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# Intimin from Enteropathogenic *Escherichia coli* Restores Murine Virulence to a *Citrobacter rodentium eaeA* Mutant: Induction of an Immunoglobulin A Response to Intimin and EspB

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**The formation of attaching and effacing (A/E) lesions is central to the pathogenesis of enteropathogenic *Escherichia coli* (EPEC)-mediated disease in humans and *Citrobacter rodentium* (formerly *C. freundii* biotype 4280)-mediated transmissible colonic hyperplasia in mice. Closely related outer membrane proteins, known as intimins, are required for formation of the A/E lesion by both EPEC ($Int_{EPEC}$) and *C. rodentium* ($Int_{CR}$). A secreted protein, EspB (formally EaeB), is also necessary for A/E-lesion formation. Here we report that expression of a cloned $Int_{EPEC}$, encoded by plasmid pCVD438, restores murine virulence to an intimin-deficient mutant of *C. rodentium* DBS255. Replacement of Cys937 with Ala abolished the ability of the cloned EPEC intimin to complement the deletion mutation in DBS255. Ultrastructural examination of tissues from wild-type *C. rodentium* and DBS255(pCVD438)-infected mice revealed multiple A/E lesion on infected cells and loss of contact between enterocytes and basement membrane. Histological investigation showed that although both wild-type *C. rodentium* and DBS255(pCVD438) colonized the descending colon and induced colonic hyperplasia in orally infected 21-day-old mice, the latter strain adhered to epithelial cells located deeper within crypts. Nonetheless, infection with the wild-type strain was consistently more virulent, as indicated by a higher mortality rate. All the surviving mice, challenged with either wild-type *C. rodentium* or DBS255(pCVD438), developed a mucosal immunoglobulin A response to intimin and EspB. These results show that *C. rodentium* infection provides a relevant, simple, and economic model to investigate the role of EPEC proteins in the formation of A/E lesions in vivo and in intestinal disease.**

Enteropathogenic *Escherichia coli* (EPEC) is a subgroup of pathogenic *E. coli* of specific serotypes that are a major cause of infant diarrhea (18). Analysis of intestinal biopsy specimens from children suffering from EPEC-induced diarrhea show that binding of EPEC to the brush border induces a specific lesion termed attachment and effacement (A/E). This lesion is characterized by effacement of cellular microvilli and the intimate adherence of bacteria to cup-like pedestals at the bare apical enterocyte cell membrane (28). Experiments using cultured epithelial cells have implicated several genes in the formation of A/E lesions. These genes map to a 35-kBp chromosomal locus termed the LEE region (21) that encodes a type III secretion system (14) which is necessary for the export of secreted LEE-encoded proteins (the so-called EPEC-secreted proteins). Two of the most important proteins encoded by the LEE region are intimin ($Int_{EPEC}$), a 94-kDa outer membrane protein encoded by the chromosomal *eaeA* gene (15, 16) and EspB (formally EaeB) (9), a secreted protein that binds eukaryotic cells and is involved in activation of host cell signal transduction pathways and induction of tyrosine phosphorylation of host cell proteins (17).

Homologous LEE regions are present in other bacteria (21), including *Citrobacter rodentium* (formerly *C. freundii* biotype 4280 [26]) (24), enterohemorrhagic *E. coli* (EHEC) (29), and *Hafnia alvei* (2, 11). Comparative analysis of the amino acid sequences of intimins from the different strains has shown that these proteins share a high degree of homology within the amino termini but a greater degree of diversity in the carboxy termini. However, two Cys residues in the carboxy-terminal region are conserved among all family members and a eukaryotic cell-binding domain resides within the 280 amino acids at the carboxy terminus of the different intimins (11). Based on the structural and functional similarities between the different intimins and the reported ability of intimin from a human EPEC isolate (O127:H6 strain E2348/69) to complement an *eaeA* mutation of a human EHEC isolate (7), we have investigated the ability of the plasmid-encoded EPEC *eaeA* gene (pCVD438) (6) to complement an *eaeA* deletion mutant of *C. rodentium* (strain DBS255) (25). *C. rodentium* is the causative agent of transmissible murine colonic hyperplasia (3), a disease of laboratory mice characterized by increased epithelial cell proliferation in the mucosa of the descending colon (4). During early stages of infection *C. rodentium* produces colonic damage similar to the A/E lesions caused by EPEC in humans (24). By mutation analysis, the chromosomal *eaeA* gene of *C. rodentium* was recently shown to be necessary for colonic colonization and hyperplasia (25). Colonic colonization, histopathological lesions of the colon, and mortality rates of the infected mice were used as criteria to determine successful

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TABLE 1. List of strains and plasmids

| Bacterial species, strain, or plasmid | Description | Reference(s) |
|---|---|---|
| *C. rodentium* (wild type) | Wild type | 3, 24 |
| DBS255 | *C. rodentium* Δ*eaeA* mutant | 25 |
| E2348/69 | Clinical EPEC isolate | 19 |
| CVD206 | E2348/69 Δ*eaeA* mutant | 6 |
| JPN15 | E2348/69 cured of the pMAR2 EAF plasmid | 16 |
| pCVD438 | pACYC184 vector containing *eaeA* from E2348/69 | 6 |
| pCVD438CA | pCVD438 in which Cys-937 was replaced with Ala | 13 |

complementation. We found that DBS255(pCVD438) colonized only the large bowel of orally infected mice and induced histological changes similar to those induced by the wild-type *C. rodentium* strain. An immunoglobulin A (IgA) response to both intimin and EspB was detected in all the mice that survived challenge with either wild-type *C. rodentium* or DBS255(pCVD438); however, in mice infected with the former strain the IgA antibodies could be detected earlier. Hence, this simple murine model can provide an in vivo system for evaluating the molecular and immunological properties of EPEC-derived gene products.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Nalidixic acid-resistant ($Nal^r$) variants of wild-type *C. rodentium* and DBS255 were selected on L agar containing 100 μg of nalidixic acid per ml. Wild-type EPEC E2348/69 (O127:H6), JPN15, and CVD206 were kindly provided by J. Kaper (Center for Vaccine Development, University of Maryland). Plasmid pCVD438, a recombinant plasmid containing the *eaeA* gene, which encodes surface-expressed and biologically active intimin from E2348/69, was also provided by Dr. Kaper. Plasmid pCVD438CA is a pCVD438 derivative in which Cys-937 was substituted with Ala.

**Western blot (immunoblot) analysis.** *C. rodentium* and EPEC derivatives were grown for 3 h at 37°C in Dulbecco's modified Eagle's medium. The bacterial cultures were harvested by centrifugation and resuspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer to an absorbency at an optical density of 650 nm ($OD_{650}$) of 1, and 10 μl was loaded on a 7.5% SDS-PAGE gel. The electrophoresed proteins were transferred to a nitrocellulose membrane, and immunodetection of intimin was performed as described previously (15), using rabbit polyclonal antiserum raised against the carboxy-terminal 280 amino acids of intimin ($Int_{EPEC280}$), diluted 1:500. Rabbits were immunized with $Int_{EPEC280}$ after the fragment encoding this domain was transferred from pMal c2 (11) to pET28a (Novagen, Madison, Wis.), and the antigen was purified on a nickel column as suggested by the manufacturer (our unpublished data). Fifty micrograms of the histidine-tagged polypeptide was used for immunization in complete Freund's adjuvant. Detailed preparation and characterization of this antiserum will be described elsewhere (1).

