CLINICAL MICROBIOLOGY REVIEWS, Apr. 1994, p. 152–173
0893-8512/94/$04.00+0

Vol. 7, No. 2

Adhesion and Its Role in the Virulence of Enteropathogenic *Escherichia coli*

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INTRODUCTION AND OVERVIEW

Historical Aspects

Escherichia coli is the major constituent of the human aerobic fecal flora. Despite its occurrence as a commensal in the intestine, it was proposed that certain *E. coli* strains may be responsible for outbreaks of infantile diarrhea (163). Although evidence accrued to support this hypothesis, acceptance of the role of *E. coli* as an enteric pathogen came in 1945 when Bray demonstrated that a distinct serological strain of *E. coli* was responsible for an outbreak of infantile diarrhea (29). When a serotyping scheme for *E. coli* was developed, Bray's strain was shown to belong to serogroup O111. Epidemiological and microbiological studies in many countries followed, and *E. coli* isolates belonging to a number of O serogroups were implicated in outbreaks of infantile diarrhea. By the mid-1950s, at least 13 *E. coli* O serogroups were considered to be causative agents of the disease (124, 163, 171). Not all *E. coli* isolates within these O serogroups are pathogenic (126, 206); certain O:H serotypes in each O serogroup are associated with disease (124, 171, 210). A clonal theory has been proposed as a reason for the restriction of pathogenicity to certain O:H types (87, 153, 195). The *E. coli* serogroups and serotypes traditionally associated with infantile diarrhea are shown in Table 1. Evidence will be presented in this review to show that pathogenicity is not restricted to serogroup or serotype.

The term enteropathogenic *E. coli* (EPEC) was first used by Neter et al. in 1955 to describe *E. coli* isolates epidemiologically linked to infantile diarrhea (149). Detection and identification of EPEC isolates in laboratories were, and still are, carried out by slide agglutination tests, using O-group antisera on *E. coli* colonies (124, 171). Ideally, the isolation of an EPEC organism should be confirmed by tube agglutination tests for the O antigen followed by H-antigen determination (126, 142, 171, 210); in practice, these tests are rarely performed.

Early attempts to clarify the pathogenicity of EPEC involved feeding strains to adult volunteers. The pathogenic potential of EPEC was confirmed by showing that strains isolated from outbreaks were able to produce diarrhea when fed to these volunteers (66, 105, 213). However, attempts to examine pathogenicity in animal models showed variable results (121, 124). The lack of suitable animal models for human EPEC infection and reliable in vitro tests to demonstrate pathogenicity prevented the confirmation of EPEC isolates as being truly

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TABLE 1. Serogroups and serotypes of *E. coli* associated with infantile diarrhea

Serogroup	Serotype(s)
O18	O18:H7, O18:H14
O20	O20:H-, O20:H26
O26[a]	O26:H-, O26:H11
O28	O28:H-
O44	O44:H18, O44:H34
O55[a]	O55:H-, O55:H6, O55:H7
O86	O86:H34
O111[a]	O111:H-, O111:H2, O111:H12, O111:H5, O111:H7
O112	O112:H-
O114	O114:H2
O119[a]	O119:H6
O124	O124:H-, O124:H30
O125	O125:H21
O126	O126:H-, O126:H27
O127[a]	O127:H-, O127:H6, O127:H21
O128[a]	O128:H2, O128:H7, O128:H12
O142	O142:H6
O158	O158:H23

[a] Most common serogroups associated with disease.

virulent. For many years, serogrouping remained the only means of identifying such isolates and distinguishing them from nonpathogenic *E. coli* (124).

In some studies, strains belonging to EPEC O serogroups did not appear to be associated with disease (74, 155). Although the serotyping methods in some of these studies were inadequate, as H types were not confirmed and in some cases full O-antigen determination was not performed (91), these reports led to confusion as to the role of EPEC in disease. At the same time, microbiologists became aware of *E. coli* strains that produced potent enterotoxins (enterotoxigenic *E. coli* [ETEC]) or possessed invasive properties (enteroinvasive *E. coli* [EIEC]) (163). Confusion arose as to the clinical significance of EPEC strains because such properties could not be detected in these organisms (79, 88). Some workers argued that EPEC strains were nonvirulent, that bacteriological investigations for EPEC should be abandoned, and that laboratories should look for ETEC and EIEC isolates with clearly defined virulence properties (74, 173). Others proposed a more cautious approach, suggesting that until more information was available, EPEC should be regarded as true pathogens (91, 172).

In a conclusive study in 1978, Levine et al. showed that two EPEC isolates of different serogroups lacking enterotoxic and enteroinvasive properties were virulent for volunteers (125), confirming the pathogenicity of EPEC by mechanisms distinct from those of both ETEC and EIEC. This important study set the scene for intensive research to identify and characterize the novel virulence attributes of these organisms. In recent years, two other categories of pathogenic *E. coli* have been identified: enterohemorrhagic *E. coli* (EHEC) and enteroaggregative *E. coli* (EAggEC). The pathogenicity mechanisms of these groups are considered below since there is some overlap with the virulence mechanisms of EPEC.

Current Aspects of EPEC Infection

In developed nations during the 1940s and 1950s, EPEC organisms were the main cause of outbreaks of infantile diarrhea in nurseries and an important cause of cases of sporadic infant diarrhea (126, 171). The incidence of EPEC infection has been decreasing since the 1960s such that EPEC is not now considered a major cause of epidemic infantile diarrhea (141, 171). However, sporadic cases of diarrhea and occasional outbreaks are still reported (26, 37, 95, 119, 142, 169). In many studies carried out in the 1940s and 1950s in the United Kingdom and United States, a high fatality rate was observed. Modern reports, however, do not document high fatality numbers (126, 171). The reason for this is unclear, but it is probably related to improvements in rehydration and the use of antibiotics (126). The marked decline in the incidence of EPEC infection in the United Kingdom has led to suggestions that screening infants for EPEC infection is neither cost-effective nor necessary (141, 214).

In underdeveloped countries, the situation is much different; EPEC organisms are a major cause of endemic infantile diarrhea, exacerbated by seasonal outbreaks (171, 200). This situation has been confirmed in numerous recent studies in many different locations (41, 42, 56, 58, 75, 80, 90, 106, 128, 158, 184, 194, 201), some of which have identified EPEC as the commonest bacterial enteropathogen of infants (5, 81, 97, 108, 110, 143, 200). The association between EPEC and disease is particularly strong among infants <6 months old (126). EPEC infection is often more severe than nonbacterial gastroenteritis, ETEC diarrhea, and *Shigella* diarrhea (126) and carries a high mortality (185), and the illness may be protracted (37, 64, 95, 126, 169, 200). EPEC infection is usually acquired by ingestion of organisms in contaminated food or water (126). The substitution of bottle feeding for breast-feeding enhances the risk of contracting diarrhea (171, 200), and the term weanling diarrhea has been used to describe the high incidence of infection at this time of life (200). The problem of EPEC infection in these areas is often aggravated by malnutrition (200). An episode of diarrhea can exacerbate malnutrition disorders, and malnutrition itself can perpetuate diarrhea. In underdeveloped countries, too, the fatality rate can be low provided adequate hospital facilities are available (200). As will become evident in this review, some of the methods used to identify EPEC are inadequate and may underestimate the number of cases; hence, it is likely that EPEC infection is a larger problem than realized in some areas.

Most epidemiological data suggest that infection in adults is rare, as during outbreaks mothers, nurses, and contacts of ill infants rarely become ill (126). However, data derived from volunteer studies with adults (52, 66, 105, 125, 127, 213) and from a small number of documented outbreaks among adults show that disease does occur but the infective inoculum is high (126). The reason for the marked resistance of adults to infection is not clear; the antibody response to the O antigen of certain O serogroups is age related, and Neter et al. (149) proposed that exposure to EPEC during childhood conferred immunity in later life. Antibodies to EPEC produced during infection, however, may not be protective. In a group of adult volunteers fed an EPEC strain who were rechallenged at a later date, diarrhea occurred at both challenges (52), indicating that significant protection may not be afforded by infection.

Pathophysiology of EPEC Infection

The incubation period in natural infection of infants is unknown (126, 200); in adult volunteers it is short, 12 to 24 h after the ingestion of large numbers (10^9 to 10^{10}) of organisms (66, 105, 125, 127). The disease is characterized by watery diarrhea of varying severity which may be persistent, and vomiting and fever are common. In infants, a "toxic" appearance (a lethargic deteriorated condition) has been described

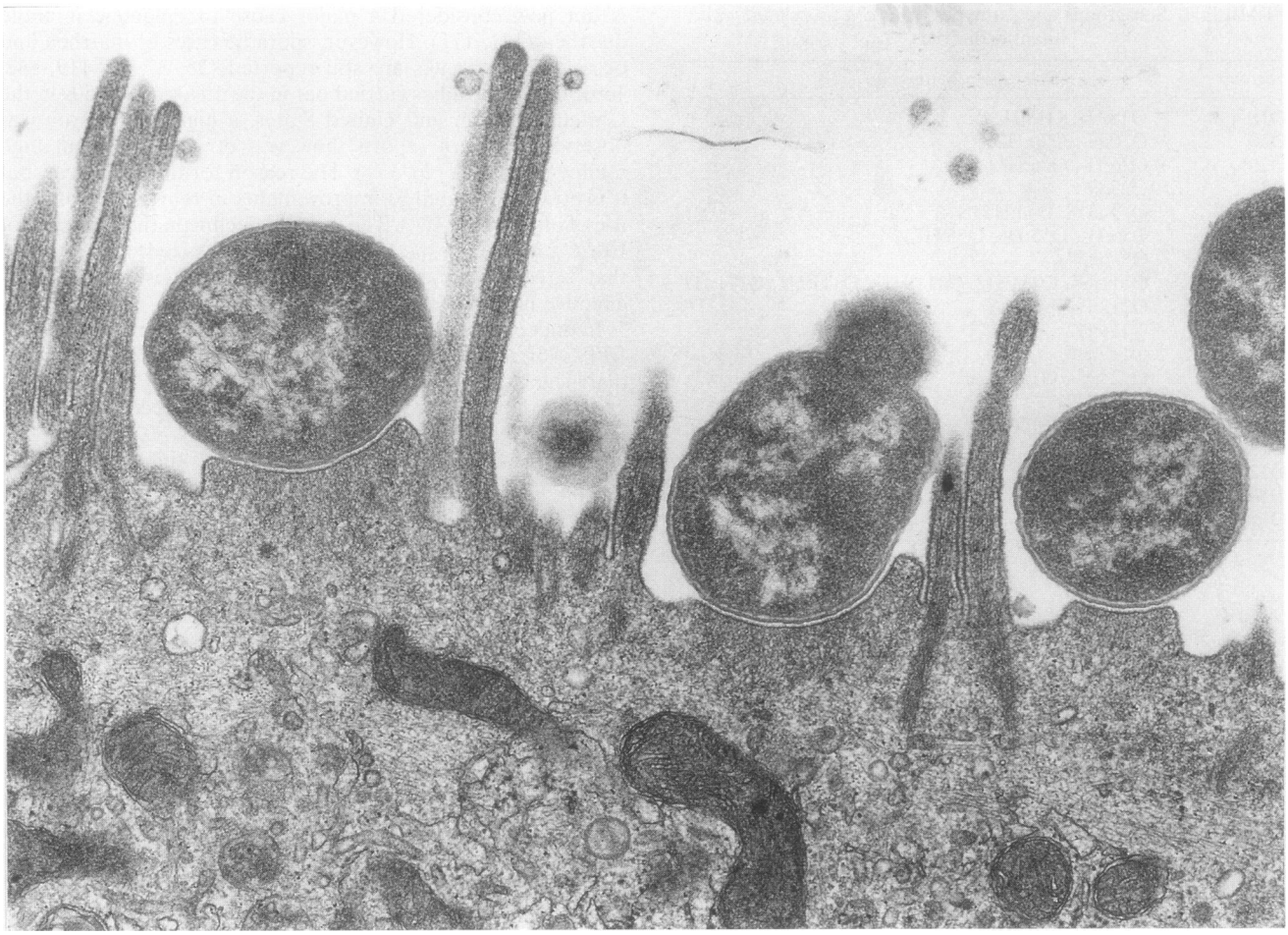

FIG. 1. Electron micrographs showing the characteristic A/E lesion produced by EPEC E2348/69 when adhering to intestinal mucosa. Note intimate attachment of bacteria to cuplike projections of the apical membrane and absence of brush border microvilli. Beneath the attached bacterium there are dense aggregates of fibrillar material. Magnification, ×45,000. Reprinted from Knutton et al. (115) with permission of the publisher.

