INFECTION AND IMMUNITY, Oct. 1992, p. 3953–3961
0019-9567/92/103953-09$02.00/0

Vol. 60, No. 10

MINIREVIEW

Enteropathogenic *Escherichia coli*

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INTRODUCTION

Organism. *Escherichia coli* strains currently recognized to cause human diarrhea can be distinguished on the basis pof pathogenic mechanisms and separated into five categories: enterotoxigenic, enteroinvasive, enterohemorrhagic (EHEC), enteroaggregative, and enteropathogenic (EPEC). Additionally, strains exhibiting diffuse adherence to tissue culture cells have been proposed to be diarrheagenic but, unlike members of the other categories, have not yet been consistently shown in epidemiologic (36, 38) or challenge (91) studies to be pathogenic. Despite the fact that EPEC strains were the *E. coli* organisms recognized as causing diarrhea, understanding of the pathogenic mechanisms of EPEC disease initially lagged behind that of some of the other categories of diarrheagenic *E. coli*. EPEC strains have long been defined by exclusion as those *E. coli* strains of serotypes epidemiologically linked to diarrheal disease that do not belong to any of the other categories. However, as our understanding of EPEC pathogenesis has improved, it has become apparent that serotyping is an outmoded method of identifying EPEC. It has recently been appreciated that EPEC strains represent a group of clonally derived organisms that may be more related to each other than to other *E. coli* organisms with the same O-antigen type (7, 72, 90). In addition, organisms which do not belong to traditional EPEC serotypes but possess putative virulence traits characteristic of EPEC have been cultured from children with diarrhea and may be pathogenic (52). Conversely, mere membership within an enteropathogenic serotype does not guarantee virulence. It has now become more appropriate to define EPEC on the basis of unique genetic determinants that encode characteristic pathogenic properties. In light of increased knowledge regarding these genetic determinants, it is no longer necessary to distinguish among classes of EPEC (69). Similarly, since the diffuse adhering and enteroaggregative *E. coli* strains lack the major putative virulence determinants described for classic enteropathogenic strains and differ in their epidemiologic features, they should not be grouped in the same category as EPEC. The purpose of this review is to summarize recent advances in our understanding of the genetic foundation of EPEC pathogenesis that allow us to describe EPEC strains, on the basis of their characteristic virulence determinants, as a unique and fascinating category of diarrheal pathogens.

Early history. Credit for the first epidemiologic description of the diarrheagenic potential of *E. coli* is often given to John Bray (9), although prior reports exist (for more extensive reviews of early EPEC history, see references 56 and 79). Investigators in the 1940s and 1950s demonstrated that *E. coli* from nosocomial and community outbreaks of neonatal diarrhea could be agglutinated by rabbit antiserum that did not react with *E. coli* from control infants. These outbreaks had extraordinary mortality rates often exceeding 50% (9, 34, 93). With the advent of the serotyping system of Kauffmann (48), the EPEC strains were defined as strains of somatic (O) and flagellar (H) antigen types epidemiologically incriminated in these outbreaks. Improved hospital practices led to the gradual elimination of neonatal diarrhea caused by EPEC from maternity wards of developed countries. Once the enterotoxigenic and enteroinvasive strains of *E. coli* were defined, it was decided that EPEC strains belonged to neither of these categories. Some investigators questioned EPEC pathogenicity or postulated that EPEC had lost plasmids necessary for disease (37, 82). These doubts were allayed by volunteer studies conducted by Levine et al. demonstrating that EPEC strains that were neither enterotoxigenic nor enteroinvasive caused diarrhea (55).

Current epidemiology. Nosocomial neonatal diarrhea due to EPEC, although now rare in developed countries, still occurs in developing nations and still has high mortality (86). In addition, diarrheal disease due to EPEC has been reported in child-care settings in the United States (8, 73). However, EPEC seems to take its greatest toll on infants in the community setting in developing countries. Reports within the past 5 years from Chile, Mexico, Brazil, Thailand, China, and Yugoslavia emphasize the continued importance of EPEC on a global scale (15, 17, 28, 38, 47, 58). EPEC has been reported to be the principal cause of infant diarrhea in locations such as São Paulo, Brazil, and Johannesburg, South Africa, exceeding even rotavirus in some studies (38, 78). EPEC causes disease in the very young, rarely affecting children over 1 year of age and most closely associated with diarrhea in those under 6 months (39, 56). In addition, EPEC infection is a recognized cause of chronic diarrhea (8, 14, 29, 41, 73, 81, 98).

