Piglets were autopsied within 40 to 72 h after challenge with the appearance of neurological symptoms, coma, or death.

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sequenced recently (1, 23), shares considerable homology with the *eaeA* gene of EPEC (23).

In an earlier publication (4), we reported that mutation of the *eaeA* gene from O157:H7 EHEC strain 86-24 prevented intimate bacterial attachment to colonocytes in conventional piglets. This intimate attachment, however, was restored when the EHEC *eaeA* gene or the EPEC *eaeA* gene was introduced into the *eaeA* mutant on a plasmid. In this communication, we have compared in gnotobiotic piglets the pathogenicities of the wild-type O157:H7 strain 86-24 and of its *eaeA* mutant UMD619 with those of the two derivatives of the *eaeA* mutant strains complemented with either the EHEC or the EPEC *eaeA* gene introduced on a plasmid.

MATERIALS AND METHODS

Bacterial strains. The origins of EHEC strain 86-24, an O157:H7 SLT-II-producing isolate; the mutant; and the two constructed strains used in this study are detailed elsewhere (4). Briefly, the chromosomal EHEC *eaeA* gene was inactivated in strain 86-24 by replacement of an internal fragment with the tetracycline resistance gene cassette, creating the mutant described as 86-24 *eaeA1*::Tc^r and referred to as UMD619. Strain UMD619 has lost the ability to attach to colonic epithelium and to cause bacterial A-E lesions in newborn piglets, a characteristic of the parent strain (4). The UMD619 mutant was transformed with recombinant plasmids containing cloned *eaeA* genes from EHEC (pCVD444) and EPEC (pCVD438). Strains UMD619(pCVD444) and UMD619(pCVD438) are mutants complemented with the *eaeA* gene derived, respectively, from wild-type EHEC (O157) or EPEC (O127) strains (18). *E. coli* C600 was used as the control strain. Plasmid pCVD444 carries an ampicillin resistance gene, and pCVD438 carries a chloramphenicol resistance gene. For animal inoculations, bacterial strains were grown in Luria-Bertani broth overnight from a single colony at 37°C with shaking, and 1% of the inoculum from the overnight broth was then grown for approximately 2 h at 37°C with shaking. Bacterial cultures were thoroughly washed to remove residual toxin.

Experimental animals and design. Twenty-four gnotobiotic piglets were derived from 3 litters by cesarean section and maintained inside microbiological isolators for the duration of the experiment. They were fed twice daily with 250 ml of reconstituted cow's milk. The piglets were divided into five uneven groups and inoculated orally with one of five *E. coli* strains within 24 h after birth, when it was clear that they were healthy and drinking well. Each animal received orally with a feeding needle a suspension of 10 ml containing 4×10^9 washed viable organisms. Group 1 (six animals) received the wild-type strain 86-24, group 2 (four animals) received the *eaeA* mutant strain UMD619, group 3 (six animals) received strain UMD619 expressing Intimin$_{O157}$, group 4 (six animals) received strain UMD619 expressing Intimin$_{O127}$, and group 5 (two animals) received strain C600. The piglets were monitored several times daily and were euthanized and autopsied when severe illness developed. Selection for plasmids was maintained by inclusion of 50 mg of ampicillin per kg of body weight for group 3, 75 mg of chloramphenicol per kg for group 4, twice daily with the milk diet. Group 2 was treated with 25 mg of tetracycline per kg twice daily. Formalin-fixed sections from the stomach, from three sites along the small intestine, and from the cecum, spiral colon, and brain were taken for examination by light and electron microscopy (4). Colonic contents and blood specimens were also taken, for quantitative bacterial culture.

Immunoblotting. Overnight cultures of each strain in Luria-Bertani broth containing appropriate antibiotics were diluted 100-fold in Eagles's minimal essential medium containing antibiotics and shaken at 225 rpm for 3 h at 37°C to enhance expression of Intimin (9). Bacterial pellets were suspended in 0.1 volume of loading buffer (60 mM Tris-HCl [pH 6.8], 2% sodium dodecyl sulfate, 10% glycerol, 0.025% bromphenol blue, 5% 2-mercaptoethanol) and boiled for 10 min. Samples containing approximately 4 μg of protein were separated by discontinuous 7.5% acrylamide gel electrophoresis and transferred to nitrocellulose membranes by electroblotting. Membranes were blocked with phosphate-buffered saline (PBS) containing 10% nonfat dry milk and 0.1% Tween 20 and incubated with primary antisera in PBS containing 0.1% Tween 20. The antisera used were a rabbit polyclonal antiserum raised against a glutathione *S*-transferase–Intimin$_{O157}$ fusion at a 1:100 dilution (13) and a rabbit polyclonal antiserum raised against an Intimin$_{O127}$-alkaline phosphatase fusion at a dilution of 1:1,000 (10). Bands were developed by using an enhanced chemiluminescence kit (Amersham). Protein concentration was measured by using the bicinchoninic acid kit (Pierce, Rockford, Ill.).