(24, 200). In severe cases, the fluid loss may resemble that of classical cholera (199). In one study, one volunteer passed a total of 5.6 liters of watery stool (125).

Studies in which bowel biopsies of infected infants have been examined have shown that EPEC bacteria adhere to the mucosa of the small and large bowel, and close attachment between EPEC and the small bowel mucosa has been demonstrated in many cases of EPEC diarrhea (37, 54, 169, 170, 188, 198, 205). In some cases, biopsies have not demonstrated EPEC adhering to the bowel (188). This may be due to the adhesion occurring in discrete areas rather than throughout the small intestine (37).

A characteristic histopathological lesion is produced by EPEC when the organisms adhere to human intestinal tissue (37, 169, 198, 205). This lesion was named the attaching and effacing (A/E) lesion (140) and was first described by Staley et al. (193) in 1969. A/E lesions have also been observed in a variety of EPEC-infected animal models (1, 140, 156, 193, 203, 204) and in EPEC-infected cell and organ cultures (16, 61, 62, 103, 113–115). EHEC also produce the A/E lesion in animal models (203) and in cell cultures (114).

The A/E lesion is characterized by dissolution of the brush border membrane and loss of microvillus structures (effacement) at sites of bacterial attachment (169). The organisms adhere intimately to cuplike projections or pedestals of the apical membrane, with just 10 nm separating bacterial and eukaryotic membranes (113). In the cell, high concentrations of filamentous actin derived from the cytoskeleton are present beneath the site of bacterial attachment (Fig. 1) (67, 113–115). In addition, the cytoskeletal proteins actin, α-actinin, talin, and ezrin also accumulate under EPEC cells during infection of HeLa cells (67). The presence of the A/E lesion appears to be associated with the fluid secretion and diarrhea that is characteristic of EPEC infection. ETEC, EIEC, and nonpathogenic *E. coli* do not produce this lesion (114). Among *E. coli*, the A/E lesion appears to be a specific marker for EPEC and EHEC, although *Helicobacter pylori* (55), some isolates of *Citrobacter freundii* (177), and *Hafnia alvei* (2, 4) also produce similar lesions.

An in vitro test to determine the ability of EPEC to produce the A/E lesion has been developed (114). The test uses fluorescein isothiocyanate-labelled phalloidin (a fungal toxin that binds to filamentous actin) to detect polymerized actin derived from localized cytoskeletal breakdown in cell cultures to which EPEC organisms have attached (Fig. 2). A positive fluorescent-actin staining (FAS) reaction is indicative of the ability of an organism to produce A/E lesions in vivo and is highly sensitive and eliminates the need for electron micros-

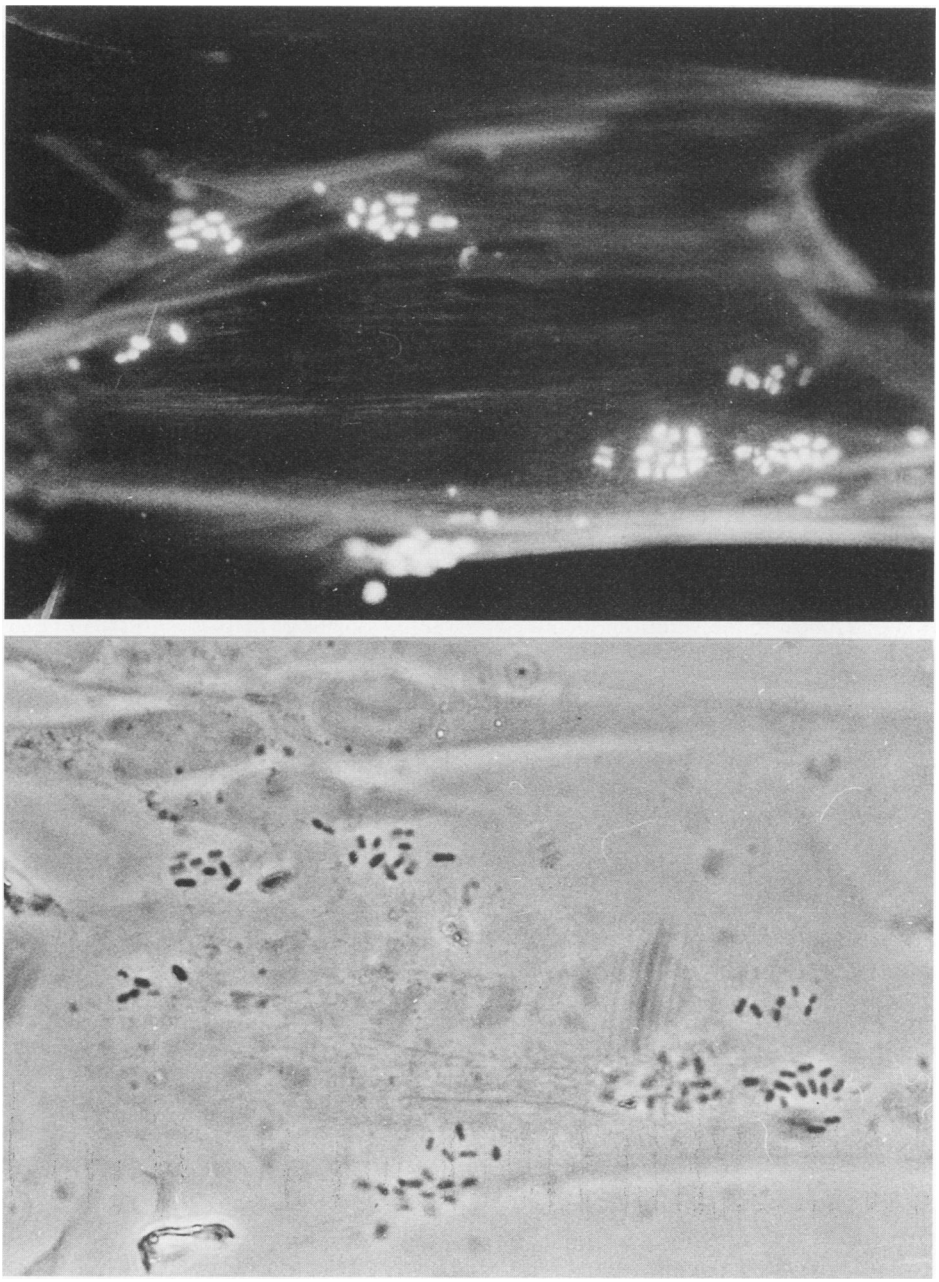

FIG. 2. Fluorescent and phase-contrast micrographs of EPEC E2348/69-infected HEp-2 cells. Intense spots of actin fluorescence stained by fluorescein isothiocyanate-phalloidin which correspond to adherent bacteria can be seen. Magnification, ×600. Reprinted from Knutton et al. (114) with permission of the publisher.

copy to identify the A/E lesion (114). EPEC serogroups that have been shown to produce the A/E lesion in vivo or in vitro or are FAS positive are O26, O55, O86, O111, O114, O119, O125, O127, O128, and O142 (103, 114, 116). However, not all isolates belonging to traditional EPEC serogroups are FAS positive and produce the A/E lesion (103, 114, 116); those that do not should not be classed as EPEC as they lack the major virulence determinant of EPEC.

There may also be edema, neutrophil infiltrate, and a moderate disordered arrangement of enterocytes in the intestinal mucosa following EPEC infection (24). Coincident with damage to the mucosal surface, there is a marked increase in the release of brush border enzymes into culture media from EPEC-infected explants (16, 62), and the enzymatic activity of EPEC-infected infant intestinal mucosa has been shown to be reduced (198, 205). In rabbits infected with a rabbit enteropathogenic *E. coli* strain, RDEC-1, bacterial adherence to the mucosa elicited abnormal myoelectric responses in the bowel which developed prior to the onset of diarrhea (189). The same strain also elicited changes in ion transport. Sodium absorption was abolished, and chloride absorption was reversed to secretion (197). It is likely that similar changes may occur with human EPEC and may account for the secretory diarrhea typical of EPEC infection.

Role of Toxins in EPEC Infection

The basic clinical features of EPEC disease, i.e., profuse watery diarrhea with little inflammation of the intestinal mucosa, suggest an enterotoxin-mediated illness (163, 171, 200). However, despite intensive investigation, no conventional or unconventional toxins have been reliably and consistently detected in EPEC isolates (79, 88, 121, 123, 129, 165). Indeed, EPEC may be characterized by the lack of production of typical heat-labile and heat-stable enterotoxins (121). In some animal systems, such as perfused rat jejunum or canine jejunal loops, "enterotoxic" activity has been detected in EPEC sonicates or concentrated culture supernatants (111, 125). Further characterization of these products has not been described.

In 1977, Konowalchuk et al. showed that some isolates belonging to EPEC serogroups produced a toxin cytotoxic to Vero cells that was termed verotoxin (118). Further studies by other groups showed a strong association between verotoxin production and EPEC of serogroup O26 (178, 211). O'Brien et al. demonstrated that some EPEC strains, particularly those of serogroup O26, produced high levels of a cytotoxin similar to Shiga toxin, the toxin produced by *Shigella dysenteriae* type 1 (151). This toxin was termed a shiga-like toxin (SLT) and was subsequently shown to be identical to the verotoxin identified by Konowalchuk et al. (118). Two main forms of SLT have been identified from *E. coli* isolates; the first, SLT-I, is almost identical to Shiga toxin. The second, SLT-II, has a structure and mode of action similar to those of Shiga toxin and SLT-I but is immunologically distinct (86, 150). Isolates producing these toxins are known as SLT- or verotoxin-producing *E. coli.*

In 1982, an *E. coli* isolate of serotype O157:H7, which produced high levels of SLT, was implicated as the causative agent of an outbreak of hemorrhagic colitis in the United States (162). A new category of pathogenic *E. coli*, EHEC, was formed, of which *E. coli* O157 is the prototype (86). EHEC is a subgroup of SLT-producing *E. coli* composed of isolates that produce high levels of SLT, cause an illness similar to that caused by *E. coli* O157, and produce A/E lesions (86). Many isolates of EPEC serogroups O26 and O111 produce SLTs, produce A/E lesions both in vitro (114) and in vivo (203), and are classed as EHEC rather than EPEC (86, 124, 150, 183). Some *E. coli* isolates belonging to EPEC serogroups such as O55, O119, and O126 also produce SLTs (23, 34, 59, 109, 190, 191, 215). EPEC isolates of most other classical serogroups do not hybridize with probes for *SLT-I* or *SLT-II* genes, and although they are reported to be cytotoxic, the activity is low (38, 133, 151) and the cytotoxin is active on cell lines that are unaffected by Shiga toxin (34, 190). The nature of this EPEC cytotoxin is unknown, but on the basis of its spectrum of activity and the probe data, it is unlikely to be Shiga toxin or an SLT.

Although some toxic products are expressed by EPEC, the role of these toxins in EPEC disease is unclear. Intensive studies in the last 10 years have provided important insight into aspects of EPEC infection. EPEC is now recognized as a group of *E. coli* bacteria that cause infantile diarrhea by distinct pathogenetic mechanisms, of which adhesion is a critical feature. This review describes current knowledge of the adherence mechanisms of EPEC and the role of adhesion in pathogenicity.

EPEC ADHERENCE

Adherence to Cell Cultures

Because of the demonstration that EPEC adhere to intestinal mucosa in vivo (37, 54, 169, 170, 188, 198, 205), initial work attempted to characterize adhesive mechanisms. Cravioto et al. (40) demonstrated that 80% of 51 EPEC strains isolated from outbreaks showed mannose-resistant adhesion to HEp-2 cells, whereas normal flora *E. coli* isolates rarely adhered. EPEC isolates that were nonadherent were often those that had been stored for considerable periods of time, and it was suggested that loss of a plasmid-mediated adhesin was responsible (40). With HeLa cells and by altering the conditions of the adhesion assay, two distinct patterns of EPEC adherence, localized adhesion (LA) and diffuse adhesion (DA), were observed (176). The LA pattern is characterized by organisms attaching to one or two small areas of the cell surface in microcolonies (Fig. 3). Organisms showing the DA pattern attach in a scattered pattern to the whole of the cell surface (Fig. 3). Although use of HEp-2 and HeLa cells is thought to give identical results in adhesion assays (134), Moyenuddin et al. (142) and Nataro et al. (148) have shown that some isolates adhere to HeLa cells but not to HEp-2 cells. The significance of this observation is unclear.