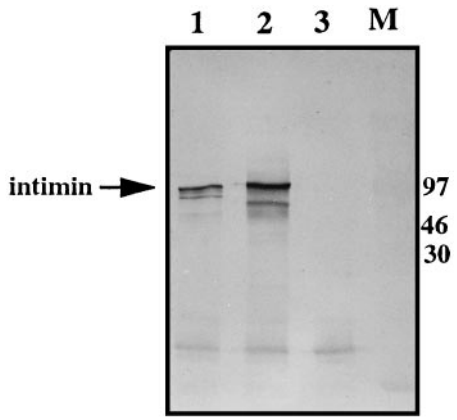



FIG. 1. Western blot of whole-cell extracts probed with the anti-intimin polyclonal antiserum. Higher level of intimin expression is detected in the wild-type *C. rodentium* strain (lane 2) compared with DBS255(pCVD438) (lane 1), while no reaction was observed with DBS255 (lane 3). Size of molecular weight markers (lane M) are indicated in thousands on the right.

TABLE 2. Colonic colonization of 21-day-old mice by *C. rodentium* and DBS255(pCVD438)

| Mouse no.[a] | CFU/mg of tissue | |
|---|---|---|
| | Wild type | DBS225(pCVD438) |
| 1 | $8 \times 10^3$ | 0 |
| 2 | $1.4 \times 10^2$ | 0 |
| 3 | $2.3 \times 10^0$ | $1.8 \times 10^3$ |
| 4 | $5.7 \times 10^3$ | $8.9 \times 10^3$ |
| 5 | $8.5 \times 10^2$ | $5.2 \times 10^2$ |
| 6 | $8 \times 10^3$ | $6.3 \times 10^2$ |
| 7 | $5.5 \times 10^3$ | $3.5 \times 10^3$ |
| 8 | $1.4 \times 10^3$ | $5.7 \times 10^{-1}$ |
| 9 | 0 | $8.3 \times 10^{-1}$ |
| 10 | $6.5 \times 10^{-1}$ | $1 \times 10^3$ |

[a] For histological examination, samples of upper large intestine were taken from mice 1 to 5 and samples of descending colon were taken from mice 6 to 10.

**Challenge of mice with bacteria.** Thirty 21-day-old female Swiss NIH mice (Harlan/Olac, Scunthorpe, United Kingdom) were orally challenged with a gavage needle with either wild-type *C. rodentium* DBS255 or DBS255(pCVD438), grown with shaking at 37°C. For inoculations, bacteria were grown overnight in L broth containing 100 μg of nalidixic acid per ml or 30 μg of chloramphenicol per ml. The bacteria were diluted with phosphate-buffered saline (pH 7.2) (PBS) to an $OD_{600}$ of 1.7 and delivered to mice in a volume of 100 μl (ca. $1.5 \times 10^7$ CFU). At 15 days postchallenge, 10 of the surviving mice, picked at random, were used to determine the level of colonic colonization and the degree of the histopathological changes. The remaining mice from each group were used to follow the disease progression and to determine the mortality rate. Results of a typical challenge experiment are presented. Additional groups of 10 mice were similarly infected with $1.5 \times 10^7$ CFU of DBS255(pCVD438CA), E2348/69, JPN15, and CVD206 and tested for colonic colonization at 15 days postchallenge. Ten mice were mock infected with PBS.

**Histology.** The 10 mice picked at random from the groups infected with the different bacterial strains were divided randomly into two groups of five mice, which were sacrificed at 15 days postchallenge. The large intestine was removed, opened longitudinally, and rinsed in PBS. From the first group of mice a 0.5-cm section of the upper colon was removed and processed for histology, while from the second group of five mice a 0.5-cm section of the lower colon was processed for histology. Small samples of the pieces were processed for electron microscopy. The remaining colon was used for determining levels of bacterial colonization (see below). Samples were fixed in 10% phosphate-buffered formaldehyde, wax embedded, and stained with hematoxylin and eosin (H&E) (10).

**Colonization.** Following opening of the large intestine and removal of samples for histology, the remaining intestine was homogenized mechanically by using a Colworth Stomacher 80. Serial dilutions were plated on L agar plates containing 100 μg of nalidixic acid per ml, and the number of CFU in each sample was determined by direct counting. This method was also used to determine bacterial recovery both in the small intestines, livers, and spleens from two mice inoculated with wild-type *C. rodentium* in which colonic hyperplasia, and hence colonization, was apparent and in the small and large intestines of EPEC-derivative-inoculated mice and mock-infected mice.

**Morphometry.** A computer-based image analysis system (Kompira Imagan 2 video system with a Dell 320SX personal computer) was used to measure total mucosal thickness in H&E-stained sections. Video images were obtained from an Olympus BHB light microscope via a JVC TK-1085E color video camera head and digitized by using a Summa Sketch plus graphics tablet. Vertical lines were overlaid on the video image of the intestinal sections to allow careful orientation perpendicular to the muscularis mucosae. Areas of the tissue showing well-oriented, longitudinal sections were selected, and the distance between the junction of the mucosa with the muscularis mucosae and the mucosal surface was measured along the superimposed lines by using a 16× objective lens at a total magnification of ×535. A minimum of 10 measurements were made per sample and stored on the computer for subsequent analysis.

**Statistics.** A Wilcoxon ranking test was performed with commercially available software (Statgraphics Plus; Manugistics, Rockville, Md.) to assess differences between the groups; $P < 0.05$ was taken to be significant.

**Transmission electron microscopy.** Samples were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% aqueous osmium tetroxide, and

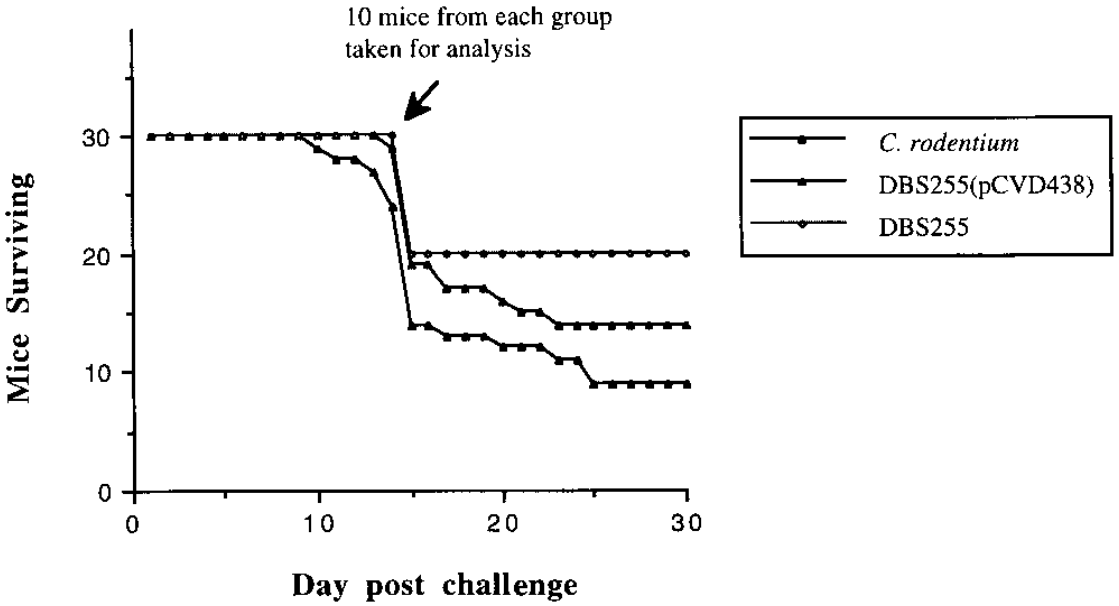



FIG. 2. Mortality rate of Swiss NIH mice challenged with the wild-type *C. rodentium*, DBS255, and DBS255(pCVD438). Mice infected with the wild-type strain died faster and in greater numbers than those infected with DBS255(pCVD438), while mice infected with DBS255 survived throughout the experiment, which lasted 50 days postchallenge. On day 15 postchallenge, 10 of the surviving mice from each group were selected at random and used for histological and ultrastructural examinations and to determine the level of colonic colonization.

embedded in TAAB resin (TAAB Laboratories, Reading, United Kingdom). Thin sections were double stained with uranyl acetate and lead citrate and viewed in a JOEL 1200 EX II transmission electron microscope at 80 kV.