PATHOGENESIS OF EPEC INFECTION

Localized adherence. Shortly after Levine et al. reestablished the virulence of EPEC (55), Cravioto et al. showed that unlike many *E. coli* strains, EPEC strains are able to adhere to epithelial cells in vitro (16). Rather than uniformly covering the cells, EPEC strains produce distinct microcolonies in a pattern that has been called localized adherence (85). Localized adherence is highly correlated with specific EPEC serotypes in strains isolated from patients with diarrhea (84). Baldini et al. reported that localized adherence in E2348/69, an O127:H6 EPEC strain, is associated with the presence of a 60-MDa plasmid denoted pMAR2 (3). Curing

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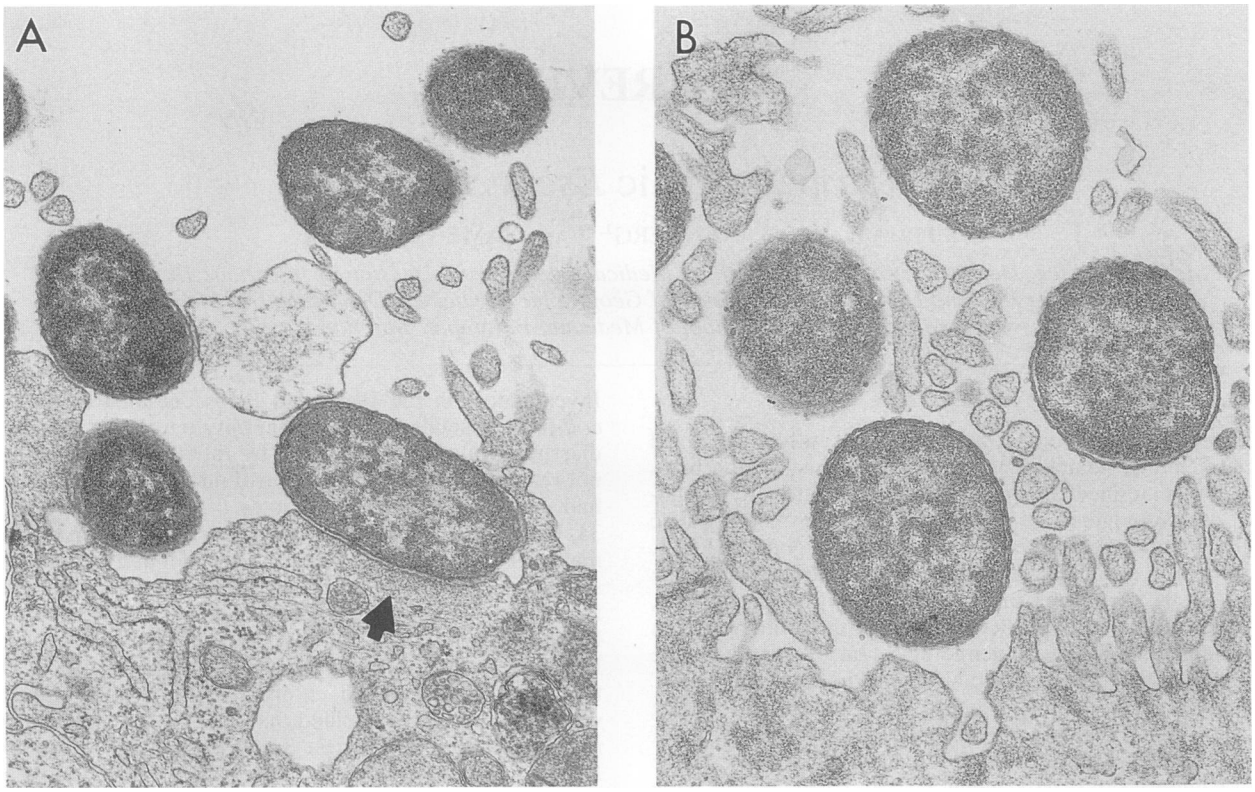

FIG. 1. Caco-2 cells infected with wild-type EPEC E2348/69 (A) or isogenic *eaeA* deletion mutant CVD206 (B). Note the bizarre thickened, vesiculated, and even branching microvilli seen with both strains. The arrow shows the site of intimate attachment and cytoskeletal disruption.