Because of the strong association between EPEC isolates and LA, the nature of this form of adhesion has been intensively investigated. Baldini et al. (9) found that 31 of 32 EPEC isolates contained a large plasmid of 50 to 70 MDa. In one EPEC isolate of serotype O127:H6 (E2348/69), virulent for volunteers, a 60-MDa plasmid encoded the HEp-2-adhesive properties. This was demonstrated by transfer of the plasmid to *E. coli* K-12, which then acquired the ability to adhere to HEp-2 cells. Deletion of the plasmid from the host EPEC resulted in loss of adherence to HEp-2 cells, the intestine of colostrum-deprived piglets (9), and human adult-derived duodenal enterocytes (115). In addition, the plasmid-cured isolate was found to be considerably less virulent than the parent isolate when fed to humans, confirming an important role for the plasmid and its associated virulence properties in EPEC infection (127).

The adhesin property encoded on this plasmid was designated the EPEC adhesive factor (EAF) (127), although at that time its identity was unknown. A DNA probe composed of a 1-kDa portion of the EAF plasmid was developed by isolating plasmid fragments associated with adhesion (10, 144). This probe has been used to detect EPEC in various epidemiological investigations (56, 65, 80, 82, 106, 128, 144), and hybridization with the probe correlates well with the production of LA (10, 33, 36, 56, 65, 71, 128, 146). In two EPEC isolates, the EAF probe hybridized with chromosomal DNA, indicating a chromosomal location for this sequence in these isolates (148).

The ability of EPEC to adhere to HEp-2 cells in a localized manner and to hybridize with the EAF probe was most commonly found among EPEC of serogroups O55, O111, O119, O127, O128, and O142; such serogroups were called class I EPEC as they were the strains most commonly associated with outbreaks of infantile diarrhea (148). Strains showing DA and those showing no adhesion to HEp-2 cells belonged to serogroups less often incriminated in outbreaks of diarrhea and were termed class II EPEC, composed of serogroups O44, O86, and O114 (148). Many EPEC bacteria of serogroup O114 are HEp-2 adherent and should be included in class I (20, 35, 102, 114, 179, 182, 191).

Isolates hybridizing with the EAF probe but which belong to serogroups outside the recognized classical EPEC O serogroups have been isolated in various geographical locations (1, 36, 42, 56, 81, 82, 128). On the basis of epidemiological evidence, such isolates were not considered pathogens as they were isolated with similar frequencies from control and ill infants (36, 56, 81, 82, 128). In one of these studies, FAS activity was also determined, and EAF- and FAS-positive

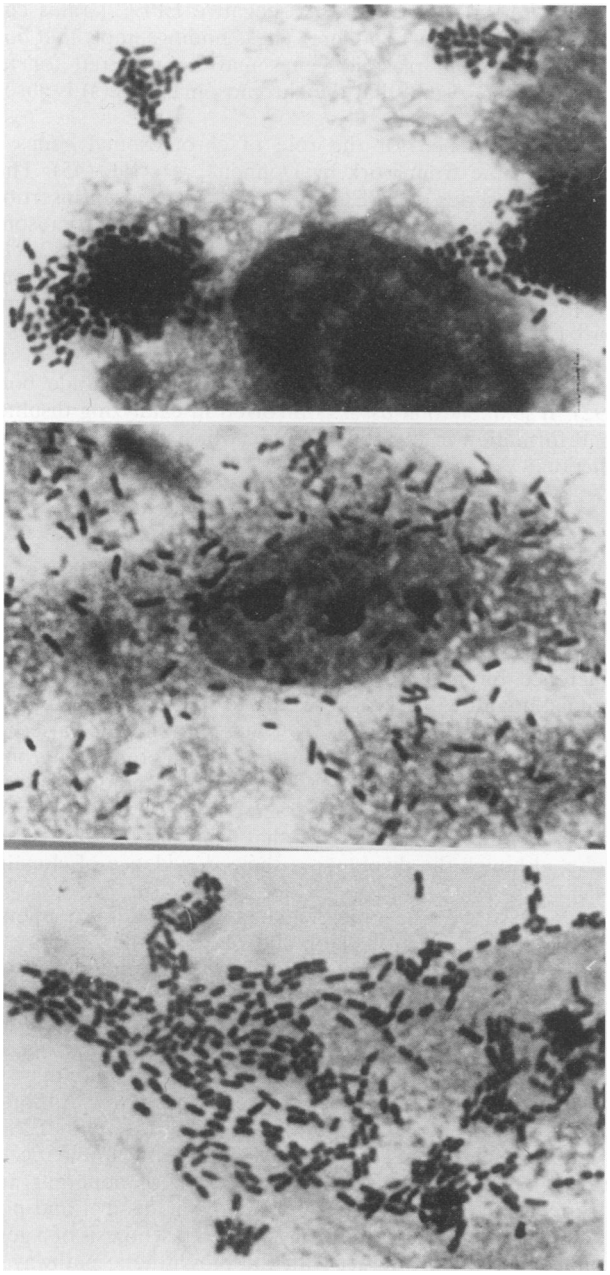

FIG. 3. Light micrograph of Giemsa-stained HeLa cells showing the three types of *E. coli* adherence patterns: localized (top), diffuse (middle), and enteroaggregative (bottom) adhesion. Note the discrete clusters of organisms in the LA pattern. In the DA pattern, organisms adhere to all of the cell surface. In the enteroaggregative pattern, organisms attach to both cells and coverslip in a stacked brick appearance. Magnification, ×500. (Photomicrograph provided by S. Knutton.)

isolates belonging to non-EPEC serogroups were isolated with similar frequencies from cases and controls (56). However, in other studies, EAF- and FAS-positive *E. coli* organisms of non-EPEC serogroups were isolated and considered possible pathogens (1, 42, 116).

EPEC isolates cured of the EAF plasmid show some residual adherence to HEp-2 cells, albeit at very low levels and detectable only after prolonged incubation (72, 114, 115, 138, 164). The nature of this adhesion is discussed in more detail below.

EAF

In two studies, EPEC strains were examined by electron microscopy (7, 179). No fimbrial structures other than type 1 fimbriae were found, and the authors concluded that the EAF was nonfimbrial in nature. In addition, no relationship between LA and hemagglutination of erythrocytes of various animal species was found (179). Levine et al. (127) showed that volunteers fed EPEC E2348/69 developed diarrhea and mounted an antibody response against a 94-kDa outer membrane protein of this isolate. These antibodies also reacted with a similar protein in EPEC isolates of other serogroups. One volunteer had antibodies to the 94-kDa protein prior to challenge and did not develop diarrhea following ingestion of the organism, suggesting a protective role for antibodies to this protein. The virulence of EAF plasmid-cured E2348/69 was considerably reduced in volunteers, and those volunteers who developed diarrhea did not mount an antibody response against the 94-kDa protein (127). This study confirmed an important role for the plasmid and the EAF in virulence and implied that the 94-kDa protein was plasmid encoded and possibly the EAF.

Chart et al. (35) immunized rabbits with a formalinized EPEC suspension. The resulting antibodies reacted with the 94-kDa protein from the immunizing EPEC isolate but did not react with a 94-kDa protein of EPEC of other serogroups, indicating possible interstrain antigenic variation of this protein. In addition, the antibodies did not inhibit HEp-2 adhesion of EPEC isolates even though the 94-kDa protein was surface exposed.

Cravioto et al. showed that purified secretory immunoglobulin A from breast milk reacted with the 94-kDa protein of six different EPEC serogroups (43). In contrast to the results of Chart et al. (35), secretory immunoglobulin A inhibited adhesion of EPEC strains to HEp-2 cells, and the inhibition was specific for LA. The antibody did not inhibit adhesion of DA strains.

These discrepancies may be explained by the existence of a common epitope on the 94-kDa protein of EPEC strains; antibodies to this epitope produced during natural infection may be protective and prevent bacterial adhesion to cell cultures. Vaccination of rabbits with formalinized EPEC may not induce antibodies to this epitope (104), due to either conformational changes in the protein caused by formalin treatment or the inability of rabbits to mount a response to this epitope. Alternatively, the antibody preparations used by Cravioto et al. (43) and Levine et al. (127) may contain antibodies to EPEC structures other than the 94-kDa protein that are involved in adhesion.

Scaletsky et al. (175) provided evidence that a 32-kDa protein produced by an EPEC strain was the EAF, but Chart and Rowe (32) showed that this protein was a porin protein, OmpF, and was not involved in adhesion. However, some recent data have shown that production of a 32-kDa protein may be regulated by the EAF plasmid (209). The function of this protein is unknown, but it may be identical to the protein detected by Scaletsky et al. (175).

Identification of a Fimbrial Adhesin in EPEC

The absence of fimbriae on the surface of some adherent EPEC strains (7, 179) and the identification of the plasmid-associated 94-kDa outer membrane protein (127) led to a hypothesis that this protein was the EAF and that fimbriae

were not involved in LA (43, 134). Mapping of the EAF plasmid showed that two distinct regions were necessary to encode HEp-2 adhesion, a phenomenon seen with some fimbrial adhesins (147). Knutton et al. (113), using specialized electron microscopy techniques involving ruthenium red staining, detected 7-nm-diameter fimbrial structures mediating adhesion between EPEC and cell cultures and between the EPEC cells themselves. A non-fimbriated *E. coli* K-12 strain transformed with the EAF plasmid produced fimbriae and adhered to HEp-2 cells, implying a role for plasmid-encoded fimbrial structures in adhesion. The *E. coli* K-12 strain is now known to be fimbriate (112).

A candidate for the elusive EPEC adherence factor was finally detected by Girón et al. (77). After culturing a serotype O111:NM EPEC strain on sheep blood agar, these authors identified a 3-nm-diameter pilus that was not expressed by an EAF plasmid-cured derivative. On electron microscopy, pili were seen to aggregate to form bundles that were intertwined, forming a three-dimensional mesh in which individual bacteria were embedded. This pilus structure has been termed the bundle-forming pilus (BFP). BFPs are inducible in nature, and EPEC cells grown under conditions that permit their expression cluster together and form aggregates. EPEC may not infect cells as single organisms but as infectious units composed of multiple organisms aggregated together by the BFP, explaining the clusters of bacteria observed with LA (209). An antibody raised to the BFP reacted with pili of other EPEC strains, indicating that they may be a common feature of EPEC and that antigenic relatedness exists between BFP of EPEC belonging to different serogroups. Antibodies raised to BFP also partially inhibit LA (77).

The N-terminal amino acid sequence of the BFP subunit shows some homology to the toxin-coregulated pilus of *Vibrio cholerae* (77) and other members of the type IV pilin family (48, 192), and the DNA sequence of the BFP gene designated *bfpA* is 41% identical to the toxin-coregulated pilus gene of *V. cholerae* (48). EPEC E2348/69 also possesses an EAF plasmid-associated gene whose product shows predicted amino acid homology at 25 of 28 residues with the N terminus of the BFP of the O111:NM isolate (48). Cloning of the *bfpA* gene from E2348/69 suggests that the BFP subunit is an 180-amino-acid protein with a molecular weight of 18,370 (48). When the *bfpA* gene is introduced into a laboratory *E. coli* strain, it does not produce any BFP-like fibers; instead, the *bfpA* product is a 21-kDa protein consisting of the BFP subunit and a 13-amino-acid signal peptide. This precursor molecule requires processing, and other genes probably located on the EAF plasmid are required for its normal maturation (192).

Culture conditions play an important role in the expression of BFP, with expression being repressed in conventional bacteriological media but induced by growth on sheep blood agar (77) or in cell culture medium (209). The medium components responsible for the induction of BFP are unknown, but their absence may explain the failure to identify a pilus structure in EPEC when organisms were grown in nutritionally rich media (7, 179). Because of the inducible nature of the BFP, other genetic loci must be involved in their production and regulation (51).