**Detection of *eaeA* by PCR.** PCR (22) was used to amplify a segment of *eaeA* encoding the carboxy-terminal 280 amino acids of intimin (Int280). Thirty amplification cycles (95°C, 20 s; 50°C, 1 min; and 74°C, 1 min) were employed using 25 pmol of each of the forward (5′ GCCAGCATTACTGAGATTAAG) and reverse [5′ TTTTACACAA(A/G)(C/T)(G/T)GCA(A/T)AAGC] primers and 1.5 U of *Taq* DNA polymerase (Appligene, Durham, United Kingdom). Representative colonies recovered from the colon of colonized mice (10 colonies per mouse) were tested for the presence of the gene. For each reaction, about one-third of a colony was transferred to a 0.5-ml tube containing the PCR reaction mixture and *eaeA*-specific primers and the tubes were incubated at 95°C for 5 min prior to the PCR cycling. Ten microliters from each reaction was analyzed by agarose gel electrophoresis.

**Preparation of IgA antibodies from fecal pellets.** Measurement of IgA in fecal pellets was performed by collection of between 3 and 5 fresh stools from individual mice at different time points postchallenge. These were soaked overnight at 4°C in 1 ml of PBS containing 0.1% bovine serum albumin and 1 mM of phenylmethylsulfonyl fluoride. The solid material was then disrupted by vortexing, and the suspension was centrifuged for 5 min at 15,000 rpm. The resultant supernatants were collected and stored at −20°C prior to analysis.

**ELISA.** EPEC E2348/69 DNA was used as template to amplify the entire *espB* gene (9) by PCR using 25 pmol from each of the forward (5′ CTGAATTCAT CGCAGTTAATTCTGTTTTGA) and reverse (5′ CTTCTAGAACACTTCAT CATTAAACGTATC) primers as described above. The amplified DNA fragment was cleaved with restriction endonucleases *Eco*RI and *Xba*I and cloned into a similarly cleaved pMal c2 vector before being subcloned in pET28a in *E. coli* BL21. The histidine-tagged EspB polypeptide was purified on a nickel column as suggested by the manufacturer. The purified intimin and EspB polypeptides were used to detect specific IgA antibodies in the fecal pellets by enzyme-linked immunosorbent assay (ELISA). In brief, antigen (2.5 μg/ml) was coated onto 96-well plates overnight at 4°C. Plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and blocked for 1 h at 37°C with 1% bovine serum albumin. Supernatants from individual animals were diluted 1:3 from the neat sample to a dilution of 1:2,178. These were added to the wells, and the plates were incubated overnight at 4°C. Plates were washed as described above and incubated with biotinylated goat anti-mouse α-chain-specific antibodies (Sigma) for 90 min at 37°C. After washing, horseradish peroxidase streptavidin conjugate (Sigma) was added for 90 min at 37°C, and bound antibodies were detected by adding *O*-phenylendiamine substrate (Sigma) and hydrogen peroxide. Absorbencies were read at 490 nm, and ELISA titers were determined arbitrarily, after subtraction of the reading obtained from fecal pellets of prechallenged mice, as the dilution of supernatant which gave an OD value of 0.3.

## RESULTS

**Colonic colonization of mice challenged with different *C. rodentium* and EPEC derivatives.** Preliminary experiments with Swiss NIH mice challenged with wild-type *C. rodentium* revealed that a clear macroscopic colonic hyperplasia was evident at 15 days postchallenge while a lesser degree of macroscopic damage was seen at 10 days postchallenge. For this reason, the level of colonic colonization (and histological examination) was routinely tested at 15 days postchallenge. Thirty mice were infected orally with wild-type *C. rodentium*, DBS255(pCVD438), and DBS255. Ten mice were mock infected with PBS. The importance of Cys937 for the biological activity of intimin in vivo was also determined by inoculation of mice with DBS255(pCVD438CA). Qualitative analysis of whole-cell extracts by Western blots revealed that the wild-type *C. rodentium* strain exhibited a higher level of intimin expression compared with DBS255(pCVD438), while no reaction was seen with DBS255 (Fig. 1). Comparable level of intimin expression was seen in DBS255(pCVD438) and DBS255 (pCVD438CA) (not shown). Ten mice which survived the infection on day 15 were selected at random from each of the experimental groups. Nine of the 10 mice infected with wild-type *C. rodentium* were colonized as determined by the number of Nal$^r$ bacteria recovered from the large intestine (Table 2). Although the level of colonization in two of the mice was low ($2.3 \times 10^0$ and $6.5 \times 10^{-1}$ CFU/mg of tissue for mice 3 and 10, respectively), the level of *C. rodentium* recovered from the other seven mice varied between $10^2$ and $10^4$ CFU/mg of tissue (Table 2). Nal$^r$ bacteria were also recovered from 8 of the 10 mice infected with DBS255(pCVD438). Two mice (no. 8 and 9) were poorly colonized, and no bacteria were recovered from mice 1 and 2. By using PCR, it was possible to show that all representatives of the Nal$^r$ colonies recovered from the mouse tissue contained the *eaeA* DNA fragment encoding Int280 (data not shown). No Nal$^r$ bacteria were recovered from mice