RESULTS

Clinical observations. Table 1 summarizes the clinical outcome of this experiment. Only groups in which extensive bacterial A-E lesions were observed consistently experienced moderate diarrhea. These groups include those infected with wild-type EHEC strain 86-24 and those infected with UMD619 expressing Intimin$_{O127}$. Conversely, animals with little (UMD 619 expressing Intimin$_{O157}$) or no (*eaeA* mutant UMD619) A-E lesions at all had either mild or no diarrhea. Control piglets inoculated with strain C600 had no symptoms of diarrhea, nor did they have any gastrointestinal (GI) tract lesions. Diarrhea began within 2 days after inoculation and lasted till the onset of neurological symptoms, recumbency, or death. Diarrhea generally began earlier and was more watery and severe in piglets infected with strain UMD619 expressing Intimin$_{O127}$ than in piglets infected with the parent strain, 86-24. Regardless of diarrheal symptoms, 21 of the 22 piglets challenged with EHEC strains developed severe neurological symptoms of incoordination, ataxia, or recumbency, which in some piglets resulted in an overnight death. Neurological symptoms probably preceded recumbency, which was not observed when it developed overnight. The severe illness and death, even when neurological symptoms were not observed, could not be attributed to the extent of diarrhea or dehydration. The edema disease-like neurological symptoms we attribute to the systemic translocation of SLT-II produced in the gut lumen by each of the four EHEC strains. Blood cultures

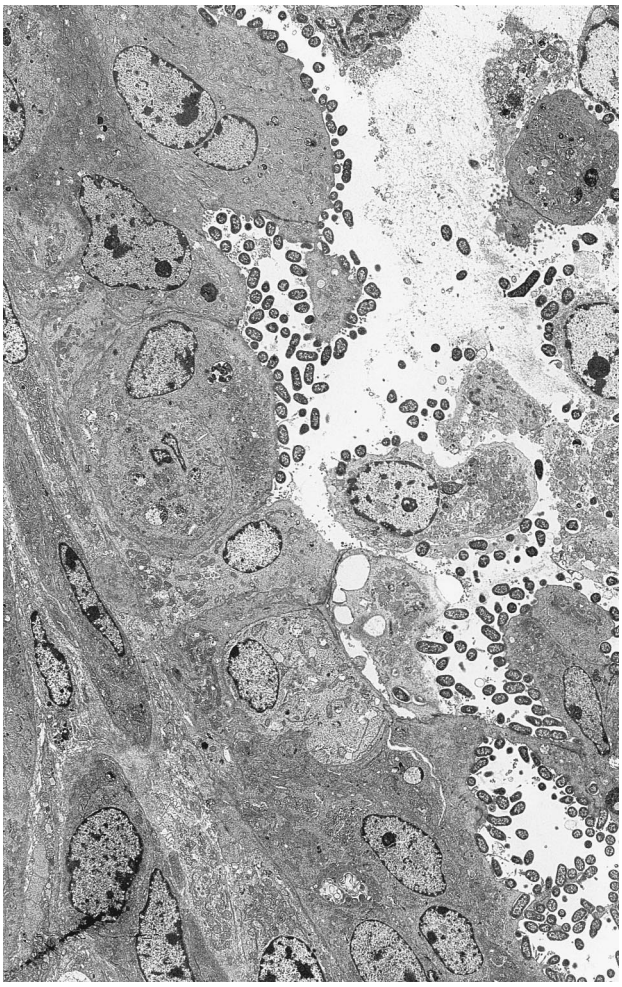

FIG. 1. An electron micrograph of a colonic crypt from a piglet infected with O157:H7, the wild-type strain 86-24, showing intimate bacterial A-E of the glandular epithelium. Magnification, ×860.