Role of Chromosomal Genes in LA

McConnell et al. (138) found that *E. coli* K-12 and other non-EPEC isolates transformed with an EAF plasmid from one of three different EPEC isolates did not always show LA, and when adhesion was expressed, it was at a much lower level than that of the plasmid donor strain. Transfer of an EAF plasmid into a number of EAF-negative EPEC strains conferred the full ability to adhere. These findings imply that both chromosomal and plasmid genes may be required for full expression of LA and that the chromosomal gene(s) is absent from non-EPEC strains.

Further evidence for the role of chromosomal genes in adhesion came from work by Donnenberg et al. (45). They generated a series of EPEC mutants by transposon insertion. In two instances, the transposon inserted into the chromosome and the mutants lost the ability to produce LA on HEp-2 cells. The chromosomal locus has been identified as the *dsbA* gene, which catalyzes disulfide bond formation in the periplasmic space (219). The BFP subunit contains two cysteine residues in the last half of the molecule, which for some members of the type IV pilin family form an intramolecular disulfide bond (192). It is likely that *dsbA* is involved in catalyzing disulfide bond formation in the BFP subunit, which is essential for its function.

EAF Plasmids

Studies on EAF-encoding plasmids have shown that they are usually large plasmids of 50 to 70 MDa (9, 10, 147, 182, 185). The genes encoding LA occur in two distinct regions of the EAF plasmid; neither region alone is sufficient to confer adherence, but adherence occurs when both are present. The minimal quantity of DNA necessary to specify localized adhesion is 35 kb (147). Plasmids of strains from a wide variety of geographical locations and belonging to different serogroups showed conservation of DNA sequences with some divergence of restriction sites. Restriction fragment length polymorphism was observed even in the region that hybridized with the EAF probe, although the divergences showed evidence of clonality (147).

In most studies, no other biochemical markers or phenotypes have been identified on the plasmid, the majority of which remains cryptic (147). In a Brazilian study, antibiotic resistance genes were found on the EAF plasmid in some isolates of serotype O55 (120), but this appears to be a rare phenomenon (147). Genes encoding O-specific lipopolysaccharide (LPS) occur on some EAF plasmids and may be associated with genes for antibiotic resistance (160). Although plasmid genes encoding O antigens may be rare in EPEC isolates, plasmid genes that modify the O antigen may occur. Bradley et al. showed that the core LPS of adherent and nonadherent EPEC of serogroup O119 was distinct and postulated that the EAF plasmid may cause the synthesis of a gene product that could interact with the biosynthetic pathway of the core sugars (27). The possible significance of this is discussed below. In one EPEC isolate of serogroup O111, a high-molecular-weight plasmid encoded O-LPS, antibiotic resistance, HEp-2 adherence, A/E activity, and invasive properties (70).

It has been shown that a locus on the EAF plasmid of E2348/69 controls expression of the 94-kDa protein (104). Indeed, two EAF plasmid-encoded regulating loci have now been identified. One locus increases expression of the 94-kDa protein by 100-fold and also increases the expression of other outer membrane proteins (84). A second locus increases expression of the 94-kDa protein by 10-fold (84). The gene for the first regulator has been termed *perA* (plasmid-encoded regulator) (51, 84) and specifies a 30-kDa protein that is similar to the *virF* gene of *Shigella* spp. which positively regulates plasmid-encoded virulence determinants (93). The *perA* locus is present in all EPEC that carry the EAF plasmid (83).

EAF Receptor

Little is known of the eukaryotic receptor to which the EAF binds. Using adhesion inhibition studies, Chart et al. (33) showed that, of five different fatty acids, linoleic acid was able to inhibit adhesion to HEp-2 cells of 20 different EPEC isolates. Oligosaccharide fractions of breast milk also inhibit EPEC adhesion to HEp-2 cells (43). Among purified oligosaccharide fractions, pentasaccharides and difucosyllactose showed the highest degree of adhesion inhibition. Fucose residues on these oligosaccharides appeared to be of crucial importance, as the defucosylated groups were poor inhibitors. A change from a localized to a diffuse adhesion pattern was observed after incubation of EPEC strains with hexa- or heptasaccharide fractions. These fractions may have an effect on interbacterial associations that may be responsible for the LA phenotype, or they may have a direct effect on the expression of the LA genes (43).

While these findings are of interest and suggest possible mechanisms for preventing or modifying EPEC infection by preventing adhesion, the identity of the EAF receptor is still unclear. Linoleic acid and the oligosaccharides may not directly inhibit adhesion by binding to the EPEC adhesin but rather may bind to either bacterial or HEp-2 cell components not involved in adhesion and block adhesion by steric hindrance. It may be more relevant to measure binding of these substances to adhesive EPEC isolates and their plasmid-cured derivatives to confirm the specificity of adhesion inhibition. Using this approach, Wadström and Baloda (212) showed that EPEC strains bound fibronectin and that an EAF plasmid-cured strain bound fibronectin to a lesser extent. However, as fibronectin binding is a characteristic of other classes of *E. coli* (212), this would argue against fibronectin being an EAF receptor.

Jagannatha et al. (100) examined LA EPEC and a DA *E. coli* isolate for the ability to bind glycolipids. DA *E. coli* did not bind any of the glycolipids tested, while LA strains bound asialo-GM1, asialo-GM2, globoside, and lacto-*N*-neotetraose in decreasing order of affinity. The minimum common sequence among the four glycolipids that bound was galactose-*N*-acetyl-β1,4-galactose. However, GM1 and GM2 were not bound by LA EPEC strains; steric blocking of the galactose-*N*-acetyl-β1,4-galactose moiety by sialic acid groups was thought to account for this. Adhesion-negative mutants of an LA EPEC strain showed markedly reduced binding to asialo-GM1, indicating that recognition of galactose-*N*-acetyl-β1,4-galactose correlated with HeLa cell adherence and suggested a role for glycolipids as receptors for EPEC adherence. A similar disaccharide sequence is present in many of the breast milk oligosaccharides that inhibited adhesion (43), although in that study fucose was identified as the important residue. The usefulness of plasmid-containing and plasmid-cured EPEC pairs to identify adhesin receptors may be limited. Not only will plasmid-cured, nonadhesive strains lack an adhesin, but other properties regulated by genes on the EAF plasmid may also be altered, e.g., the 94-kDa protein (104) and, possibly, LPS (27).

It is difficult to interpret these varied and discrepant findings concerning potential receptors except by postulating that EPEC organisms possess a number of distinct adhesins that bind to distinct receptors. Now that a potential adhesin, the BFP, has been recognized, identification of the receptor for LA may be achieved.

The demonstration that EPEC adhesion may be inhibited by various substances offers a potential therapeutic strategy and is worthy of further study, particularly as infusions of plant materials containing complex carbohydrates can inhibit bacterial adhesion (89). Such approaches may allow inexpensive control of EPEC infection that will be extremely beneficial in underdeveloped countries.

LA EPEC That Are EAF Probe Negative

In certain EPEC isolates, the EAF probe sequence is absent from the EAF plasmid, without any deleterious effects on LA expression. It is likely that the region of the plasmid containing the EAF probe sequence is closely linked to adhesion genes but is not directly involved in adhesion. A DNA probe for a 3-kb region of the *bfpA* structural gene has been prepared and gives better correlation with the ability to produce LA than the EAF probe (76). Some LA-positive strains that are EAF probe negative are BFP probe positive (76). The EAF DNA probe used to detect EPEC will not recognize some true EPEC isolates, and the frequency of these organisms in infection may have been underestimated. Scotland et al. (181) proposed that LA-positive, EAF probe-negative EPEC may be of greater importance in the United Kingdom than LA-positive, EAF probe-positive strains.

Most studies have shown good correlation between LA and reactivity with the EAF probe (9, 35, 36, 58, 65, 71, 128, 144, 147). Other studies have shown a poorer correlation between the two methods (42, 180–182). This discrepancy may be due to the order in which the tests are performed. If the probe is used first to identify adherent organisms and then results are confirmed by HEp-2 adhesion assays, correlation is generally very good. However, when HEp-2 assays are performed first, followed by EAF probe hybridization studies, LA EPEC that do not hybridize with the probe are encountered and the correlation is lower. An alternative explanation may be that the prevalence of EAF probe-negative, LA-positive EPEC differs in the geographic locations or populations studied.

Scotland et al. (182) found 17 EPEC strains of serogroup O128 isolated from sporadic human infections in the United Kingdom that showed LA to HEp-2 cells but did not hybridize with the EAF probe. In a further study, only 4 of 29 serogroup O128 strains that showed LA to HEp-2 cells hybridized with the EAF probe (180). In one isolate that did not hybridize with the probe, a 50-MDa plasmid was found to encode LA (180). Some isolates belonging to EPEC serogroups O55 and O111 show LA but are EAF probe negative (70, 181). A study in Mexico detected four isolates belonging to EPEC serogroups and 59 *E. coli* isolates of non-EPEC serogroups that were FAS positive and showed LA but were EAF probe negative (42).

HEp-2-Nonadherent EPEC

EPEC isolates showing either DA or no adhesion to HEp-2 cells were termed class II EPEC by Nataro et al. (148). A putative class II EPEC strain (E128010) of serotype O114:H2 produced diarrhea in three of five volunteers with symptoms similar to those produced by a class I EPEC strain (127). Other isolates of this serotype are often both HEp-2 adhesive and EAF probe positive (20, 35, 102, 114, 179, 182, 191). Gomes et al. proposed that E128010 may possess an adhesin distinct from the EAF (80). Knutton et al. have shown that strain E128010 does adhere to HEp-2 cells but to a lesser extent than most EAF-positive EPEC strains (114). E128010 has since been shown to be HEp-2 adherent and reactive with the EAF probe (102). Therefore, this strain is in fact a class I EPEC, and the virulence in humans of a class II EPEC has not been determined.

In a United Kingdom study, only 8% of 449 isolates belonging to EPEC serogroups were EAF positive (191). In Germany, only 26% of EPEC isolates showed LA (107), and in an

African study, 52% of EPEC showed LA (71). The lack of HEp-2 adhesion and probe reactivity may be due to three factors. First, the nonadherent isolates may not be true EPEC. Second, plasmid loss in stored strains may have occurred. Plasmid loss occurs with a high frequency in vivo (127), and spontaneous loss of plasmids in stored strains has been documented (40, 142), although many stored strains still possess the EAF plasmid (142, 166). Third, nonadhesive EPEC may have adhesive structures distinct from BFP that are not detected in HEp-2 adhesion assays. Karch et al. (107) have shown that some EPEC bacteria that do not adhere to HEp-2 cells can adhere to other cell lines. Furthermore, *E. coli* isolates that belong to EPEC or non-EPEC O serogroups and that are FAS positive but EAF probe negative and adhere poorly to HEp-2 cells have been detected (116). When tested for adherence to human intestinal mucosa, these strains showed levels of adherence similar to those of HEp-2-adherent EPEC strains (116). These findings suggest that other adhesins may be present in some EPEC isolates that are not detected by either the HEp-2 adhesion assay or the EAF probe.

Other adhesins have been identified in some *E. coli* isolates belonging to EPEC O serogroups, but since many of these isolates have not been tested for FAS activity, it is not clear whether these strains are true EPEC. One study demonstrated extended type 1 fimbriae in some EPEC, although they did not appear to mediate adherence to Henle 407 gut epithelial cells (28). Two EPEC isolates of serotype O111:H12 were shown to possess fimbriae that appeared to mediate adherence to HEp-2 cells and also conferred ability to hemagglutinate ox and sheep erythrocytes (216). These structures had a diameter of less than 3 nm, and their thinness made it difficult to determine their distribution in a population of cells or on a single cell. In two serogroup O86 isolates, fimbriae mediating hemagglutinating activity and HEp-2 adherence were detected (154). It is now apparent that these isolates are probably not true EPEC but are representatives of a new group of pathogenic *E. coli*, EAggEC (117), described below.

DAEC

DA *E. coli* (DAEC) belonging to classical EPEC serogroups were first described by Scaletsky et al. (176) and were termed class II EPEC by Nataro et al. as they did not hybridize with the EAF probe (148). The situation has become more confusing since the identification of a third group of HEp-2-adherent *E. coli*, EAggEC (117, 146). Some organisms previously thought to be LA *E. coli* or DAEC have now been shown to be EAggEC (92, 146, 207). The differences among the three adhesion patterns are usually quite distinct, but their detection is highly dependent on the adhesion assay method (Fig. 3). The method described by Scaletsky et al. recognizes only two patterns, LA and DA (176). The alternative method, first used in the United Kingdom and used at the Center for Vaccine Development in the United States, recognizes three patterns, LA, DA, and aggregative adhesion (146), and strains showing either LA or DA in the Scaletsky method (176) may be aggregative in the Center for Vaccine Development method.