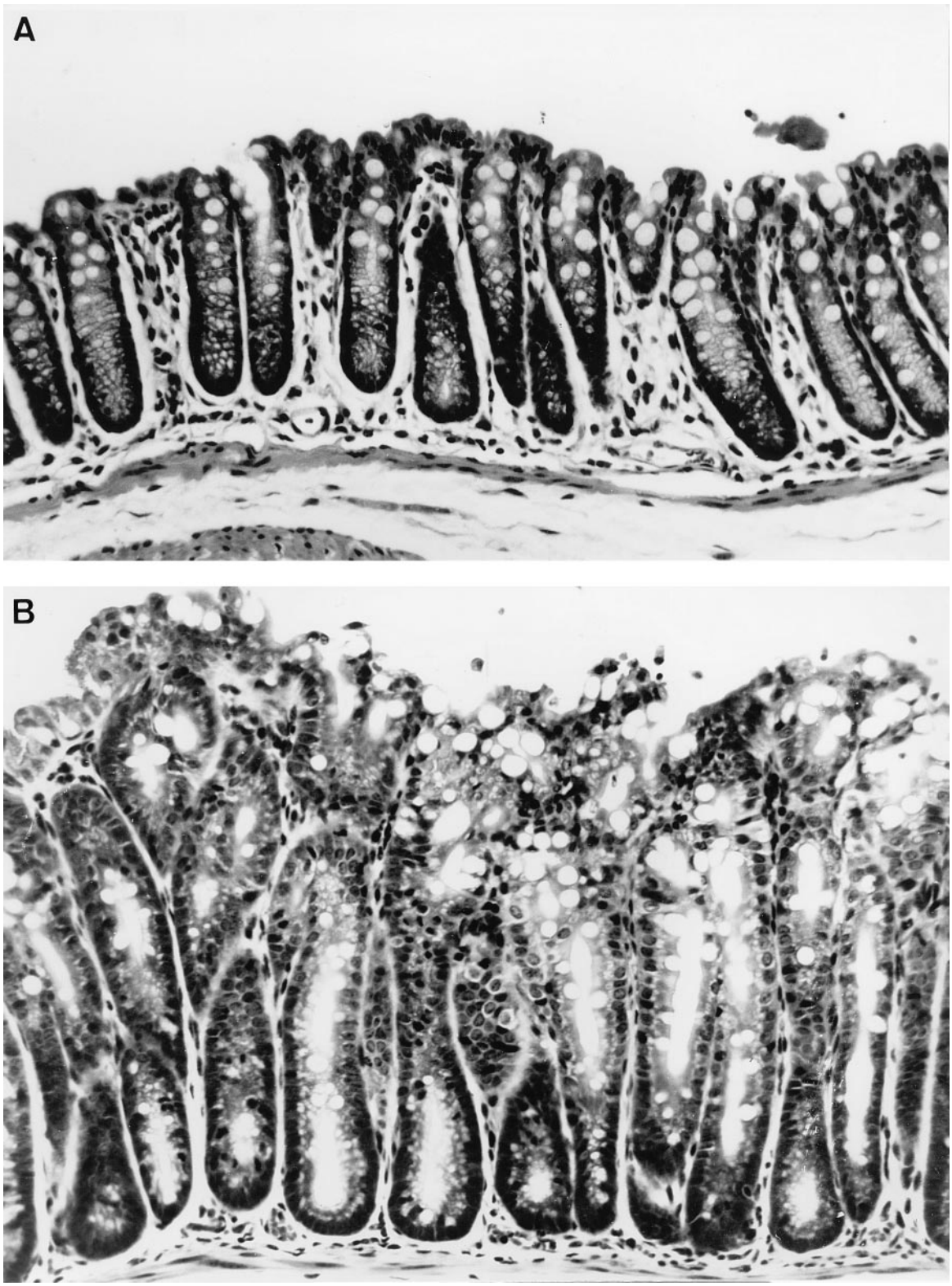
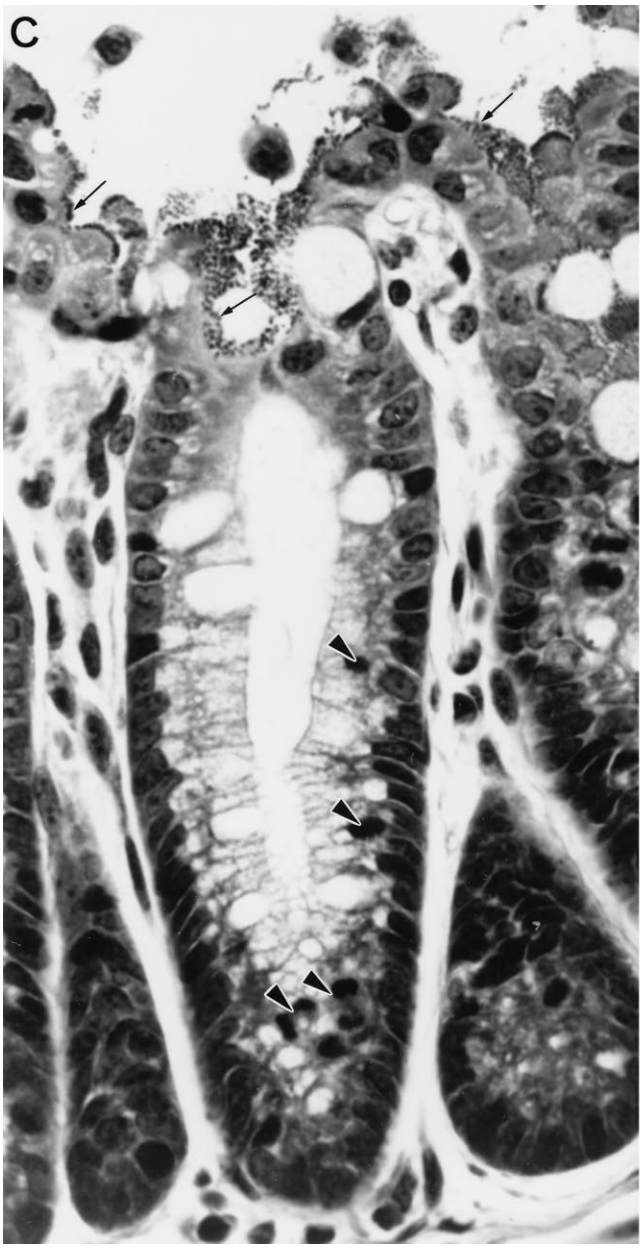



FIG. 3. Lower colon from a mouse infected with DBS255 (A) and from a mouse inoculated with wild-type *C. rodentium* showing crypt hyperplasia with ragged surface epithelium (B); both were H&E stained (magnification, ×300). (C) Detail of crypt from a mouse inoculated with wild-type *C. rodentium*; note bacteria adhering to surface and upper crypt epithelia (small arrows) and increased mitotic figures (arrowheads). (D) Epithelia of a mouse inoculated with DBS255(pCVD438) demonstrating bacterial adherence from surface to mid-crypt epithelia (arrowheads). (E) As in panel D but a crypt lumen dilated with enclosed bacteria (arrowhead). Panels C to E were all H&E stained (magnification, ×550).