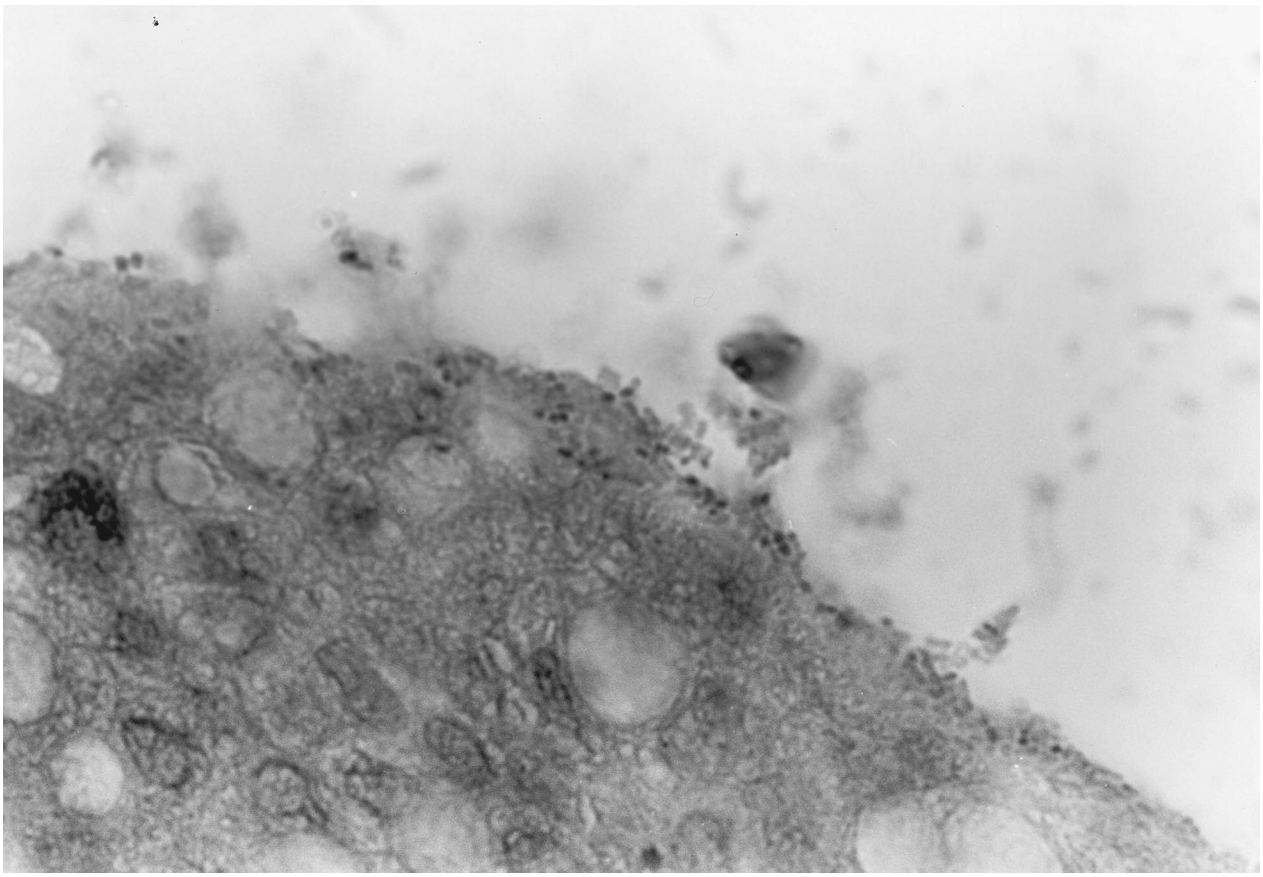

FIG. 2. A light micrograph of colonic surface epithelium from a piglet infected with UMD169 containing plasmid pCVD444 expressing $Intimin_{O157}$, showing mild colonization with A-E bacteria (hematoxylin and eosin; magnification, ×400).

indicated that the severe neurological symptoms were not associated with systemic bacterial invasion.