Because of variations in the methods used in different studies, there is considerable confusion in the literature concerning the adherence patterns of *E. coli*. Mathewson et al. proposed that HEp-2-adherent *E. coli* of non-EPEC serogroups, enteroadherent *E. coli*, were a cause of travellers' diarrhea in adults (135). Two such isolates produced very mild diarrhea in some volunteers (136). One isolate showed the DA pattern and one showed the LA pattern, although the LA isolate showed enteroaggregative adhesion by the Center for Vaccine Development method (146). The term enteroadherent *E. coli* has been used widely in the literature, but it is imprecise as it includes LA EPEC, DAEC, and EAggEC. This group encompasses *E. coli* isolates pathogenic by varying mechanisms and strains that may not be pathogenic. The term enteroadherent *E. coli* adds to the general confusion regarding pathogenic *E. coli* and should no longer be used.

Two distinct adhesins have been identified from DAEC strains: a plasmid-encoded 100-kDa protein from a serotype O126:H27 *E. coli* isolate (19), and a chromosomally encoded fimbrial adhesin from a serogroup O75 isolate (22). Many O126:H27 isolates show the aggregative adhesion pattern (181), and it is not clear whether this isolate is DAEC or EAggEC. A DNA probe prepared from the chromosomal adhesin gene has been used in epidemiological studies, but it does not show perfect correlation with the results of HEp-2 assays (78, 128), and it is likely that a number of different genetic determinants may specify DA.

The isolation rate of EPEC O-serogroup strains showing DA as determined by HEp-2 cell adhesion and/or a DNA probe is similar in infants with diarrhea and control infants, implying that this adhesion pattern is not a characteristic of pathogenic *E. coli* (36, 42, 80, 128, 146). However, two recent reports have documented a strong association between DAEC and diarrhea in Mayan and Bangladeshi children (15, 78). It is possible that only a subpopulation of DAEC may have the necessary factors in addition to adherence properties to produce disease (78, 196). When two true DAEC isolates were fed to volunteers, very mild diarrhea was induced in some volunteers, but as it did not appear to be related to the inoculum ingested, the isolates were considered avirulent (196).

True DAEC bacteria are clearly and unequivocally distinct from EPEC as they are uniformly negative in the FAS test (114) and have a different mode of adherence; also, their pathogenesis is inconclusive. If DAEC organisms cause diarrhea, then they do so by as yet uncharacterized mechanisms. DAEC bacteria have been shown to produce a unique adherence feature on HeLa cells. Adherence is characterized by dimple formation at sites of bacterial attachment, with locking of bacteria by elongated microvilli at the edge of the dimple (217). These findings warrant further study on DAEC pathogenesis.

EAggEC

Using a modification of the HEp-2 adhesion assay, Nataro et al. detected a third pattern of *E. coli* adherence, enteroaggregative adhesion (146). This pattern was characterized by aggregates of bacteria with a "stacked brick" appearance adhering to both the surface of the HEp-2 cells and the glass coverslip (Fig. 3) and distinct from the LA of EPEC. Isolates showing this pattern of adhesion were termed EAggEC (146).

Numerous epidemiological studies in a variety of geographical locations have shown that EAggEC bacteria are a common cause of infantile diarrhea which may be bloody or protracted (15, 21, 42, 81, 128, 137, 146). However, some studies have shown no association between EAggEC and diarrhea (57, 78, 80). Although EAggEC bacteria were initially isolated from adult travellers with diarrhea (135), this association has not been confirmed in a recent study (39). However, Scotland et al. isolated EAggEC of serotype O44:H18 from elderly patients with diarrhea (181). The role of EAggEC in adult diarrhea must be evaluated.

Examination of EAggEC has shown them to be clearly distinct from EPEC in that they are EAF probe and FAS negative and do not produce the A/E lesion (114, 146, 181).

When EAggEC organisms are tested in ligated rabbit and rat ileal loops, a distinct histopathological lesion is seen (208). This is characterized by shortening of villi, hemorrhagic necrosis of villus tips, and a mild inflammatory response with edema and mononuclear infiltration of the submucosa. These effects are similar to those of SLTs, although EAggEC bacteria do not hybridize with probes for *SLT-I* or *SLT-II* (208). Neither supernatants nor sonicated cell pellets induce cytopathic effects on a variety of cell lines, although a toxin that induces fluid secretion, antigenically related to the heat-stable toxin of ETEC has been detected in a rabbit intestinal loop model (174). In addition, a toxin similar to the α-hemolysin of *E. coli* has been demonstrated (12).

Examination of EAggEC for fimbrial structures revealed at least two different morphological and immunological types (208). Knutton et al. (117) identified four morphologically distinct types of fimbriae, including a form consisting of bundles of fine filaments morphologically similar to the BFP of EPEC. Isolates possessed various combinations of the four fimbrial types, although 43 of 44 isolates possessed the bundled fibrils. A region of an EAggEC plasmid that encodes fimbriae and mediates aggregative adhesion and hemagglutination has been cloned, although the region which specifies the fimbrial subunit has not yet been identified (145). The BFPs of EAggEC have been termed aggregative adherence fimbriae I. Hybridization with a 1-kb DNA probe prepared from the 60-MDa plasmid of EAggEC (17) correlated excellently with the presence of the fibrils and hemagglutinating activity (145).

Some EAggEC isolates are O nontypeable, and the O type, when present, is often encoded on a high-molecular-weight plasmid (208). Many *E. coli* bacteria belonging to EPEC serogroups O44, O111ab, O86, and O126 are enteroaggregative (117, 181). It is likely that these serogroups originally classified as EPEC are EAggEC and produce disease by a mechanism distinct from those of EPEC, EHEC, EIEC, and ETEC (124).

THREE-STAGE MODEL OF EPEC PATHOGENICITY

Using cultured adult human intestinal mucosa and E2348/69 and its EAF plasmid-cured derivative, Knutton et al. showed that mucosal adherence of EPEC could be divided into two distinct stages (115). The first stage involves initial attachment of bacteria to cells mediated by plasmid-encoded adhesins. The second stage consists of effacement of brush border microvilli and intimate attachment.

Donnenberg and Kaper suggested that intimate adherence and effacing activity are separate and distinct phenomena and extended the two-stage mechanism of EPEC pathogenesis (115) to a three-stage mechanism (51). The three stages of EPEC infection are (i) nonintimate attachment to intestinal cells mediated by BFP; (ii) effacement of microvilli mediated by uncharacterized gene products; and (iii) intimate attachment allowing the cytoskeletal altering effects of the effacing genes to be amplified and resulting in accumulation of phosphorylated proteins around the organism, pedestal formation, and, eventually, invasion.

A/E LESION

Characteristics

The A/E lesion is a characteristic feature of EPEC infection, but how are these profound changes in cellular architecture and the intense fluid secretion characteristic of EPEC diarrhea produced?

Transient elevation of calcium in intestinal brush border cells by treatment with calcium ionophores or hormones causes breakdown of cytoskeletal actin in the microvillus core, leading to loss of microvilli (60). Microscopically, there are strong similarities between these calcium-induced effects and the A/E lesion, and this lesion may result from increased calcium levels in infected cells (60, 115). Measurement of calcium levels inside EPEC-infected cell cultures has shown elevated levels of calcium due to release from intracellular stores, particularly in areas of bacterial colonization (14). A crucial role for calcium in the development of the A/E lesion can be seen when intracellular calcium is chelated, with a consequent reduction in actin aggregation (13).

In addition to changes in intracellular calcium levels, a variety of eukaryotic proteins become phosphorylated during EPEC infection of cell cultures (11). These include a 20- to 21-kDa protein identified as a myosin light chain (131, 132), the proteins vinculin and α-actinin (159), and an unidentified 90-kDa HeLa cell protein that becomes phosphorylated at tyrosine residues (167). Using monoclonal antibodies to identify proteins involved in cytoskeletal rearrangement, Finlay et al. (67) noted that the cytoskeletal proteins actin, α-actinin, talin, and ezrin accumulated under EPEC-infecting HeLa cells. These proteins are known either to cross-link actin filaments or to link actin filaments to the plasma membrane. The phosphorylated 90-kDa protein also accumulates under EPEC cells (167).

Before discussing the possible mechanisms for the production of the A/E lesion and fluid secretion, it is necessary to examine the bacterial components that are thought to be involved.

Characterization of EPEC Factors Responsible for Development of the A/E Lesion

Two techniques have assisted in the characterization of the factors responsible for development of the A/E lesion. The first technique is transposon mutagenesis, the introduction of an alkaline phosphatase gene randomly into bacterial DNA from a transposon. If the transposon inserts into a gene specifying an exported protein, then colonies appear blue on a medium containing an alkaline phosphatase substrate. Such colonies can then be screened for the absence of the property under investigation (45, 103). Second, the FAS test, using fluorescein isothiocyanate-conjugated phalloidin, enables screening of large numbers of isolates for defects in A/E genes (114).

Both Jerse et al. (103) and Donnenberg et al. (45) found that transposon insertions into chromosomal sites of E2348/69 markedly reduced but did not abolish development of the A/E lesion as detected by the FAS test. A "shadow" pattern was produced by these mutants, indicating that some actin polymerization was taking place. In addition, two mutants with chromosomal transposon inserts that completely abolished FAS activity but had no effect on HEp-2 adherence were identified (45). These inserts mapped to two different chromosomal sites, distinct from those that caused the shadow FAS pattern. A number of distinct genes therefore appear to be crucial for production of the A/E lesion.

eae Gene Cluster

The gene inactivated in mutants that showed a shadow FAS pattern was termed the *eae* gene (EPEC attaching and effacing) (103). Cloning of the *eae* gene from E2348/69 suggested that it encoded a 107-kDa protein; however, the product is, in fact, a 94-kDa protein and is the same protein against which volunteers mount an antibody response following EPEC infec-

tion and which was originally hypothesized to be the EAF (104). Differences in molecular weight between the native protein and that deduced by DNA sequencing may be explained by posttranslational modification (104). When a DNA fragment containing the *eae* gene is introduced into a non-EPEC isolate, it does not confer A/E activity upon the recipient, but it restores A/E activity to an EPEC isolate containing an insertion in the natural gene (103). The *eae* locus is therefore necessary for production of the A/E lesion but alone is insufficient to confer this activity. When the *eae* gene was sequenced, it was found to share considerable homology (overall, 31% identical residues and 50% conserved residues) with the invasin gene of *Yersinia pseudotuberculosis* (103).

Additional loci downstream of the *eae* gene that have a role in development of the A/E lesion have been identified; transposon insertions in these sites also produce a shadow FAS pattern (49). In these mutants, a 94-kDa protein is produced and localized to the cell surface. The function of these genes is unknown, but they do not appear to influence the localization of intimin in the outer membrane or modifications that result in major changes in size (49). The identification of a cluster of genes involved in the A/E lesion has resulted in the need for altered nomenclature: the *eae* gene is now termed the *eaeA* gene; subsequent genes will be termed *eaeB*, etc. (51).

A DNA probe prepared from a *SalI-KpnI* fragment of the E2348/69 *eaeA* gene gives excellent correlation with the FAS test (103). Of 99 EPEC isolates that were FAS positive, all were *eaeA* probe positive. ETEC, EIEC, and normal flora *E. coli* did not hybridize with the probe (103). Two EPEC isolates hybridized with the *eaeA* probe but were FAS negative. One of these isolates was EAF probe negative, and when it was transformed with an EAF plasmid, this strain became FAS positive (102). Of 30 EHEC strains belonging to serogroups O157 and O26, 29 hybridized with the *eaeA* probe, suggesting that similar virulence properties occur in both EPEC and EHEC strains (103).