pMAR2 results in the loss of localized adherence, a phenomenon confirmed by others with different EPEC strains (65). Moreover, transfer of the plasmid to HB101, a nonadherent laboratory *E. coli* strain, confers adherence to HEp-2 cells (3), although adherence of HB101 transformed by using this plasmid is poor in comparison to that of EPEC (23, 65). Furthermore, *E. coli* strains capable of localized adherence which have been isolated during outbreaks of infantile gastroenteritis and from children with diarrhea almost invariably possess 55- to 70-MDa "EPEC adherence factor" (EAF) plasmids (71) that share significant regions of DNA homology (70). The EAF DNA probe, developed from an uncharacterized region of pMAR2 necessary for localized adherence (4), correlates well with the localized adherence phenotype and has been used extensively in epidemiologic studies (28, 38, 47, 58, 69). Further supporting the role of the EAF plasmid in pathogenesis, Levine et al. reported that MAR20, an EAF plasmid-cured strain, is significantly less pathogenic in volunteers than E2348/69, the parental strain from which it was derived (57).

The molecular nature of localized adherence is not yet understood. Recent reports have described inhibition of localized adherence by linoleic acid (13) and by factors present in both the carbohydrate and immunoglobulin fractions of breast milk (18). Candidates for the adhesin involved have been proposed (57, 83) and rebuffed (12, 44). Girón et al. have recently described a bundle-forming pilus (BFP) purified from EPEC O111:NM strain B171 after passage on solid medium containing sheep blood (35). These fimbriae tend to aggregate, forming bundles that can be visualized by transmission electron microscopy. Furthermore, expression of the fimbrial gene product is induced in tissue culture medium, and under these conditions the bacteria tend to clump (99). It has not yet been determined whether the BFPs are produced in vivo. The fimbriae have been visualized in several EPEC strains but never in strains cured of the EAF plasmid (35). The N-terminal amino acid sequence of the major fimbrial protein resembles that of the *Vibrio cholerae* toxin-coregulated pilus and members of the type IV fimbrial family (87). However, like the toxin-coregulated pilus, BFP lacks an *N*-methylphenylalanine at its amino terminus.

Attaching and effacing. Even before the localized pattern of EPEC adherence was appreciated, the unique effect of EPEC on epithelial cells at the ultrastructural level had been reported (74, 89). Numerous reports have confirmed that EPEC strains are capable of inducing profound cytoskeletal alterations in epithelial cells in animal models (67, 96), tissue culture models (45, 49), and indeed in humans infected with EPEC (81, 92, 98). Affected enterocytes exhibit a dramatic loss of microvilli and a rearrangement of cytoskeletal elements, with a proliferation of filamentous actin beneath areas of intimate bacterial attachment (50) (Fig. 1A). This effect has been termed attaching and effacing (67) and is characteristic of EPEC. Similar effects have also been described for animal models of EHEC infection (33, 97), for an isolate of *Hafnia alvei* from a child with diarrhea (1), and for murine colonic hyperplasia caused by *Citrobacter freundii* (46). Clinical *E. coli* isolates that display diffuse adherence or aggregative adherence do not exhibit attaching and effacing activity (52), supporting the concept that they are not EPEC. Recently Knutton et al. have developed a highly specific method for identifying *E. coli* organisms that demonstrate

attaching and effacing activity (50). The fluorescent-actin staining test uses fluorescein isothiocyanate (FITC)-conjugated phalloidin, which specifically binds to filamentous actin, to identify the high concentrations of actin in epithelial cells directly beneath areas of bacterial attachment. In addition to actin, myosin (62), α-actinin, talin, and ezrin have been identified beneath attaching and effacing EPEC (30). The last three components are of special interest because of their putative roles in linking cytoskeletal proteins such as actin to transmembrane proteins such as integrins (10, 75). In contrast to localized adherence, attaching and effacing occur in the absence of the EAF plasmid, albeit in reduced numbers and with delayed kinetics (51, 95). However, two groups have reported the transfer of attaching and effacing ability with large plasmids from an EPEC (31) and an EHEC (94) strain.