Bacterial counts in the spiral colon in all infected piglets ranged between 10^9 and 10^{10} viable pure *E. coli* organisms. This range indicated that the much reduced bacterial A-E lesions in the GI tract of group 3 were not due to poor bacterial proliferation and establishment in the gut lumen. Treatment of the inocula, infected animals, and their culture plates with the relevant antibiotics ensured that there was selective pressure for maintenance of the plasmids and the tetracycline resistance cassette.

Histopathological observations. Animals infected with wild-type *E. coli* 86-24 developed the characteristic bacterial A-E lesions in the cecum and colon which involved the surface and glandular epithelia. The extent of the lesions depended on the time after challenge. In severely affected mucosa, the surface epithelium appeared flat, irregular, or absent and depleted of goblet cells. The glandular epithelial surface was equally affected (Fig. 1). The mucosa of the GI tracts of animals infected with the *eaeA* mutant UMD619 (group 2) appeared normal, and no A-E lesions were seen. Piglets of group 3, which were infected with the recombinant strain UMD619 expressing $Intimin_{O157}$, showed only one or two foci of bacterial A-E lesions (Fig. 2). In contrast, the recombinant strain UMD619 expressing $Intimin_{O127}$ colonized the distal half of the small intestine in all animals (Fig. 3) and the large intestine, in a distribution characteristic of EPEC strains. Furthermore, the site of bacterial A-E lesions in the large intestine, which occurred more extensively on the colonic surface and less in the crypts, also resembled that caused by EPEC strains in pigs (Fig. 4). Characteristic brain lesions observed previously (18), consisting of focal microhemorrhages in the cortex (Fig. 5), were seen in all animals with neurological symptoms. They were absent in the control piglets and in the single infected animal which did not display any such signs.

Immunoblotting. As expected, Intimin was not detected in the *eaeA* mutant of EHEC strain 86-24. However, expression of $Intimin_{O157}$ and that of $Intimin_{O127}$ were restored to EHEC *eaeA* mutant UM619 when either the EHEC or the EPEC *eaeA* gene was introduced on a plasmid (Fig. 6). Unfortunately, since different antibodies were used to detect $Intimin_{O157}$ and $Intimin_{O127}$, and affinities of these antibodies are not known, we cannot comment on the relative expression levels of the two proteins.

DISCUSSION

In a previous communication, we had demonstrated the crucial role of the *eaeA* gene of EHEC in the intimate attachment of bacteria to colonocytes in newborn conventional piglets. It was also shown that this attachment can be restored when cloned *eaeA* genes from either EPEC or EHEC strains are reintroduced on a plasmid (4). In this study, we have investigated and compared in gnotobiotic piglets the clinical and pathological consequences of infections with (i) EHEC strain 86-24 (the wild-type parent strain) (ii) its *eaeA* mutant UMD619, and (iii) the *eaeA* mutant UMD619 complemented with plasmids expressing either $Intimin_{O157}$ or $Intimin_{O127}$.