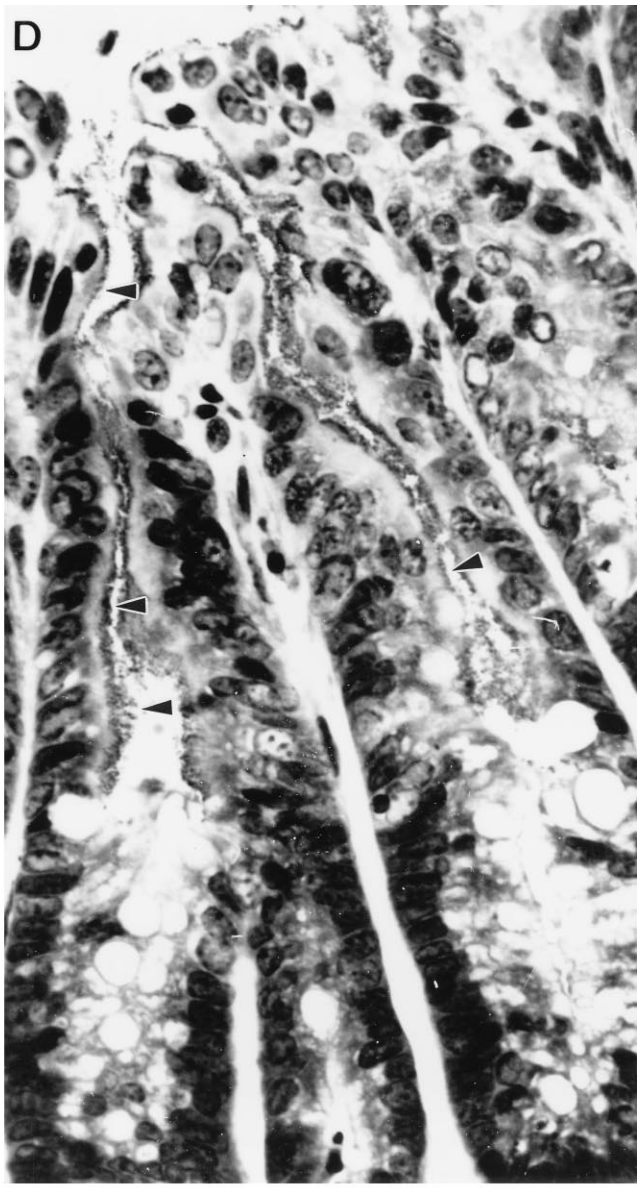




FIG. 3—*Continued.*

inoculated with either DBS255, DBS255(pCVD438CA), or PBS. These results indicate that a functional *eaeA* gene is necessary for *C. rodentium* colonic colonization and that the *eaeA* gene of EPEC can substitute, in vivo, for the *C. rodentium eaeA* homolog. In mice infected with either of the two virulent strains no bacteria were recovered on day 49 postchallenge (data not shown). In addition, no bacteria were recovered from the small intestines, livers, or spleens of two colonic-colonized mice challenged with wild-type *C. rodentium.*

In view of the proficiency of the cloned EPEC intimin to complement the mutation in DBS255, the ability of E2348/69, JPN15, and CVD206 to colonize Swiss NIH mice was assessed. When the same infection regimen described for *C. rodentium* was used, neither the small nor large intestine was colonized by any of the EPEC derivatives.

**Mouse mortality following challenge with the *C. rodentium* derivatives.** By day 15, 6 of the 30 mice challenged with the wild-type *C. rodentium* and 1 of the 30 mice inoculated with DBS255(pCVD438) died as a result of the infection, while no death was recorded for the mice challenged with DBS255 (Fig. 2). At this stage, 10 of the surviving mice were removed for the histological examination and determination of the level of colonic colonization (Fig. 2). Of the remaining 14 mice challenged with the wild type and 19 mice challenged with DBS255(pCVD438), 5 and 4 more mice died by day 25 postchallenge, respectively (Fig. 2). Therefore, substitution of $Int_{CR}$ with $Int_{EPEC}$ appeared to delay the onset of a fatal infection, as mice infected with DBS255(pCVD438) died later then those infected with the wild-type strain. No deaths were recorded after day 25 postchallenge. None of the mice infected

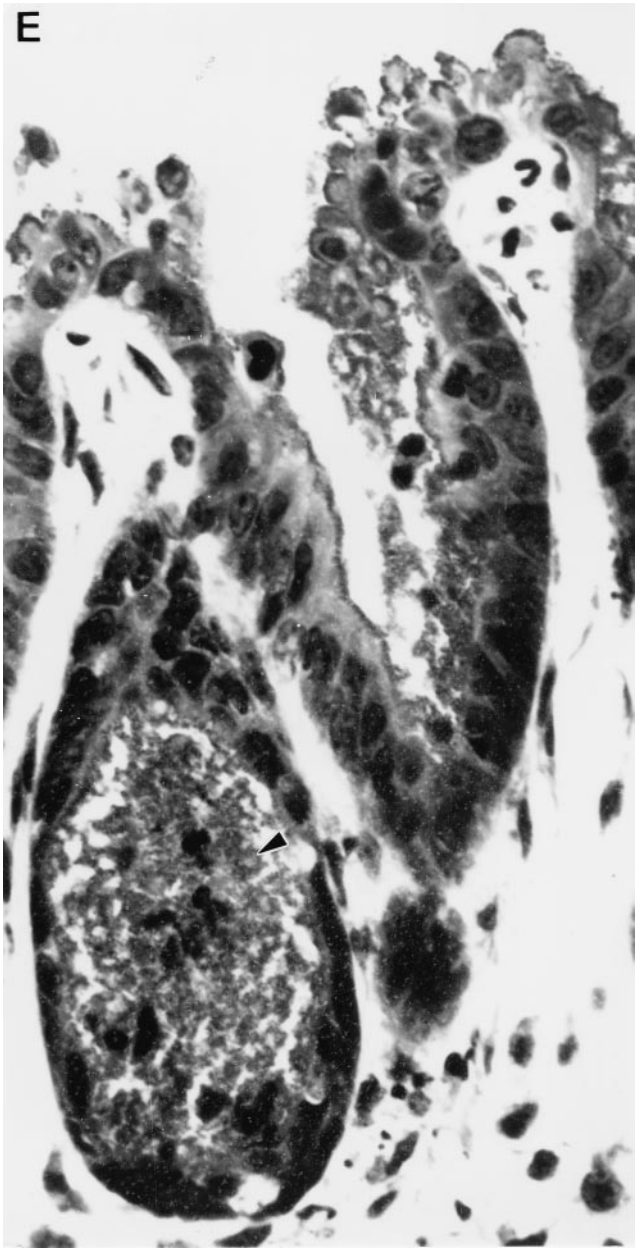



FIG. 3—*Continued.*

with DBS255 died throughout the whole course of the experiment (>50 days).

**Histological examination of the upper and lower colon.** The upper parts of the colons from any of the groups were indistinguishable. The mucosa was thrown into folds which projected into the gut lumen and showed the presence of villus/crypt structures. No bacteria were seen adhering to the mucosal surface (data not shown). In the lower part of the colon, mucosal folds were not apparent and no villus structures were seen in the three groups tested. No bacteria were identified in the mice infected with DBS255 (Fig. 3A). In a typical experiment mice infected with either wild-type *C. rodentium* or DBS255(pCVD438) showed the presence of bacteria adhering to the epithelium in four of five and three of five mice, respectively. Samples exhibiting bacterial adherence showed crypt hyperplasia in the crypt epithelium, and an uneven surface epithelium with increased examples of cell extrusion (Fig. 3B). Wild-type *C. rodentium*-inoculated mice showed bacteria adhering to surface and upper crypt epithelia (Fig. 3C). Interestingly, DBS255(pCVD438)-inoculated mice consistently showed bacterial adherence further into the crypt (Fig. 3D) and one individual showed dilated crypt lamina full of bacteria (Fig. 3E). There was an uneven increase in neutrophil infiltration of the lamina propria and submucosa. The overall histological picture of individual mice challenged with wild-type *C. rodentium* and DBS255(pCVD438) that showed no evidence of bacterial adherence appeared identical to that of mice inoculated with DBS255.

Morphometric examination of the mucosal thickness confirmed subjective observations. Mice with bacteria on the mucosal surface had a significantly thicker mucosa ($P > 0.05$) than those without evidence of bacterial adherence, regardless of experimental group (Fig. 4).

**Ultrastructure examination of the lower colon following *C. rodentium* challenge.** No bacteria were identified in mice inoculated with DBS255, and the epithelial brush border appeared normal (Fig. 5A). Bacteria involved in A/E lesions were seen in mice infected with wild-type *C. rodentium* and with DBS255 (pCVD438) (Fig. 5B to F). No difference in the appearance of adhering bacteria or the A/E lesions were apparent between these two groups. The apical surface of enterocytes was variably convex (Fig. 5B and E) or concave (Fig. 5C), producing an uneven epithelial surface. Some cells with adhering bacteria showed signs of separation from the underlying basement membrane (Fig. 5C). Interepithelial cell lateral membrane interdigitations were clearly visible, as the interdigitations were organized at right angles to the lateral membrane (Fig. 5B and C), rather than in parallel configuration. Individual bacteria were seen attached to two enterocytes (Fig. 5D), causing A/E lesions on two apical membranes. This was not an artifact of section angle, as a tight junction was clearly visible and the cytoplasmic densities of the two epithelial cells were different (Fig. 5D).