Two-stage model of EPEC pathogenesis. The separation of localized adherence from the attaching and effacing effect, the former associated with the EAF plasmid and the latter usually not, led to the proposal of a two-stage model of EPEC infection (51). According to this model, initial (localized) adherence, in which the bacterium adheres at some distance from microvilli, is mediated by the EPEC adherence factor encoded on the plasmid. Subsequently, chromosome-encoded loci allow the organism to adhere intimately to the epithelial membrane (an approximately 10-nm distance; Fig. 1A), destroy microvilli and exert profound effects on the epithelial cytoskeleton. A similar model had earlier been proposed for RDEC-1 (11), a rabbit attaching and effacing diarrheagenic *E. coli* strain that possesses its own plasmid-mediated fimbrial adhesin (100).

Invasion. EPEC organisms had long been considered to be noninvasive, principally because they do not cause keratoconjunctivitis in guinea pigs, a property associated with invasion by enteroinvasive *E. coli*, *Shigella*, and *Yersinia* organisms (37, 54, 55). However, many published electron micrographs, including clinical specimens, appear to demonstrate intracellular bacteria (67, 74, 89, 96, 98). Recently, several investigators have challenged the traditional notion, publishing descriptive (2, 66) and quantitative (20, 32) studies demonstrating that EPEC strains are capable of efficient invasion of a variety of epithelial cell types. EPEC invasion in vitro can be inhibited by cytochalasins that block host cell microfilaments and, in contrast to invasion by many other invasive pathogens, by compounds that block host cell microtubules (21, 32). Like attaching and effacing activity, invasion is augmented by the presence of the EAF plasmid (20, 32). However, for one EPEC strain whose EAF plasmid also encodes O-antigen specificity, the plasmid-cured rough strain invades better than the parent (77). The clinical significance of invasion in the pathogenesis of EPEC infection is not clear. EPEC strains do not cause dysentery or typhoidal syndromes. However, it had been noted long ago that EPEC can disseminate late in infection (26, 34), and, although it must be extremely rare, sepsis due to EPEC has been reported (8).

EPEC-induced signal transduction in epithelial cells. While much progress has been made in describing the adherence of EPEC to epithelial cells, little is known about the events leading to fluid secretion and diarrhea. The loss of microvilli induced by EPEC infection could lead to malabsorption and osmotic diarrhea, but malabsorption alone does not explain the impressively short (4 to 12 h) incubation period seen in experimental human EPEC infection (25, 55). Recently, several investigators have studied the effect of EPEC on eukaryotic second messengers. There is no evidence that EPEC affects cyclic nucleotide levels (59). However, two groups have found that EPEC induces elevations of intracellular calcium (6, 27). Baldwin et al. found that this increase was inhibited by dantrolene, suggesting that the calcium was released from intracellular stores (6). Dytoc et al. found that buffering intracellular calcium prevented formation of attaching and effacing lesions (27).

Three different groups using different methods have identified different host cell proteins that become phosphorylated during EPEC infection in vitro. Baldwin et al. have found that after EPEC infection of HEp-2 cells, eukaryotic proteins with apparent molecular masses of 21 and 27 kDa become phosphorylated (5). Similar proteins are phosphorylated by activators of protein kinase C, suggesting that this regulatory protein might be involved. The lower-molecular-mass phosphoprotein was recently reported to be a myosin light chain (61). Riley et al. report phosphorylation of proteins of 130 and 100 kDa upon infection of HeLa cells with EPEC, EHEC, or enteroinvasive *E. coli* (76). By immunoprecipitation, these proteins were identified as vinculin and α-actinin. Finally, Rosenshine et al., by using a monoclonal antibody against phosphotyrosine, have identified a 90-kDa HeLa cell protein that becomes phosphorylated upon infection with EPEC (80). These authors have made the additional functional observation that EPEC invasion is blocked by inhibitors of tyrosine kinases. In addition, by using the same monoclonal antibody for fluorescence microscopy, they demonstrate that EPEC organisms become surrounded by proteins that are phosphorylated at tyrosine residues upon infection of epithelial cells.

These observations have led to the hypothesis that protein phosphorylation and increases in intracellular calcium are responsible for many of the ultrastructural changes seen in epithelial cells infected with EPEC. Baldwin et al. speculate that EPEC might activate phospholipase C, leading to cleavage of phosphatidyl inositol into inositol triphosphate and diacyl glycerol (6). Inositol triphosphate causes release of calcium from intracellular stores, and diacyl glycerol activates protein kinase C. Elevated calcium concentrations cause microvilli to vesiculate as actin is cleaved within the microvillous core (64). The putative phospholipase enzyme could be either produced by the bacterium or activated in the epithelial cell. However, we have been unable to demonstrate bacterial phospholipase activity in EPEC (23).