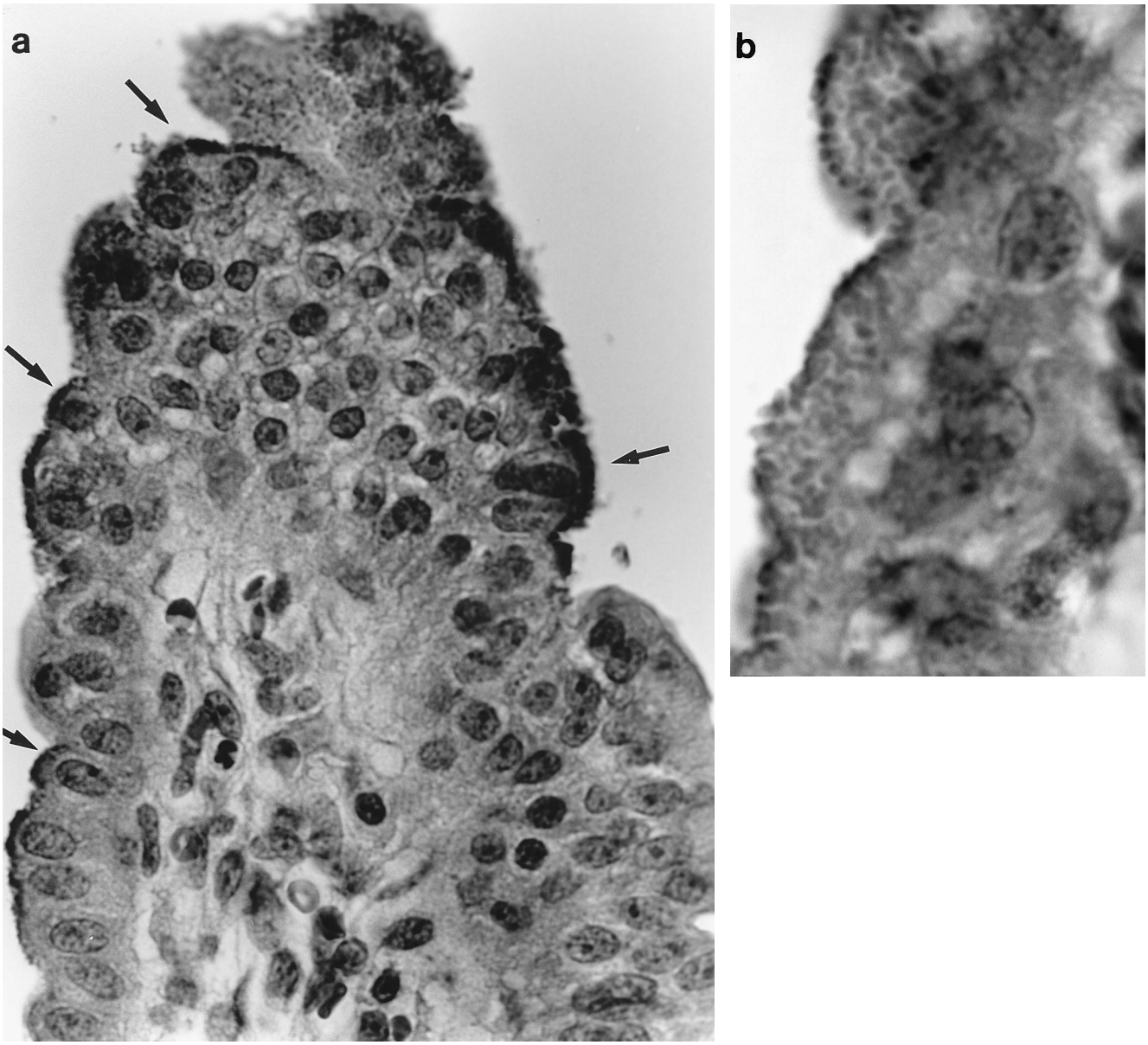

FIG. 3. (a) A light micrograph of villus from the middle of the small intestine from a piglet infected with UMD619 containing plasmid pCVD438 expressing $Intimin_{O127}$, showing extensive intimate bacterial attachment covering most of the villus surface (arrows). The group of cells at the villus tip contain numerous bacteria, invading presumably dying or dead cells (hematoxylin and eosin; magnification, ×80). (b) An area of the section shown in panel a enlarged to show A-E bacteria associated with the villus surface. Magnification, ×320.

The study not only confirmed the role of the *eaeA* gene in intimate bacterial attachment but also demonstrated that, at least in piglets, intimate attachment was necessary for full development of diarrhea as well. Diarrhea was either absent, inconsistent, or mild in animals which lacked bacterial A-E lesions in the gut. These data are consistent with the role of Intimin in EPEC infection in a randomized double-blind volunteer trial in which diarrhea developed in all 11 adults who ingested a wild-type EPEC strain but developed in only 4 of 11 adults who ingested an isogenic *eaeA* mutant (12). Bacterial attachment and epithelial injury in the distal half of the small intestine was associated with a rapid and more serious diarrheal illness than in piglets in which A-E lesions were confined to the large intestine. This distribution of bacterial A-E lesions on the surfaces of the small and large intestines, seen in piglets challenged with strain UMD619 expressing $Intimin_{O127}$, was previously observed only in the GI tracts of piglets infected with EPEC strains (14, 18, 20). In contrast, the lesions induced by EHEC strains in piglets are normally confined to the large intestine and involve extensive intimate bacterial attachment and A-E lesions of both surface and glandular epithelia (6, 18, 19, 21).