Although both EPEC and EHEC strains possess the *eaeA* gene, there is evidence of genetic diversity in this gene in the two groups of organisms. The *eaeA* gene sequence of an *E. coli* O157 isolate had 86% similarity at the nucleotide level with the *eaeA* gene of E2348/69 (218). However, relatedness was 94% at both the nucleotide and amino acid levels in the first 704 amino acid residues (75% of total residues) but only 60 and 49%, respectively, in the remaining 25% of the gene (218). A probe prepared from a 1-kb fragment of the conserved central region of an *E. coli* O157 *eaeA* gene hybridized with most SLT-producing *E. coli* of non-O157 serogroups, but a 0.5-kb probe prepared from the C-terminal end hybridized only with DNA of *E. coli* O157, suggesting that in non-O157 SLT-producing *E. coli* the C terminus of the *eaeA* gene is genetically heterogeneous (130).

Antigenic diversity between the 94-kDa proteins as well as genetic diversity in the *eaeA* gene are found in different groups of *E. coli*. Although Levine et al. showed that convalescent sera from a volunteer fed E2348/69 reacted with the 94-kDa protein of EPEC isolates of different O serogroups (126), Jerse and Kaper showed that the EHEC 94-kDa protein does not react with antibody to an EPEC 94-kDa protein-alkaline phosphatase fusion protein (104). Sherman et al. (187) found that antibodies raised to the 94-kDa protein of an *E. coli* O157 isolate did not inhibit FAS activity of EPEC E2348/69, although they inhibited FAS activity of the *E. coli* O157 isolate. It is likely that the major epitopes are located at the heterogeneous C terminus, which functions as the tissue-binding portion of the molecule (218) and implies functional heterogeneity between the EPEC and EHEC proteins. However, there is evidence that the proteins encoded by the *eaeA* gene of EPEC and the *eae* gene of EHEC may be functionally homologous. An *eae*-deficient mutant EHEC was constructed, and unlike the parent, this isolate did not adhere intimately to colonic epithelia in newborn piglets. Intimate attachment was restored when the EHEC *eae* or EPEC *eaeA* gene was introduced into the mutant (53).

Although the 94-kDa proteins of most EPEC are antigenically related, neither convalescent volunteer sera nor antibodies raised to a 94-kDa protein-alkaline phosphatase hybrid reacted with a 94-kDa protein of E128010 (104, 127). This isolate may have an antigenically distinct protein or it may produce very low quantities of this protein.

Intimin, the 94-kDa Protein, and Its Role in EPEC Virulence

The only product so far identified as having a functional role in the development of the A/E lesion is the 94-kDa protein encoded by the *eaeA* gene which shows some similarity with the *Y. pseudotuberculosis* protein invasin. The gene for invasin when transformed into *E. coli* K-12 confers the ability to adhere to and invade epithelial cells (99). Invasin recognizes multiple β-chain integrin molecules on eukaryotic cells. The integrin receptor family is a group of related proteins that promote cellular attachment to extracellular matrix proteins such as fibronectin. Latex particles coated with invasin bind to and are internalized by cells in culture, and high-affinity binding of invasin to integrin results in internalization (99). Integrins bind cytoplasmic proteins that may be linked to actin filaments, and these molecules may be a means of linking the extracellular environment to the cytoskeleton.

The function of the 94-kDa protein of EPEC is quite distinct from that of invasin in that it does not confer invasive properties when transformed into non-EPEC isolates (103). This difference in function between intimin (the 94-kDa protein) and invasin most likely reflects differences in structure. Although the *eaeA* and invasin genes are 50% related at the genetic level overall, there is little relatedness at the C terminus, the receptor binding domain of the invasin molecule (18, 218).

To fully assess the contribution of the 94-kDa protein to EPEC virulence, in vivo and in vitro studies were performed with a mutant of E2348/69 in which 60% of the *eaeA* gene had been deleted and which did not produce a 94-kDa protein (50). When fed to volunteers, the mutant produced diarrhea in 36% of recipients, whereas the wild-type strain produced diarrhea in all recipients (52). Diarrhea was milder in the volunteers receiving the mutant, as evidenced by lower stool weight and the absence of fever, a symptom seen in 45% of volunteers receiving wild-type EPEC (52). Like the EAF factor, the 94-kDa protein increases EPEC virulence, but strains lacking either product are still able to cause disease, although with reduced attack rates and severity (52, 127), and neither gene is indispensable.

In vitro, the *eaeA* deletion mutant showed the normal pattern of LA to HEp-2 cells but did not show intimate adherence or produce a true A/E lesion. It possessed the ability to produce some microvillus disruption, cause alteration in the cytoskeleton, and produce a weak FAS reaction (49). Further studies have shown that *eaeA* mutants are still able to induce tyrosine phosphorylation of a 90-kDa protein in HEp-2 cells, although the phosphorylated proteins are not organized in the same manner as those induced by the wild-type EPEC (167). Mutants with transposon insertions at chromosomal loci which completely abolish FAS activity are able to adhere intimately

to cells but are devoid of cytoskeletal altering activity and do not induce host cell tyrosine phosphorylation (167). From these data, it was suggested that the 94-kDa protein promoted intimate adhesion of EPEC to cells, and it was therefore called intimin to reflect its function (51). Support for this protein's function as an adhesin are the observations that an EAF plasmid-cured EPEC derivative adheres to Caco-2 cells, whereas a mutant with an inactivated *eaeA* gene does not (103), and the ability of the EPEC *eaeA* gene to restore adhesion to piglet intestinal epithelium of an *eae*-deficient EHEC isolate (53). The adhesive function of intimin may explain the residual HEp-2 adherence seen with EAF plasmid-cured EPEC and support the possibility of multiple adhesins described above. A further function of intimin may be that after binding to its receptor it sets up changes in the cytoskeleton which act as a focus for aggregation of phosphorylated proteins (167).

EPEC mutants with transposon insertions downstream of the *eaeA* gene show interactions with cells similar to those shown by intimin deletion mutants, despite their producing intimin and localizing it to the outer membrane (49). It is possible that one of these loci may encode the protein responsible for intimate adhesin and intimin may have only an accessory or regulatory role. Purification of intimin and other products in the *eae* gene cluster is needed to conclusively identify the function of intimin and the other products and their roles in virulence (49).

Regulation of the synthesis, localization, and function of intimin is a complex phenomenon. Chromosomal genes in the *eae* cluster may affect the function of this protein (49), and EAF plasmid-encoded loci such as *perA* regulate expression (84) by increasing the rate of transcription of both *eaeA* and *eaeB* (83). There is evidence that expression of three high-molecular-weight proteins, one of which may be the 94-kDa protein, is increased by growth in cell culture media, and these proteins may be induced by factors similar to those that induce BFP (209).

Possible Mechanisms for Development of the A/E Lesion

What are the mechanisms for the development of the A/E lesion? Although this is not yet known, it is possible to speculate regarding the nature and function of the mechanisms involved.

A number of studies have shown that the A/E lesion can be detected within 10 (44) or 15 (6) min of contact between EPEC and cell cultures, suggesting that the products required are expressed constitutively (6). Indeed, EPEC isolates treated with tetracycline to halt protein synthesis still produce A/E lesions, suggesting that bacterial replication and/or protein synthesis is not required (44). However, in other studies, incubation periods of 3 h are required before performance of the FAS test (114). Such divergent results may reflect strain or experimental differences.

The inability of culture supernatants to induce protein phosphorylation (11) and the need for viable organisms to produce invasion (6) suggest that intact organisms with a product in the correct conformation are required to induce the changes responsible for producing the A/E lesion. However, Riley et al. showed that EPEC supernatants were able to induce protein phosphorylation (159). Rosenshine et al. suggested that the product that induces tyrosine protein kinase activity may be diffusible (167).

Studies with other enteric pathogens show that eukaryotic cell invasion may be mediated by binding of bacterial components to cellular receptors. *Y. pseudotuberculosis* invasin binds to integrin receptors (99), and for *Salmonella typhimurium*, an uncharacterized cellular component is thought to bind to the receptor for epidermal growth factor, inducing tyrosine protein kinase activity in the receptor itself, which undergoes autophosphorylation (73). Bacterial binding to these components results in changes to cytoskeleton architecture and involves increases in intracellular calcium and activation of tyrosine protein kinases (73). An endocytic process follows, resulting in internalization of the bacteria. Tyrosine kinase inhibitors block invasin-promoted bacterial uptake (168) and also invasion by EPEC isolates, but not by *Salmonella* spp. (167). Certain plant lectins can damage microvilli. Although it is impossible to reproduce all of the ultrastructural changes associated with EPEC (94), the A/E lesion may be produced as a result of an EPEC component with lectin-like activity binding to a specific eukaryotic receptor.

By using data from various studies, a hypothetical pathway for the development of the EPEC A/E lesion may be formulated on the basis of observed ultrastructural, physiological, and cytoskeletal changes of the A/E and their similarity to the changes induced by hormones that increase intracellular calcium concentrations (Fig. 4) (11, 14). Binding of some hormones (e.g., angiotensin II) to membrane receptors activates the enzyme phospholipase C which cleaves membrane phosphatidylinositol lipids, generating the secondary messengers inositol-1,4,5-triphosphate and diacylglycerol (11). The first stage of the A/E lesion may involve binding of an EPEC component to a eukaryotic membrane receptor. Following binding, tyrosine protein kinase activity is induced, possibly in the receptor itself. This enzyme then begins to phosphorylate a variety of proteins, one of which may be phospholipase C, which then generates the secondary messengers inositol-1,4,5-triphosphate and diacylglycerol. Inositol-1,4,5-triphosphate causes release of calcium from calmodulin stores. The raised intracellular calcium levels activate an actin-binding protein, villin, which becomes an actin-severing protein. This causes breakdown of the structural integrity of the actin cytoskeleton with microvillus destruction (14). Raised calcium levels may also activate calcium-calmodulin-dependent protein kinases (11); these, in conjunction with calcium-phospholipid-dependent protein kinase C activated by diacylglycerol, may bring about phosphorylation of a variety of cytoskeletal proteins including myosin, vinculin, and α-actinin, affecting cytoskeletal structure and function. This reaction may contribute to microvillus vesiculation and destruction and accumulation of phosphorylated proteins.

The major substrate for tyrosine protein kinase is a 90-kDa protein, the function of which is unclear at present. This protein and the other phosphorylated cytoskeletal proteins then aggregate under EPEC in an organized manner. Intimin may serve as an organizer for aggregation of the proteins, as an *eaeA*-deficient mutant does not show the same degree of protein aggregation (167). Binding of intimin to its own specific receptor may in turn expose other receptors to which the proteins responsible for A/E activity bind, thus augmenting this activity. Alternatively, intimin binding may produce nonstructural cytoskeletal alterations that allow the cytoskeleton to be more easily modified by other products or which allow the cytoskeleton or an intracellular receptor domain to act as a focus for accumulating phosphorylated proteins. In some instances, the cytoskeletal alterations may be such that the bacteria are internalized, but this is discussed in more detail below.