While elevated intracellular calcium levels induced by EPEC may lead to actin disassembly, actin assembly also seems to play a role in EPEC pathogenesis. Cytochalasins, which inhibit actin polymerization, prevent EPEC invasion (21, 32), and actin accumulates underneath EPEC as part of attaching and effacing lesions. It appears, therefore, that EPEC organisms are able to subvert the host cell cytoskeleton, disrupting the normal architecture and inducing components to interact preferentially with the bacterium.

GENETIC BASIS OF EPEC VIRULENCE

***eaeA* gene.** The transposon Tn*5* IS50$_L$::*phoA* (Tn*phoA*), which can create active alkaline phosphatase fusion proteins if inserted in genes with signal sequences (63), has been used to identify genes encoding exported proteins potentially involved in EPEC pathogenesis. In one study, Jerse et al. screened 99 Tn*phoA* mutants of a plasmid-cured derivative of virulent O127:H6 EPEC strain E2348/69 for the ability to induce filamentous actin accumulation in HEp-2 cells (45). Three of 99 inserts were negative in this assay. One mutant was selected for further study and found to be unable to

attach intimately to epithelial cells. An oligonucleotide probe derived from the nucleotide sequence adjacent to the transposon insertion in this mutant was used to select a hybridizing clone from a cosmid gene bank. The gene interrupted by Tn*phoA* was originally designated *eae* for *E. coli* attaching and effacing but will be referred to here as *eaeA* (see below). A gene probe containing an internal 1-kb fragment of the *eaeA* gene was 100% sensitive and 98% specific for EPEC strains that induce actin accumulation and also recognized 24 of 25 EHEC strains (45). The *eaeA* gene has not been found on plasmids in any of these strains (43). The *eaeA* gene from EHEC strain EDL933 has also been cloned and sequenced and is 86 and 83% identical to the EPEC locus at the nucleotide and predicted amino acid sequence levels, respectively (101). The predicted products of both genes are remarkably similar (approximately one-third identical and one-half similar amino acids) to the invasins of *Yersinia pseudotuberculosis* and *Yersinia enterocolitica*. The invasin proteins mediate invasion by *Yersinia* organisms after high-affinity binding to members of the β_1 class of integrin receptors on epithelial cells (42). Interestingly, this family of *E. coli* and *Yersinia* outer membrane proteins displays the most sequence divergence toward their C termini, the region proposed to mediate binding of the *Y. pseudotuberculosis* protein to its receptors (53).

The product of the *eaeA* gene is a 94-kDa outer membrane protein, henceforth designated intimin, which is recognized by sera from convalescent volunteers after experimental EPEC infection (44). This protein was previously thought to be a product of the EAF plasmid, because it was consistently observed in plasmid-containing but not plasmid-cured strains of EPEC (57, 65). However, this product has never been detected in laboratory strains into which the EAF plasmid was transferred (65). Jerse and Kaper have determined that the presence of the EAF plasmid serves to enhance intimin production but that plasmid-cured EPEC strains produce low but detectable levels of the protein (44). Recently, Gómez and Kaper have cloned two loci from the EAF plasmid that enhance expression of the chromosomal *eaeA* gene (40). The nucleotide sequence of one gene, tentatively designated *per* for plasmid-encoded regulator, is related to that of *envY*, a gene involved in thermoregulation of porin expression (60). Sherman et al. have recently reported that antibodies raised against a 94-kDa EHEC outer membrane protein block EHEC adherence to epithelial cells and the ability of EHEC to induce epithelial cell actin accumulation (88). Since EHEC strains possess an *eaeA* homolog, this inhibition may be due to blocking of the EHEC intimin. However, it has not yet been determined whether the EHEC 94-kDa protein is the product of the EHEC *eaeA* gene. Nonetheless, these data, combined with the sequence similarities among invasins and intimins, suggest a possible role for intimin in adherence to epithelial cells. While it is clear that intimin is necessary for intimate adherence, definitive experiments to determine whether intimin is the actual adhesin involved, rather than acting indirectly in the process, await the purification of intimin. An alternative hypothesis is that intimin could transmit to the epithelial cell a signal that stimulates the epithelial cell to recognize another EPEC surface molecule, which in turn acts as the intimate adhesin.