While the reintroduction of cloned *eaeA* genes on a plasmid did appear to restore intimate bacterial attachment to gut epithelium, as reported earlier (4), the present study showed that the extent and distribution of the lesions induced by both recombinant strains were markedly different for the two strains and from those of the parent strain, 86-24. It is clear that the

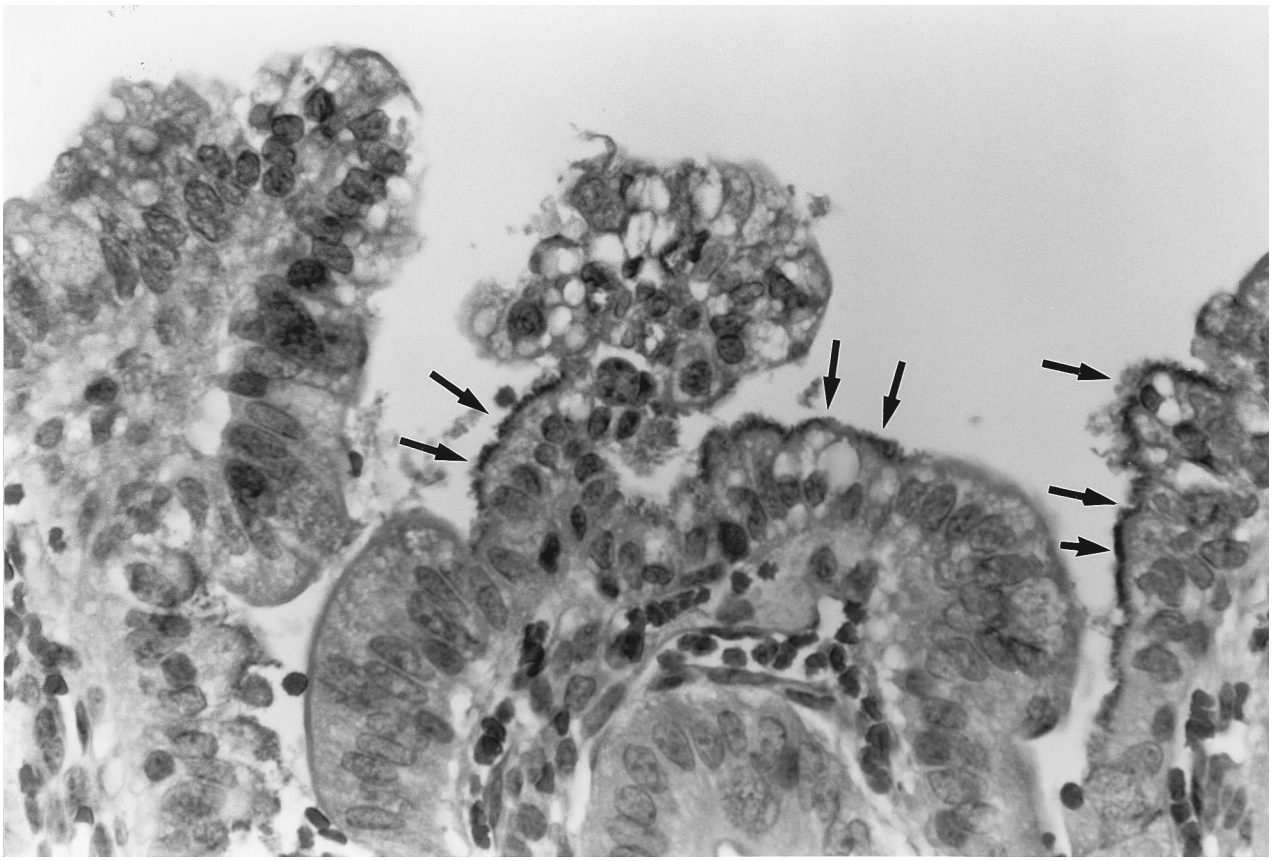

FIG. 4. A section from the colon of the same piglet described in the legend to Fig. 3, showing pronounced modification of the epithelial surface caused by lesions associated with bacterial A-E (arrows) (hematoxylin and eosin; magnification, ×80).