After performing ultrastructural studies of biopsy material from EPEC-infected infants, Rothbaum et al. (170) noted that the most prominent change in the small intestinal mucosa was

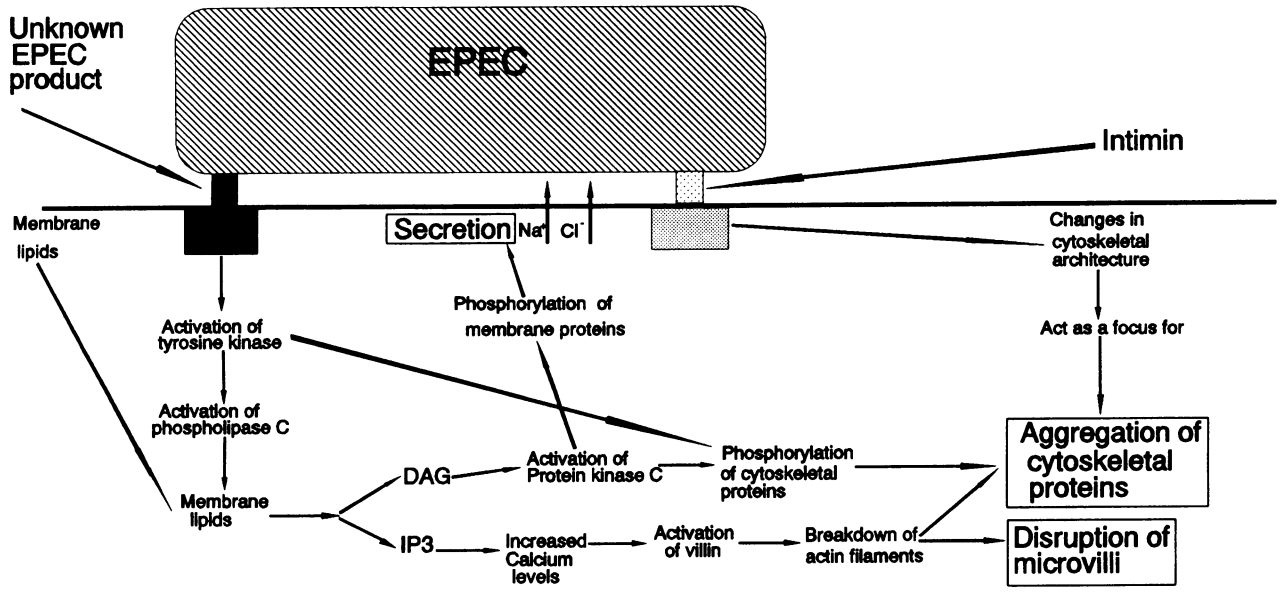

FIG. 4. Schematic representation of possible events and pathways leading to the development of the A/E lesion and fluid secretion following adhesion of EPEC to the intestinal mucosa. ■ and ⊡, eukaryotic receptors for EPEC components.

a reduction in absorptive surface area. Destruction of microvilli could reduce the activity of and bring about release of mucosal enzymes such as disaccharidases and peptidases. These alterations would prevent absorption of salt and water with resultant development of diarrhea (170). Reduced enzymatic activity has been demonstrated in infant intestinal mucosal biopsy samples (198, 205) and in in vitro EPEC-infected organ cultures (16, 62). Intracellular damage and disorganization have also been noted; these could also contribute to a defect in epithelial cell absorptive function (156, 170).

Although degeneration and effacement of microvilli may account for diarrhea through a malabsorption mechanism, the short incubation period seen in volunteer studies suggests that a direct secretory mechanism may exist (51). A possible pathway for secretory diarrhea caused by EPEC is that specific membrane proteins associated with ion transport may become phosphorylated by protein kinase C (11). In animals, stimulation of protein kinase activity produces a degree of hypersecretion similar to that produced by cholera toxin (11). Activation of protein kinases may therefore have a dual role in causing both microvillus disruption or cytoskeletal alterations and fluid secretion. A recent study (30) has shown that EPEC cause a decrease in transepithelial electrical resistance across polarized monolayers of epithelial cell cultures. This decrease is mediated by a transcellular pathway and may be involved in diarrhea. Mutants with qualitative or quantitative deficiencies in the ability to produce the A/E lesion fail to cause decreased tissue resistance.

The mechanisms described are based on findings observed when EPEC and other pathogens infect cells and are likely to require modification or adaptation as further experimental data become available. The scheme is therefore tentative, but it serves as a useful framework for planning further studies.

The difference in response between EPEC, which essentially remains extracellular, and *Y. pseudotuberculosis* and *Salmonella typhimurium*, which are rapidly invasive, may be due to the receptors to which these organisms bind, the different signal transduction pathways that are activated, and different proteins that are phosphorylated and the consequent effects on function. Alternatively, bacterial characteristics such as hydrophobicity may influence the type of bacterial-eukaryotic interaction.

As mentioned above, production of the A/E lesion is not restricted to *E. coli*; some strains of *C. freundii* (177) and *H. alvei* (2, 4) have been shown to produce this lesion. The latter have been implicated as a cause of diarrhea in infants. In both groups of organisms, a gene homologous to the *eaeA* gene has been identified (4, 177). These observations indicate that other genera of enterobacteria may be enteropathogens by mechanisms analogous to those of EPEC. *Helicobacter pylori* produces a morphologically similar lesion consisting of microvillus effacement, but it does not induce accumulation of actin or hybridize with an *eaeA* gene probe (55).

Identification of the genes responsible for the A/E lesion may be assisted by the detection of an EPEC and an *E. coli* O157 isolate in which A/E activity is encoded on high-molecular-weight plasmids (70, 202). In the EPEC isolate, cloned plasmid fragments do not confer A/E activity on laboratory *E. coli* strains, although the native plasmid does, implying that a number of physically separate plasmid genes encode A/E activity (70). The A/E activity of a laboratory *E. coli* strain transformed with the EPEC plasmid is considerably lower than that of the parent EPEC strain, suggesting that the plasmid-encoded process is distinct from the chromosome (*eaeA*)-dependent process found with most EPEC strains.

Adhesion and Its Role in Development of the A/E Lesion

Although the presence of the EAF plasmid increases the number and intensity of A/E lesions produced by EPEC isolates (115) and is essential for full virulence (127), adhesins other than BFP can cause EPEC to adhere to HEp-2 cells. An EAF plasmid-cured EPEC strain transformed with either a plasmid encoding type 1 pili or an afimbrial adhesin gene adhered to HEp-2 cells and produced a positive FAS reaction (72). The strain expressing type 1 fimbriae showed a weaker

FAS reaction and was less invasive than the strain expressing the afimbrial adhesin and the EAF-positive parent EPEC. It is unlikely that the plasmids used in the transformations have the *perA* gene; therefore, sufficient intimin may have been produced to mediate intimate adherence and allow development of the A/E lesion (72). Alternatively, the 94-kDa protein may have been induced by growth in the cell culture medium used in the FAS test (209), which may have enhanced its production in the absence of a *perA* gene. Initial adherence to cells by any adhesin may be sufficient to allow the factors necessary for the development of the A/E lesion to interact with the eukaryotic membrane. Although both type 1 fimbriae and the afimbrial adhesin produce a diffuse type of adhesion, the difference in FAS reactions observed may be due to the closer proximity of adherent cells with the afimbrial adhesin, allowing intimin and the products involved in the A/E lesion to interact with the membrane.

Rafiee et al. (157) studied EPEC RDEC-1 in the rabbit model. They found that in this strain, which produces A/E lesions in vivo, the fimbrial adhesin AFR/1 binds to a membrane glycoprotein involving sialic acid. The receptor also appears to be associated with cytoskeletal components via myosin links. Binding of the AFR/1 fimbriae may influence the development of the A/E lesion by inducing small but subtle changes in the cytoskeleton. The BFP of human EPEC may have a similar function, although as Francis et al. (72) have demonstrated, other adhesins can be substituted for BFP, and therefore, any changes induced by BFP are not essential for development of the A/E lesion.

The role of genes such as *perA* as a requirement for full A/E activity has been questioned. Cantey and Moseley (31) worked with the rabbit enteropathogen RDEC-1, which although possessing plasmid-encoded fimbriae AFR/1, does not adhere to HEp-2 cells. When RDEC-1 and an AFR/1-negative mutant were transformed with a plasmid construct containing the minimum DNA to specify DA, both strains showed DA to HEp-2 cells and actin aggregation, implying that the *perA* gene is not essential for development of the A/E lesion. However, the isolate containing both the DA and native AFR/1 plasmids invaded cells at a fourfold-higher rate than the isolate containing the DA plasmid alone. The native plasmid appears to enhance HEp-2 invasiveness of RDEC-1, although the adhesin coded for on this plasmid does not interact with these cells. Genes on the native plasmid of RDEC-1 may regulate the expression of invasion in a manner similar to *perA* regulation of intimin, as described for human EPEC. Such loci are found in many EPEC strains (83) and, although not essential for full A/E activity and invasion, may enhance both traits.

Many EPEC isolates possess type 1 fimbriae (40, 63), although the influence of these fimbriae in HEp-2 adhesion is often masked by incorporation of mannose into the adhesion assay. Andrade and de Santa Rosa (7) have shown that type 1 fimbriae and LA may interact to enhance the adhesive capabilities of EPEC and this interaction may occur in vivo.

CELLULAR INVASION BY EPEC

Compared with *Shigella* spp. and EIEC, EPEC bacteria were historically considered to be noninvasive on the basis of negative reactions in the Sereny test (79, 125). However, Sereny-negative *Shigella* strains able to invade epithelial cells have been described, and other invasive organisms such as salmonellae are Sereny negative (46). Thus, the fact that EPEC are uniformly Sereny negative does not preclude their being invasive in other systems. Indeed, numerous studies have demonstrated cellular invasion in animal models (1, 140, 156, 193, 204) and in human infant intestinal biopsy (205). In various in vitro systems in which invasion can be quantitated, EPEC bacteria have been shown to invade cells with high efficiency (7, 8, 31, 45–47, 69, 139, 161, 164). Most in vivo studies, however, have not demonstrated invasion (37, 169, 170, 198), and the extent to which this occurs in natural infection is unclear. As EPEC diarrhea is usually nonbloody, invasion is unlikely to be a major process in natural infection. If in vivo invasion does occur, it may protect the organism from clearance from the intestinal tract and by the immune system, allowing prolonged infection to occur (72).

The ability of EPEC to invade cell cultures was as great or greater than that of EIEC in some studies (46, 47); however, the methodology of some invasion assays has been criticized (164). Robins-Browne and Bennett-Wood suggested that strongly adherent bacteria such as EPEC may give artificially elevated invasion values, and they argued that EPEC organisms were less invasive than EIEC (164). However, EPEC organisms invade efficiently because many organisms adhere even though only a small proportion of adherent organisms invade. EIEC organisms do not adhere well, but a large proportion of bacteria that adhere invade. Overall, the number of EPEC able to invade is high and comparable to EIEC. Invasion of cell cultures by EPEC does not appear to be as destructive to the cell as invasion by shigellae or EIEC; presumably, EPEC isolates lack genes involved in cellular damage (72), although the killing effect of EPEC on cells is disputed (13, 30).

Riley et al. (161) questioned the invasive ability of EPEC shown in some studies. They proposed that some organisms in dense aggregates of LA may be protected from the effects of antibiotics that are added to cell cultures to distinguish internalized from external bacteria. Because of this, the invasive ability of LA EPEC may be artificially elevated, and they argued that EPEC invasion may be a rare event. However, poor recovery of EPEC from cell cultures treated with cytochalasins (which prevent invasion) (47, 72) would argue against aggregation being a protective mechanism.

There is no doubt that some internalization of EPEC takes place in in vitro systems because cytochalasin, a microfilament inhibitor; colchicine, a microtubule inhibitor; and tyrosine kinase inhibitors all reduce EPEC invasion (6, 47, 72, 168), implying roles for actin filaments, microtubules, and tyrosine kinase in the mechanism of EPEC invasion. The role of microtubules suggests that the method of entry of EPEC is different from that of other invasive enteric bacteria which do not use microtubules (47, 72). The invasive ability of EPEC appears to be related to the development of the A/E lesion because transposon insertions into chromosomal sites that affect invasion also prevent A/E lesion development and intimin-deficient EPEC mutants are noninvasive (45). Transposon insertion mutants that have normal FAS activity but are invasion deficient have been detected. Such strains may be unable to survive the intracellular environment because of a mutation in an essential gene, or they may lack other genes necessary for triggering the uptake pathway (45, 167). If the latter hypothesis is the case, then invasion may be a separate and distinct phase of EPEC pathogenesis requiring its own product and unique signal transduction pathway. Andrade et al. (6) showed that once EPEC attached to the plasma membrane they were rapidly internalized. They proposed a mechanism of endocytosis involving active mobilization of the plasma membrane by interaction between actin filaments and myosin. Others suggest that invasion may occur as an end result of A/E lesion development (67, 167). Differences in

strains or growth and assay conditions may account for these discrepant findings.

Fletcher et al. described an EPEC isolate, K798, that had the A/E ability encoded on a large plasmid (pLV501) (70). This isolate is noninvasive in a rabbit implant model; however, *E. coli* DH1 transformed with pLV501 is invasive in this system (69). A 4.5-kb DNA fragment from pLV501, pLV527, confers invasive properties, but the size of this fragment mitigates against a multigenic process for invasion such as that seen in shigellae. Invasiveness encoded by pLV527 in DH1 was 30- to 50-fold higher than that encoded by pLV501 in DH1; this may be a copy number effect. Thus, pLV527 may encode a product similar to *Y. pseudotuberculosis* invasin, although the genes are dissimilar (69). Only 8 of 46 EPEC strains examined hybridized with sequences on pLV527, and of these, only 1 strain was capable of invasion in a rabbit ileal explant model, suggesting that this gene and this invasion mechanism are rare in EPEC (69). Lack of expression of the invasive locus in the native EPEC may be explained by repression of the invasive phenotype by other EPEC genes.