We have recently constructed an isogenic *eaeA* deletion mutant of EPEC strain E2348/69 (24). When tested in a randomized, double-blind clinical trial, 4 of 11 adult volunteers who received the deletion mutant developed diarrhea, in contrast to 11 of 11 who received the wild-type strain ($P = 0.004$) (25). This result unambiguously distinguishes *eaeA* as the first virulence gene to be confirmed in EPEC. Conversely, the occurrence of illness in several recipients of the *eaeA* deletion mutant indicates that intimin is not the sole mediator of EPEC disease.

Noninvasive Tn*phoA* mutants. Since genes encoding exported products necessary for invasion might include loci involved in localized adherence and attaching and effacing, Tn*phoA* mutagenesis has been applied to the plasmid-containing parental E2348/69 EPEC strain in an effort to identify noninvasive mutants (19). Of 329 mutants screened, 22 were found to be deficient in HEp-2 cell invasion. Southern hybridization and restriction endonuclease mapping of cloned fragments containing transposon insertions indicated that the mutants could be classified into five categories. These categories correlate well with phenotypic properties, including localized adherence, intimate attachment, and cytoskeletal disruption.

(i) The *eae* gene cluster. Seven noninvasive Tn*phoA* mutants were found to have insertions on a single 13.8-kbp chromosomal *Mlu*I fragment that includes the *eaeA* gene. Thus, this region of the EPEC chromosome has been identified by two independent approaches. While five mutants have insertions within the *eaeA* gene, suggesting that intimin is necessary for invasion as well as intimate attachment, two have insertions downstream of *eaeA*. Like *eaeA* mutants, mutants with Tn*phoA* insertions downstream of *eaeA* are unable to attach intimately to epithelial cells. Unlike *eaeA* mutants, however, they produce intimin and export it to the outer membrane (23). Although both of these mutants have two Tn*phoA* insertions, restoration of attaching and effacing activity is achieved in each by introduction of plasmids containing cloned regions 3′ of *eaeA*, indicating that in both cases the Tn*phoA* insertion in the *eae* region is the mutation responsible for the loss of activity. Complementation studies have shown that the gene(s) affected in these mutants is distinct from *eaeA*, thereby indicating the presence in EPEC of an *eae* gene cluster devoted to intimate adherence. Thus, we refer to the locus encoding intimin as *eaeA* and to subsequently defined genes as *eaeB*, etc. The exact defects in mutants at these additional loci are currently under investigation.

(ii) An EPEC plasmid locus encoding a fimbria involved in localized adherence. A second category consists of seven noninvasive Tn*phoA* mutants with insertions in the EAF plasmid rendering them incapable of localized adherence. We have recently found that all seven have insertions in a single gene and that this gene encodes the major BFP protein reported by Girón et al. (35). Nucleotide sequencing of the fusion junctions reveals a 579-bp open reading frame, tentatively designated *bfpA* (22). The predicted amino acid sequence for 26 of 28 codons near the 5′ end is identical to the N-terminal sequence of the BFP protein. Using this point as the amino terminus, *bfpA* would encode a mature protein of 180 amino acids with an M_r of 18,730, which is in excellent agreement with the estimate of 18,500 to 19,500 obtained by electrophoretic mobility (35, 99). The lack of localized adherence of the *bfpA*::Tn*phoA* mutants together with the tendency of these fimbriae to aggregate suggest a mechanism for bacterium-bacterium and bacterium-host cell adherence that could result in the localized microcolonies characteristic of EPEC. Thus, this EPEC plasmid-encoded fimbrial locus is the best candidate yet described for the elusive EPEC adherence factor originally proposed by Cravioto et al. (16). Of course, the *bfpA* product itself may not be the actual adhesin. Indeed, other loci on the EAF plasmid and the