EPEC *eaeA* gene introduced on plasmid pCVD438 markedly altered the in vivo behavior of the host strain, compared with either the wild-type O157:H7 strain 86-24 or its *eaeA* mutant, UMD619. Both the nature and distribution of the intimate bacterial attachment and A-E lesions in infected piglets and the clinical outcome were altered. In contrast, the EHEC *eaeA* gene introduced on plasmid pCVD444 only minimally restored intimate colonic bacterial attachment of *eaeA* mutant strain UMD619. It certainly failed to restore intimate bacterial attachment to the level observed in animals infected with wild-type strain 86-24 and caused little or no diarrhea in these animals. Quantitative bacterial counts in the GI tracts of piglets infected with each of the four strains indicated that these results were not due to differences in the extent of bacterial proliferation in the GI tracts of the infected piglets. Likewise, immunoblotting confirmed that both the EHEC mutant containing the cloned EHEC *eaeA* gene and that containing the cloned EPEC gene expressed the corresponding Intimin molecules. Since in a previous study (4) investigation of the ability of the various strain constructs to induce A-E lesions was the primary objective, conventional animals were used. Although a similar trend was suspected in that investigation, the study design (animals were killed within 48 h) and use of conventional animals would have made the reporting of such observations highly speculative. This is because of the many variables inherently associated with conventional animals, which include (i) the presence of endogenous microflora, which in particular impacts the proliferation of bacteria derived from a different host species; (ii) some interference from maternally derived specific antibodies—piglets are born agammaglobulinemic and acquire all their passive immunity via the colostrum of which gnotobiotic piglets are deprived; and (iii) the occurrence of other potential pathogenic microorganisms in conventional animals. Indeed, the present investigation of gnotobiotic animals was designed to confirm the earlier observations as well as to determine the clinical and pathological consequences.

While the immunoblotting performed is not quantitative, we did not observe obvious differences in expression on the plasmid of the EPEC and EHEC Intimin that could account for the profound differences in the distribution of lesions and the incidence of diarrhea between these strains. The distribution of A-E lesions observed with the EHEC mutant expressing $\text{Intimin}_{\text{O127}}$ was similar to that previously observed with wild-type EPEC strains (14, 18, 20). Furthermore, our results are consistent with the observed differences in the distribution of histopathology in naturally acquired human EHEC and EPEC infections. EHEC damage in the GI tract is limited to the colon (11), while EPEC may affect the entire intestine, from the duodenum to the rectum (16). Therefore, an alternative hypothesis to explain the differences in the distribution of the lesions observed between the EHEC mutant expressing $\text{Intimin}_{\text{O127}}$ and either wild-type EHEC or the EHEC *eaeA* mutant expressing $\text{Intimin}_{\text{O157}}$ is that differences in the receptor-binding domains of the Intimin molecule dictate differences in tissue tropism. The two molecules are virtually identical for most of their length but diverge in amino acid sequence toward the C termini, so that the distal 25% portions of the proteins

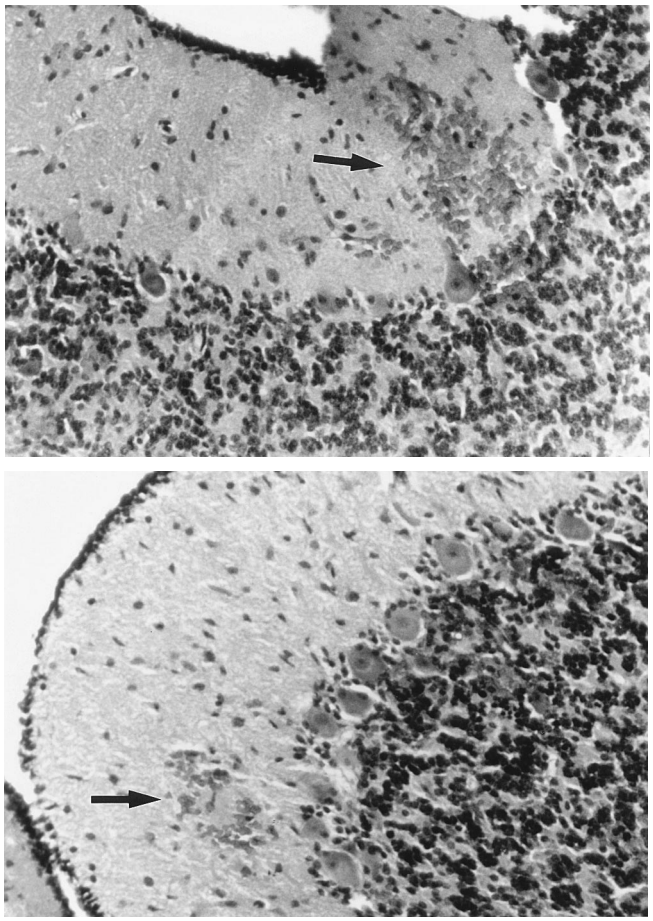

FIG. 5. Sections of cerebellum from a piglet with neurological symptoms attributed to SLT-II. Note the foci of microhemorrhages (arrows) (hematoxylin and eosin; magnification, ×40).