**IgA response to intimin and EspB.** Of the remaining mice challenged with wild-type *C. rodentium*, DBS255(pCVD438), and DBS255, fecal pellets were collected from 10 individual mice picked at random 16 days postchallenge. On days 28 and 40 postchallenge additional fecal pellets were collected from surviving mice. The presence of anti-intimin and anti-EspB IgA was measured by ELISA by using purified Int280 or EspB derived from EPEC as the coating antigens. No anti-intimin or anti-EspB IgA was detected in fecal pellet supernatants from prechallenged mice or in fecal pellet supernatants obtained from mice challenged with DBS255 at any day postchallenge. In addition, fecal samples taken at day 16 postchallenge from mice infected with wild-type *C. rodentium* and DBS255 (pCVD438) also contained no detectable anti-intimin or anti-EspB IgA antibodies (data not shown).

Seven of the 10 mice challenged with DBS255(pCVD438) survived the infection to the end of the experiment on day 50. An IgA response to intimin and EspB was detected in three mice on day 28 (Fig. 6). Feces from all mice surviving DBS255(pCVD438) challenge harbored anti-intimin IgA on day 40 postchallenge (Fig. 6), while feces from only four of these mice harbored anti-EspB IgA antibodies on day 40. In one mouse of the seven (mouse no. 3) no anti-EspB IgA antibodies were detected at any day postchallenge, although this mouse had anti-intimin antibodies on day 40 (Fig. 6). Five of the 10 mice challenged with the wild-type strain survived to day 50. In contrast to DBS255(pCVD438), an IgA response to intimin was detected in all of these mice both on day 28 and on

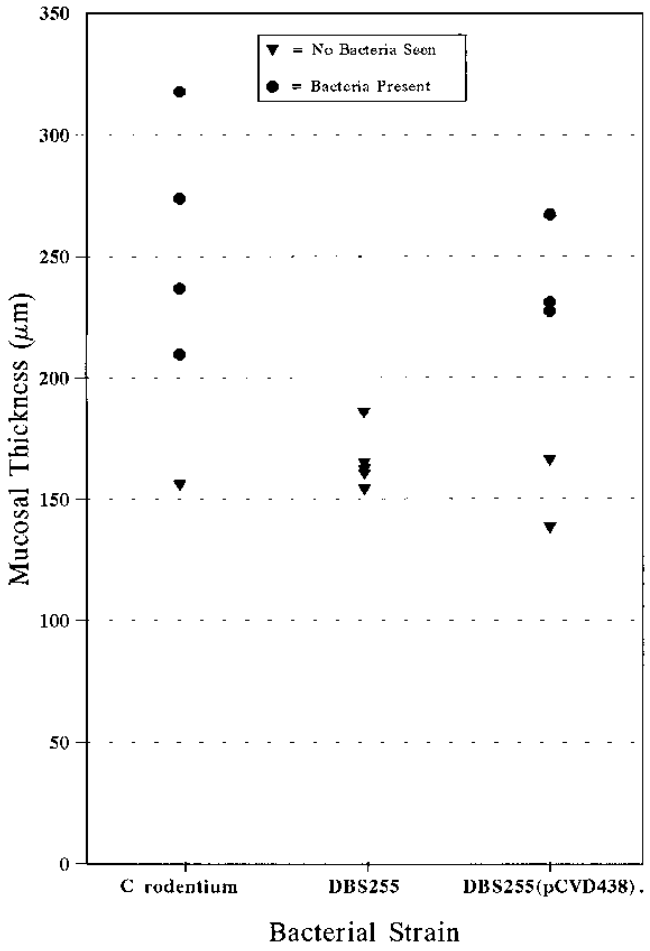



FIG. 4. Morphometry of lower colon from infected mice, examined 15 days postchallenge. Increase mucosal thickness is exhibited only in the section in which bacteria could be visualized microscopically, irrespective of the experimental group.

day 40 postchallenge. IgA antibodies directed against EspB were found in all of these mice on day 28 and in all but one mouse on day 40 postchallenge (Fig. 6).