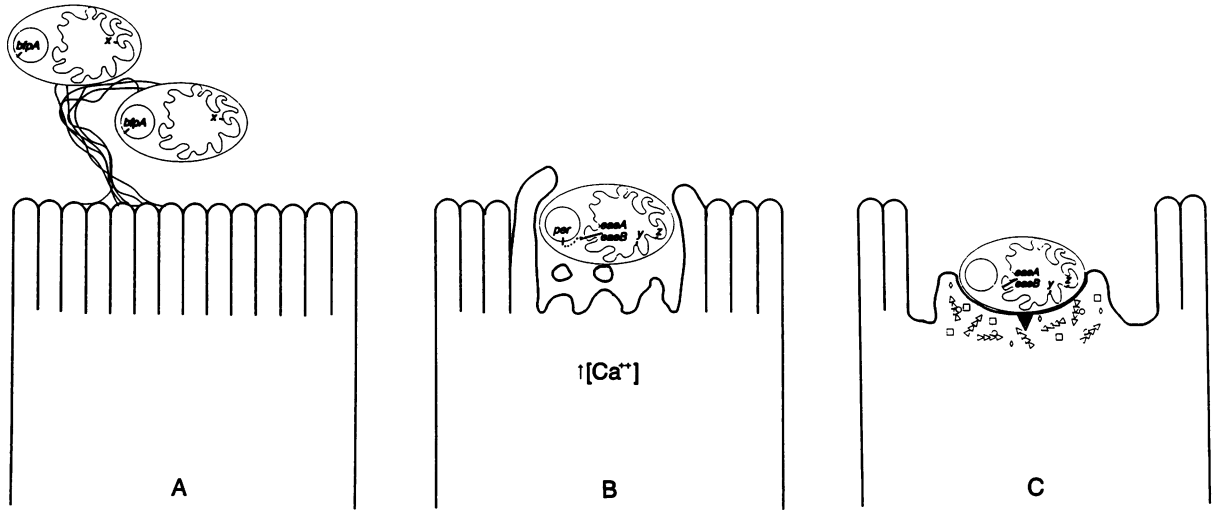

FIG. 2. Three-stage model of EPEC pathogenesis. Localized adherence (A), the initial interaction between the bacterium and the epithelial cell, is mediated by BFPs but involves additional chromosomal (gene *x*) and plasmid loci. In the second stage of infection (B), chromosomal genes (*y* and *z*) initiate a signal transduction event that results in increased intracellular calcium levels and effacement of microvilli. Simultaneously, the *eae* gene cluster is activated by the product of the plasmid *per* locus. The third stage of infection (C) results when intimin (solid triangle), the product of the *eaeA* locus, and other products of the *eae* gene cluster mediate close attachment to the epithelial cell. From this proximity, effects on the epithelial cell are amplified with accumulation of filamentous actin and other cytoskeletal proteins (geometric shapes).

EPEC chromosome (see below) are also necessary for localized adherence. The *bfpA* gene maps at some distance from the EAF probe (70), and the relationship between these loci is not yet clear. Since localized adherence is inducible (99), it follows that additional loci must be involved in the production of BFP. Indeed, Nataro described six regions of the EAF plasmid necessary for localized adherence (68); *bfpA* is located within one of these regions.

(iii) Other EPEC loci potentially involved in virulence. The isolation of noninvasive Tn*phoA* mutants of EPEC has yielded three additional categories with interesting properties. Two mutants have closely linked chromosomal Tn*phoA* insertions and have lost the ability to perform localized adherence. This implies that chromosomal genes as well as loci residing on the EAF plasmid are necessary for localized adherence and is consistent with the finding by some investigators that acquisition of the EAF plasmid by laboratory *E. coli* strains does not always confer the localized adherence phenotype (65).

Four additional Tn*phoA* mutants with unrelated insertions display normal localized adherence and attaching and effacing activity but are still impaired in the gentamicin invasion assay. These findings suggest that invasion or intracellular survival requires processes in addition to those responsible for localized adherence and attaching and effacing.