are only approximately 50% identical. Interestingly, the C terminus of the closely related Invasin protein of *Yersinina enterocolitica* and *Yersinia pseudotuberculosis* contains the domain responsible for receptor binding (12). Confirmation of the hypotheses that the C terminus of Intimin is responsible for receptor binding and that $Intimin_{O157}$ and $Intimin_{O127}$ have different receptor specificities must await studies with purified proteins.

Piglets infected with SLT-II-producing strains, regardless of the presence or extent of expression of Intimin, developed neurological symptoms. This indicates that SLT-II is capable of translocating the mucosa into the circulation in the absence of intimate bacterial attachment and A-E lesions.

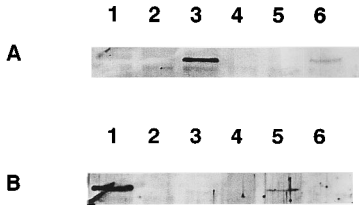

FIG. 6. Immunoblots of Intimin. Whole-cell lysates from EPEC strain E2348/69 (lane 1), E2348/69 *eaeA* mutant CVD206 (lane 2), EHEC strain 86-24 (lane 3), 86-24 *eaeA* mutant UMD619 (lane 4), UMD619 containing plasmid pCVD438 encoding $Intimin_{O127}$ (lane 5), and UMD619 containing plasmid pCVD444 encoding $Intimin_{O157}$ (lane 6) were incubated with rabbit antiserum raised against $Intimin_{O157}$ (panel A) or $Intimin_{O127}$ (panel B).

These observed neurological complications, however, could be prevented by prior administration of specific anti-SLT-II pig serum (data not shown). The occurrence of intimate bacterial attachment and induction of extensive A-E lesions in the mucosa had only a slight effect on the uptake of SLT-II from the GI tract; animals with extensive A-E lesions developed neurological symptoms earlier, which suggests that although damaged mucosa perhaps accelerated SLT-II translocation, it was not the primary mechanism. These results are consistent with the impression that other SLT-II producing but *eaeA*-negative strains (serotype O113:H21) can cause HUS and diarrhea in humans (5, 19a). Infections of piglets with SLT-I-producing O157:H7 strains, in contrast, cause no neurological symptoms, even with severely altered mucosa (data not shown). Previously, neurological symptoms were reported to occur in only 25 to 33% of newborn gnotobiotic piglets challenged with other O157:H7 strains (19). Characteristic brain lesions of cerebellar petechiae, mostly in the cortex and shrunken nuclei in the granular layer, were described in earlier studies (17). The presence of these lesions in brains correlated well with the occurrence of neurological symptoms in these animals. Immunocytochemical studies of mice infected with an O157:H− strain that developed acute encephalopathy showed that SLT-II was present in damaged myelin sheaths of neuron fibers and edematous axons in the brain cortex and spinal cord (7). How SLT-II molecules reach systemic sites remains a mystery.

In summary, these results confirm the role of Intimin in the development of A-E lesions and diarrhea in a gnotobiotic piglet model of EHEC O157:H7 infection. In addition, our studies suggest that differences in the extent and location of A-E lesions and in the incidence and severity of diarrhea may be dictated by differential tissue tropism of EHEC and EPEC Intimin molecules. Lastly, we demonstrate that A-E lesions are not necessary for neurological complications of EHEC infection, which we attribute to the effects of SLT-II.

ACKNOWLEDGMENTS

We are grateful to Melissa Paris and Keith Johnson for their technical skills and animal care.

This work was supported by NIH grant AI-20325 from NIAID, and, in part, by GRASP, PHS grant 1P30 DK39428 from NIDDK, the Office of Research & Development, Medical Research Service, Department of Veterans Affairs, and Public Health Service Award AI-32074 from NIH. F. Gunzer was supported by the Deutsche Forschungsgemeinschaft (DFG grant GU 387).

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