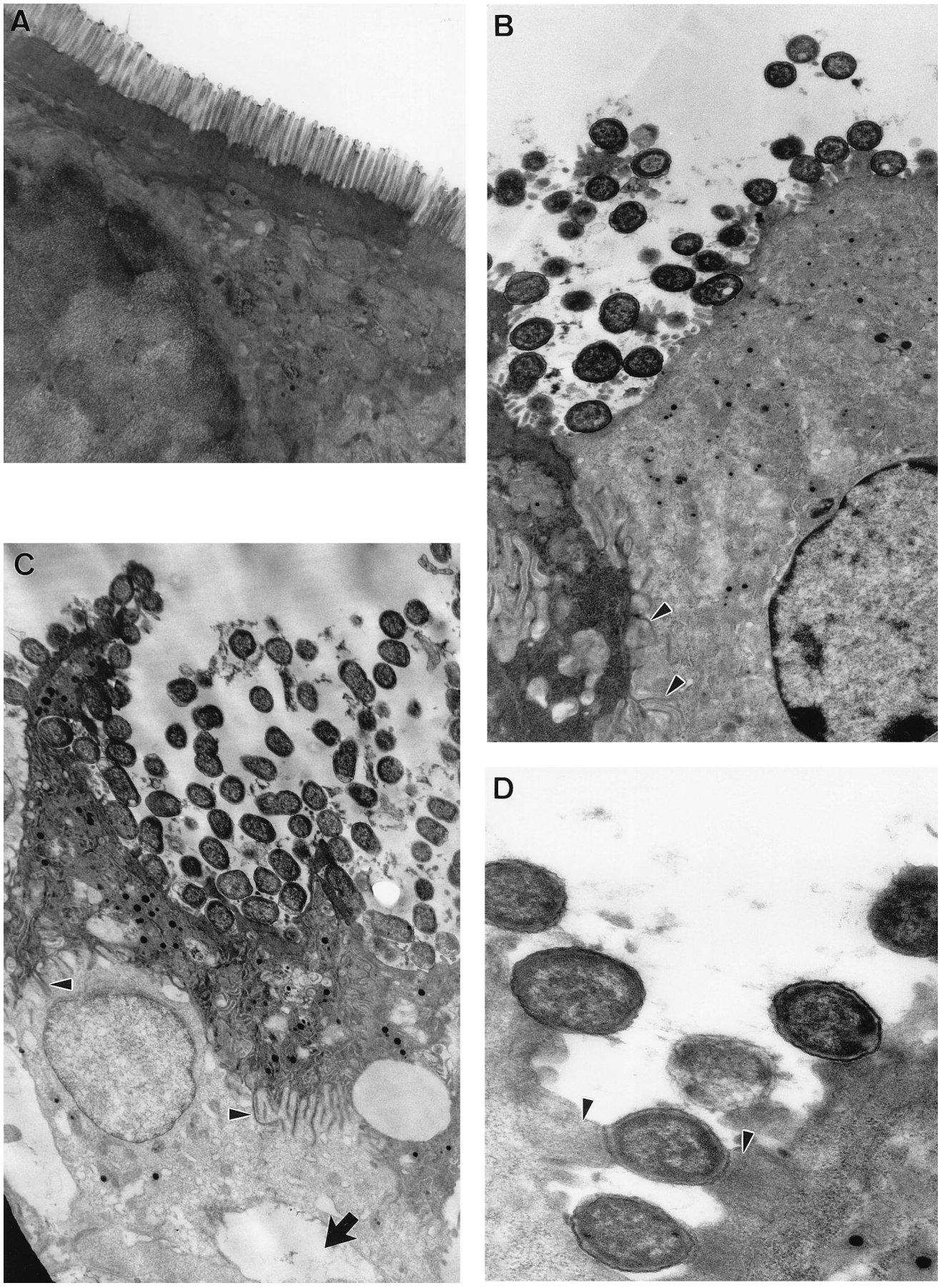
## DISCUSSION

In this report we show that the cloned *eaeA* gene of EPEC can restore the virulence for mice of *C. rodentium* DBS255 harboring a deletion mutation in the chromosomal *eaeA* gene. Moreover, expression of the cloned EPEC intimin in DBS255 also restored an IgA response by the challenged mice to both intimin and EspB. DBS255(pCVD438)-challenged mice showed a histological picture similar, but not identical, to that for *C. rodentium*-challenged mice. Like *C. rodentium*, DBS255(pCVD438) colonized only the descending colon of the mice. However, although adhesion remained tissue specific the cell specificity of DBS255(pCVD438) appeared to be subtly altered as there was a more extensive colonization of the crypts, where bacteria could be seen associated with cells which are probably at earlier stages of maturation (5). Although Western blot analysis revealed that the wild-type *C. rodentium* expresses intimin in vitro at a higher level than DBS255 (pCVD438), this may not reflect the level of expression in vivo. Therefore, the reason for the location of DBS255(pCVD438) deeper within crypts is at present not known. However, despite the subtle difference, the fact that *eaeA* genes are interchangeable between two human pathogens (i.e., EPEC and EHEC) (7) and EPEC and a mouse pathogen (*C. rodentium*) gives a strong indication that the different *eaeA* gene products are functionally equivalent. Further, Int$_{EPEC}$ can clearly cooperate in vivo with other *C. rodentium* proteins, such as EspB, required for the formation of A/E lesions (9). The *C. rodentium* murine model has limitations for EPEC infection because of the different clinical nature of the diseases caused by EPEC and *C. rodentium*, although A/E lesions appear morphologically identical. However, since the intimins are functionally homologous, this model provides a simple and economical approach to studying the role of intimin and other virulence factors shared between EPEC and *C. rodentium* (21). Further, this model may also provide a simple in vivo system for studying the role of host proteins, using knock-out-mutated mice, in colonic hyperplasia and EPEC disease.

Intimin exchange studies have been performed previously with *E. coli*. Donnenberg et al. (8), using colostrum-fed newborn piglets, found A/E lesions restricted to the terminal ileum and colon when challenged over a 24-h period with wild-type EHEC or EHEC expressing the EPEC-derived intimin. In this model *E. coli*-derived intimins did not confer regional tissue specificity. In contrast, another study (27) showed, using 24-h-old gnotobiotic piglets, that EHEC expressing Int$_{EPEC}$ produced lesions in the distal half of the ileum as well as the colon, whereas a similar EHEC strain expressing EHEC intimin showed minimal attachment. They concluded that differences in receptor-binding domain of the intimin molecules dictate differences in tissue tropism. The data in these papers suggest that host factors, including the endogenous microflora, immune status, and dietary background, could also influence colonization. Supporting this is a report which showed that hyperplasia of cecum and colon was frequently observed in *C. rodentium*-inoculated germ-free mice but was only occasionally found in Swiss NIH mice (3). Schauer and Falkow reported that DBS255 harboring a recombinant plasmid containing the wild-type *C. rodentium eaeA* gene did not colonize the colon of mice after oral inoculation (25). Differences in the level and regulation of expression of the *C. rodentium eaeA* genes encoded on either the chromosome or a plasmid may account for the difference. However, the use of different mouse strains and period of time chosen to assay for colonic colonization may provide an alternative explanation when comparing different sets of data.

Although functionally similar in terms of their ability to bind to HEp-2 cells, the cell-binding carboxy-terminal domains of the different intimins show significantly lower homology than the amino-terminal regions (24). However, two conserved Cys residues are similarly located within this region. In agreement with in vitro binding assays (12) the distal of these Cys residues, Cys-937, is essential for the biological activity of intimin in vivo as mice infected with DBS255(pCVD438CA) were not colonized. Western blot analysis showed no detectable difference in the level of expression of the wild-type and mutated intimins, and our unpublished results show that the mutated protein is expressed on the bacterial cell surface, indicating that the mutation does not interfere with intimin export functions.

Recently it was shown that despite the ability of rabbit-specific EPEC strains to colonize mice, the infection was asymptomatic and no closely adherent bacteria were detected (23). In this study we have shown that EPEC derivatives of E2348/69 could not colonize mice, although intimin derived from this strain could restore the ability of DBS255 to colonize orally challenged mice.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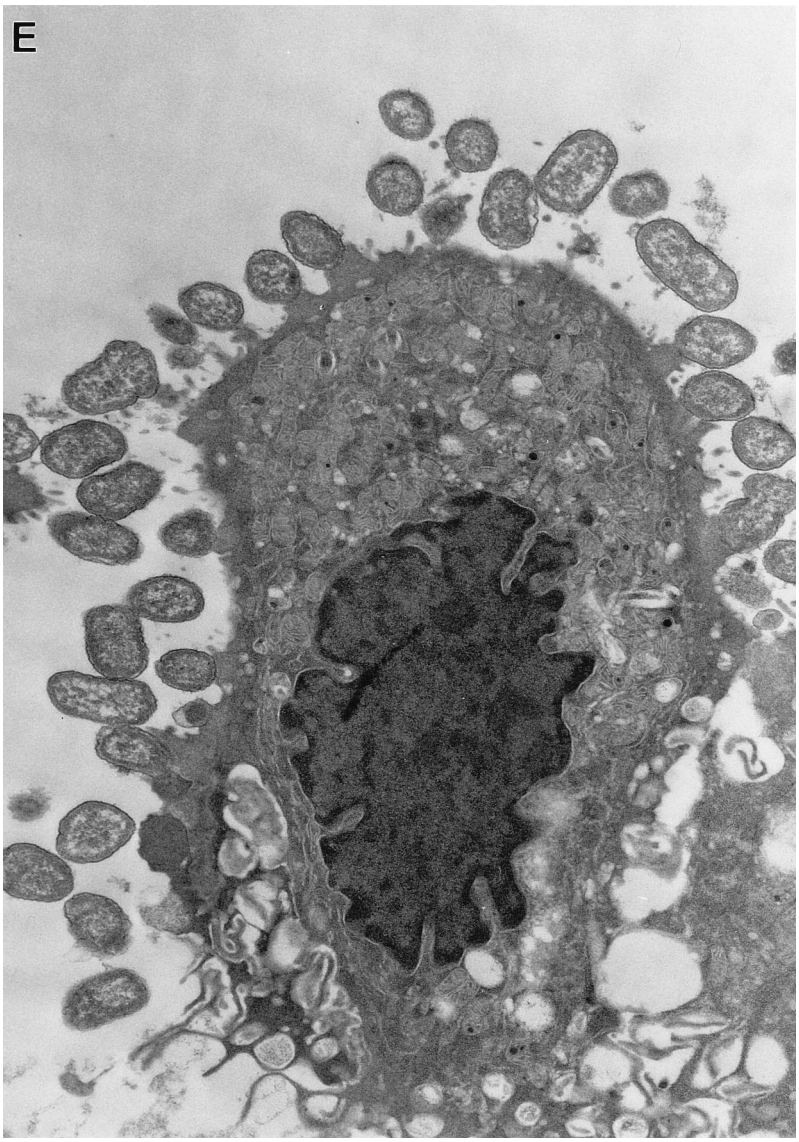