The final class of noninvasive Tn*phoA* mutants includes two that lack the ability to induce the accumulation of filamentous actin in epithelial cells beneath areas of intimate adherence. These mutants, which have Tn*phoA* insertions not obviously linked to the *eae* gene cluster or to each other, are proficient in localized adherence and intimate attachment to epithelial cells. Yet no disruption of the underlying epithelial cell cytoskeleton is seen by electron microscopy (23), and no induction of filamentous actin accumulation is detected with FITC-phalloidin (19). Furthermore, these mutants fail to induce the host cell tyrosine kinase activity seen with wild-type EPEC (80). Therefore, they seem to be defective in the ability to transduce a signal to epithelial cells that results in characteristic cytoskeletal effects. Interestingly, mutations in the *eae* gene cluster, although rendering the organism unable to attach intimately to epithelial cells, leave the residual ability to induce a faint "shadow" of filamentous actin accumulation detected with FITC-phalloidin (19, 45). In addition, *eaeA* mutants retain the ability to induce the tyrosine phosphorylation of a 90-kDa host cell protein (80). This cytoskeleton-altering activity and tyrosine kinase induction correlates with the residual ability of *eaeA* mutants to damage microvilli (Fig. 1B) (32) and the residual diarrhea seen with volunteers who ingested the *eaeA* deletion mutant strain (25). Thus, the *eae* gene cluster alone is not responsible for all of the changes seen in attaching and effacing but is necessary for intimate attachment and only augments the cytoskeletal changes induced by other, as yet uncharacterized loci. When HEp-2 cells are coinfected with the *eaeA* deletion mutant and either mutant deficient in signal transduction, complementation occurs and near-wild-type levels of invasion are restored (80). Interestingly, however, this complementation is unidirectional: only the signal transduction deficient mutants invade, and the *eaeA* mutant remains extracellular. Presumably, the failure of the *eaeA* mutant to attach intimately to the epithelial cell precludes subsequent invasion, while the signal transduction mutant lies in intimate association with the epithelial cell, poised to enter when the correct signal is sent by the *eaeA* mutant.

THREE-STAGE MODEL OF EPEC PATHOGENESIS

The genetic approach to EPEC pathogenesis has yielded sufficient information to justify a reevaluation of the two-stage model. The sequence similarities between intimin and invasin and the lack of intimate adherence of *eaeA* mutants to epithelial cells suggest that intimate adherence may be mediated by *eaeA* and other genes located immediately downstream of this locus. However, the *eae* gene cluster is not solely responsible for the attaching and effacing effect or

for virulence. A cosmid clone including the entire *eae* gene cluster does not induce actin accumulation in epithelial cells (45). Furthermore, the *eaeA* mutants retain the ability to induce protein phosphorylation, alter the epithelial cytoskeleton, and damage microvilli. In contrast, mutants at other chromosomal loci, while retaining intimate adherence, are devoid of cytoskeleton-altering activity and fail to induce host cell protein tyrosine phosphorylation. Thus, intimate adherence (attaching) and cytoskeletal disruption (microvillus effacement) can now be viewed as separate phenomena and EPEC pathogenesis can be seen as encompassing three stages (Fig. 2). The following theoretical framework is based on these observations.

Early in EPEC infection, the bacteria adhere to microvilli and to each other probably by virtue of BFPs encoded by the *bfpA* locus on the large EPEC EAF plasmid (Fig. 2A). Additional plasmid and chromosomal (gene *x*) loci are also necessary for initial localized adherence. In the second stage (Fig. 2B), other, as yet uncharacterized chromosomal loci, represented by two Tn*phoA* insertions (genes *y* and *z*), initiate signal transduction leading to protein tyrosine phosphorylation, elevation of intracellular calcium concentrations, early cytoskeletal damage, effacement of microvilli, and fluid secretion. As infection proceeds, the *eae* gene cluster is activated by *per* and an additional plasmid-encoded regulator(s). Intimin then allows the bacterium to become intimately attached to the epithelial cell membrane (Fig. 2C). From this proximity, the cytoskeletal effects are amplified, resulting in profound accumulation of actin, myosin, α-actinin, talin, and ezrin beneath the adherent microorganisms, with formation of cuplike pedestals upon which the bacteria rest. A subset of these organisms can then invade the epithelial cell.

Much needs to be done to substantiate or refute this model and to fill in gaps in the picture before a comprehensive understanding of EPEC pathogenesis emerges. Undoubtedly, many EPEC virulence genes remain to be identified. The precise nature of the epithelial cell response to EPEC infection needs to be investigated, and the pathophysiology that leads to fluid secretion and diarrhea needs to be elucidated. A better understanding of EPEC pathogenesis should lead to insights into normal and abnormal intestinal physiologies and to strategies for the prevention and treatment of diarrheal disease. With the accelerating progress made in the 14 years since the virulence of EPEC was reestablished (55), these goals seem attainable.

ACKNOWLEDGMENTS

We are grateful to Myron M. Levine and James P. Nataro for critical review and to Karen Gicquelais, Jorge Girón, Oscar Gómez, Ann Jerse, and Yu Jun for many helpful discussions.

This work was supported by Public Health Service awards AI32074 and AI21657 from the National Institutes of Health and by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs.

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