FIG. 5. A mouse inoculated with DBS255 showing normal microvilli on surface epithelium (magnification, ×8,000) (A). Mouse inoculated with wild-type *C. rodentium* (B) showing bacterial attachment with microvillous effacement on surface of epithelial cell with convex apical membrane; note lateral membrane interdigitations (arrowheads) (magnification, ×10,000). (C) As shown in panel B but on cell with concave apical membrane, prominent lateral interdigitations (arrowheads), and detachment from basement membrane (large arrow) (magnification, ×10,000). (D) Attaching effacing lesion on neighboring cells associated with a single adhering wild-type *C. rodentium* bacteria (arrowheads) (magnification, ×28,000). Mouse inoculated with DBS255(pCVD438) (E) showing A/E lesions on the surface of an enterocyte (magnification, ×10,000).

The mechanism of *C. rodentium*-induced colonic hyperplasia is unknown. However, the increased epithelial cell extrusion seen in the infected colon might suggest that direct epithelial cell damage is responsible for the hyperplastic response. Transmission electron microscopy demonstrated that epithelial cells with adhering bacteria showed effacement of microvilli which, along with the convex and concave appearance of the apical membrane, indicate changes to the absorptive capacity of the cell and its cytoskeleton. The loss of contact between the enterocyte and the basement membrane and the perpendicular appearance of the lateral epithelia cell interdigitation could be interpreted to mean that cells are in the process of extrusion. These phenomena suggest that cell damage is a direct result of bacterial attachment. The various orientations of the bacteria on the enterocyte cell membrane and the simultaneous attachment to two epithelial cells indicate the mechanism for forming attaching and effacing lesions is probably evenly distributed on the bacterial surface. The uneven neutrophil response may, in this case, reflect a secondary response to disruption of epithelial integrity. Supporting this is a report that crypt hyperplasia was present without inflammation in early stages of *C. rodentium* infection (3). Alternatively, colonic hyperplasia may result from immune activation; however, evidence for such phenomena must await further evaluation. Nevertheless, the association between the presence of bacteria and crypt hyperplasia indicate that adhesion, and not the simple presence of the bacteria in the gut lumen, is an essential part of the pathology.

Preliminary evidence from EPEC-challenged human volunteers studies suggests that a 94-kDa outer membrane protein (presumably intimin) is highly immunogenic and may confirm

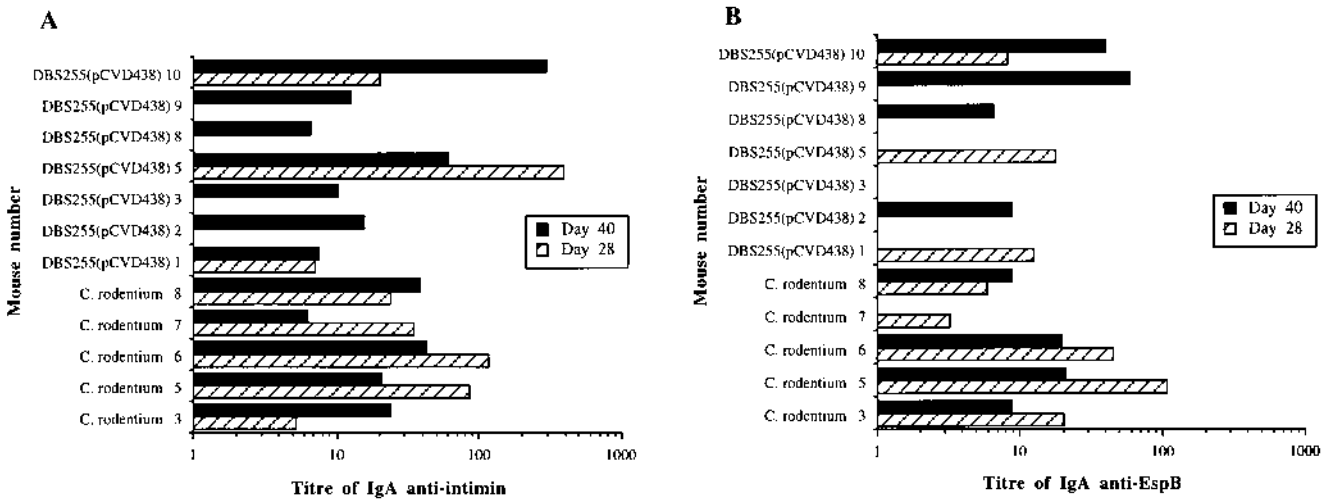


FIG. 6. IgA responses to intimin (A) and EspB (B) in fecal pellets obtained from mice challenged with wild-type *C. rodentium* or DBS255(pCVD438) strains. IgA against intimin was detected in supernatants collected from all the mice challenged with either of the virulent strains, while all but one of the mice responded to EspB. In some of the responding mice, IgA to both intimin and EspB was detected at both 28 and 40 days postchallenge; in others, antibodies were detected either at the 28-day or the 40-day point.

protective immunity (20). All the affected volunteers in this study mounted serum IgA and IgG responses to the 94-kDa protein and also contained antibodies directed against the EPEC-secreted proteins (14). In this study we demonstrated the presence of IgA in fecal pellets obtained from mice challenged with virulent *C. rodentium* derivatives, reactive against intimin and EspB. We found that in all but one mouse tested that survived the challenge with either wild-type or DBS255(pCVD438) to day 40, detectable IgA responses to both proteins were seen while no IgA response was detected in DBS255-challenged mice. This result shows that colonic colonization is a prerequisite for the mounting of this mucosal immune response, since DBS255 contains an intact *espB* gene. In any of the mice challenged with either of the virulent strains, the IgA response was not apparent at 16 days postchallenge. In all the mice challenged with the wild-type strain, IgA to intimin was detected 28 days postchallenge, compared with only three of the seven mice challenged with DBS255(pCVD438). Early response to intimin coincided with an early response against EspB. It is conceivable that in those mice who survived the challenge with the virulent strains, any IgA response at day 16 (and earlier) was too low to detect but sufficient to confer protection. It is possible that mice showing low levels of colonic colonization (Table 2) are in the process of clearing the infection while those with higher bacterial counts may result in lethal infection. Although mice challenged with the wild-type *C. rodentium* developed an early IgA response to intimin and EspB, infection by this strain appeared to be overall more virulent as mice died more quickly and in greater numbers than mice infected with DBS255(pCVD438). The reason for the variations in the immune responses and mortality are not yet clear; however, they might be attributed to detectable differences in the level of intimin expression between the wild-type *C. rodentium*- and DBS255(pCVD438)-infected mice. In general, the ELISA results show not only that intimin and EspB are both immunogenic in the *C. rodentium* mouse model, but also that intimin and EspB from EPEC and *C. rodentium* contain cross-reactive epitopes.

In conclusion, we have shown that the $Int_{EPEC}$ gene can functionally substitute for $Int_{CR}$ in vivo in a murine infection model. This simple model should facilitate future studies on the in vivo role of EPEC-derived proteins in pathogenesis and allow studies to be undertaken on assessing the potential of these EPEC proteins as future vaccine components.

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