Riley et al. (161) studied an EPEC isolate that possessed the genes for O111 antigen and LA on a large plasmid. They demonstrated that HeLa cell invasion occurred more frequently with the plasmid-cured rough strain than with the smooth parent strain. They proposed that a chromosomal factor in this isolate mediated intimate contact with the HeLa cells, initiating a phagocytosis-like response, and that a plasmid product such as the O antigen aborted or prevented complete internalization, which might, in turn, lead to the development of the A/E lesion (161).

Most EPEC isolates are hydrophilic due to the presence of neutral LPS with electrophoretic mobilities distinct from the LPS of other pathogenic *E. coli* (101). The hydrophilic properties may be responsible for preventing internalization. The O antigen may also maintain surface proteins in a particular configuration favoring development of the A/E lesion rather than invasion. Changes in the composition of the LPS core of *E. coli* have pleiotropic effects on the expression of outer membrane proteins (93). In *Shigella* spp., mutations that affect the mobility of the LPS core result in mutants that exhibit delayed Sereny reactions (93). In the rabbit pathogen RDEC-1, the O15 somatic antigen is thought to prevent uptake of the organism by M cells in the Peyer's patch lymphatic tissue (98). A recombinant RDEC-1 strain which has the same O antigen as *S. flexneri* is taken up by Peyer's patch M cells and is less virulent than the parent RDEC-1. Finlay et al. found that noninvasive salmonella mutants often possessed altered LPS (68). However, it should be noted that none of the noninvasive EPEC mutants studied by Donnenberg et al. (45) had altered LPS. Development of the A/E lesion may therefore depend on the bacterium possessing complete O antigen to maintain critical bacterial determinants in the correct conformation and inhibiting internalization by enterocytes by reducing hydrophobic interactions.

EPEC may possess two invasive mechanisms, one associated with the development of the A/E lesion and the other encoded by pLV501, which is found in a small proportion of EPEC. Both mechanisms may be repressed by possession of a complete O antigen. However, the extent and role of EPEC invasion in vivo remain to be determined.

MICROBIOLOGICAL DIAGNOSIS OF EPEC INFECTIONS

EPEC constitute a group of *E. coli* organisms which cause infantile diarrhea by a unique mechanism characterized by attachment to intestinal mucosa with effacement of the microvillus border. This capability and the lack of SLT genes differentiates them from other classes of *E. coli* and serves as a useful definition of EPEC. Diagnosis of EPEC infections has traditionally been performed by identifying organisms belonging to a number of specific EPEC O serogroups or serotypes which were epidemiologically linked to diarrhea. Although many strains belonging to these serogroups are true EPEC, many are not; some may be EAggEC (117, 181) or EHEC (122, 124, 183), and many are nonpathogens (116). In addition, some *E. coli* isolates with other O serogroups should be classed as EPEC as they show LA and A/E or FAS activity (1, 42, 116). Some O serogroups traditionally regarded as being EPEC, e.g., serogroups O18, O20, O28, O44, and O112, should be excluded from this definition of EPEC as they either are enteroaggregative (132) or have not been detected in recent studies with DNA probes or the FAS test (42, 58, 65, 82, 116, 128, 146, 191).

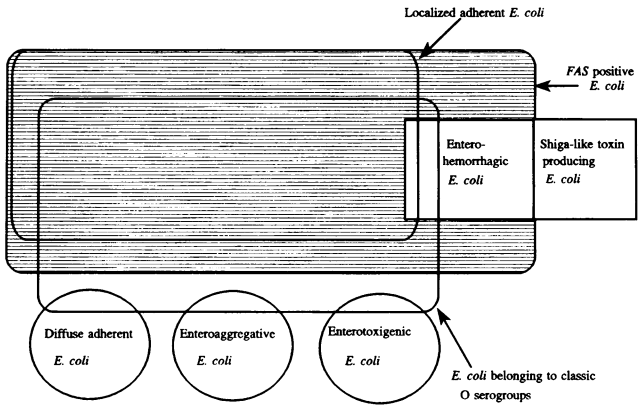

FIG. 5. Relationship between serogroup and pathogenic status of *E. coli* isolates belonging to the classical EPEC O serogroups. True EPEC bacteria (shaded area) are *E. coli* that are FAS positive and do not produce SLTs. They may or may not show LA, and not all belong to classic O serogroups. Some isolates belonging to classic EPEC O serogroups which are FAS negative may belong to the DAEC, EAggEC, or ETEC group. EHEC form a subset of FAS-positive *E. coli*; some isolates belong to classic EPEC serogroups, whereas others do not. SLT-producing *E. coli* form another group distinct from EHEC in that the organisms are FAS negative.

The relationship between possession of a classical EPEC O serogroup and pathogenic status is shown in Fig. 5. It is clear that possession of a traditional EPEC O serogroup does not make an organism pathogenic; diagnosis of EPEC will always be problematic until there is a definition of EPEC that is based on virulence properties rather than serological characteristics (116). Partly because of this fact, it has been argued that serogrouping should be discontinued (141); this raises the problem of the need for alternative methods to be used to detect and identify EPEC.

Detection methods other than serogrouping or serotyping involve screening isolates for LA using cell cultures (42, 146); this method has drawbacks in that nonadherent EPEC would not be detected. Use of the EAF DNA probe as a means of detecting adherent EPEC would suffer similar disadvantages. In addition, EAF-positive *E. coli* belonging to non-EPEC serogroups which may be nonpathogenic may be isolated (36, 56, 81, 82, 128). The EAF probe, however, has proved highly effective at detecting EPEC in many field studies (56, 65, 80, 82, 106, 128, 144, 146). A DNA probe for the BFP is likely to be more specific than the EAF probe and can detect more

EPEC than the EAF probe (76). It may also detect isolates such as those of serogroup O128, which are HEp-2 adherent but EAF probe negative (180, 181), but will fail to detect HEp-2-nonadherent EPEC.

The FAS test developed by Knutton et al. (114) is highly specific for EPEC and EHEC and has been successfully used as a screening test to detect EPEC in a number of studies (56, 116, 186). Two of these studies have shown that FAS-positive isolates may belong to serogroups other than the classical EPEC O serogroups (56, 116), a finding confirmed by others (1, 42). The hazardous nature of the phallotoxin used in this test is likely to prevent its widespread use as a diagnostic tool (116). The use of DNA probes to detect the *eaeA* gene would appear to be a promising diagnostic test as reactivity with this probe is highly specific for EPEC and EHEC (102, 103). When the effacing genes are eventually identified, DNA probes for these are also likely to be highly specific for EPEC.

Immunological tests for detecting EPEC have been developed. Albert et al. (3) described an enzyme-linked immunosorbent assay for identifying LA *E. coli* based on the detection of uncharacterized EAF plasmid-associated antigens. However, its use in field trials has not been evaluated. Immunological tests to detect intimin could be a useful means of detecting EPEC; however, in isolates that have lost the EAF plasmid, expression of this protein may be reduced and it may not be detectable (102). Alternatively, immunological tests for the products of the effacing genes may be used.

A major obstacle to the diagnosis of EPEC infection is that this disease is a problem in geographical areas with poor socioeconomic conditions and where facilities for cell culture and DNA probes are not widely available (146). In these areas, serogrouping followed by serotyping remains the only practical approach to diagnosis. However, as the distribution of EAF-positive EPEC serotypes varies from country to country (82), the choice of antisera with which to test would be dictated by the serotypes prevalent at different locations.

In developed nations, the very low frequency of infection due to EPEC of classical O serogroups has led to suggestions that it is no longer useful or cost-effective to examine fecal samples for these organisms (141, 214). However, surveys should be undertaken to evaluate the incidence and role of EPEC belonging to serogroups other than the classical O serogroups in sporadic infantile diarrhea (1). If such organisms are found to be common, diagnostic tests such as gene probes or immunological tests are likely to prove most useful for their detection.

THERAPY OF EPEC INFECTIONS

The mainstay of EPEC infection therapy is similar to therapy of other diarrheal diseases in that fluid replacement and dehydration control are of prime importance (24). Such measures alone, however, may not bring about resolution of the infection (205), and antibiotic therapy is often used, although it is not clear whether this is needed. A controlled trial in Ethiopia has shown a beneficial response to antibiotics, which brought about complete resolution of diarrhea in 76% of cases within 3 days, whereas only 7% of control infants were cured (200). Other studies have documented a beneficial response to antibiotics particularly in cases of prolonged EPEC infection (37, 169, 205). Gorbach suggested that antibiotics are unnecessary for the treatment of diarrhea except in neonates (85). There is evidence, however, that antibiotics are also beneficial in older children with EPEC infection. In one report of three infants, 7 to 8 months old, with a 3- to 4-week history of diarrhea that had become life-threatening, a 5-day course of antibiotics resulted in resolution of the diarrhea (96).

In some areas of the world, antibiotic resistance among EPEC is very common (81, 87, 142, 152, 184, 200), and susceptibility testing must be performed before the institution of therapy. Enteral feeding with infant formulas may result in worsening of diarrhea, even after EPEC eradication with antibiotics, as the damaged mucosa may require time to fully recover its functions of digestion, absorption, and defense (169). Nutritional support through intravenous alimentation may be needed to allow this recovery of the damaged mucosa to take place (169). Breast-feeding has a protective effect and should be continued during the illness (171). The protective effect may be due to a combination of immunoglobulin A antibodies to EPEC components (43), or the presence of oligosaccharides that inhibit adhesion (43), or a direct inhibitory effect of lactoferrin on EPEC (25).

At present, there is no vaccine available to prevent EPEC infection. Now that knowledge of the virulence attributes of these organisms are known, it may be possible to design vaccines to control this infection.

CONCLUSION

EPEC bacteria remain an important cause of diarrhea, particularly in underdeveloped countries; consequently, there is a need to understand the pathogenesis of infection in order to develop effective treatments or vaccines. EPEC pathogenesis is a complex mechanism involving a variety of different structures subject to complex environmental and genetic regulation. The initial stage of infection occurs by adherence of the organisms to microvilli. This is mediated by fimbriae which cause bacterial aggregation to each other and to the microvillus surface. The fimbriae are plasmid encoded, but at least one chromosomal locus as well as environmental factors may be involved in regulating expression. Following adhesion, a number of chromosomally encoded factors appear to interact with microvilli, triggering signal transduction pathways possibly through increases in tyrosine kinase activity. These bring about increases in intracellular calcium levels, activating actin-severing enzymes and protein kinases, which then leads to vesiculation and disruption of microvilli. The bacteria then attach to the epithelium in a more intimate fashion, possibly mediated by the outer membrane protein intimin, which is chromosomally encoded but is regulated by at least two loci on the EAF plasmid and possibly also by environmental factors. Intimate adherence brings the organisms into closer apposition with the eukaryotic membrane, allowing maximal activation of protein kinases and resulting in major changes to the cytoskeleton and to alterations in ion permeability of the membrane. These changes lead to the characteristic A/E lesion in which EPEC are surrounded by phosphorylated cytoskeletal proteins. Changes in ion permeability brought about by phosphorylation of the membrane result in ion secretion or reduced ion absorption, resulting in secretory diarrhea. This effect is amplified by destruction of the microvillus layer, which prevents absorption of nutrients and ions from the lumen.

The virulence determinants of EPEC are an amalgam of those found in other enteric pathogens. The BFP of EPEC are closely related to the toxin-coregulated pilus of *V. cholerae*. The intimin protein shares significant homology with the invasin protein of *Y. enterocolitica*. Intimin production and the genes involved in producing the A/E lesion are found in some other enterobacteria, such as *H. alvei* and *C. freundii*. The *perA* locus which regulates intimin production is related to a regulatory gene found in *Shigella* spp.

EPEC virulence is highly complex, and there is much yet to be learned concerning the mechanisms that take place during infection. Areas for future research include identification of the genes and their products which cause the A/E lesion and the mechanism(s) by which they cause signal transduction, identification of the membrane receptors for the BFP and intimin, examination of the regulatory mechanisms for these products, and the mechanisms and role of invasion in disease. Other areas of research are identification of host or bacterial factors that predispose to protracted infection, identification of other adhesins that may be present in HEp-2-nonadherent EPEC, and epidemiological and microbiological evaluation of the role in infection of non-EPEC O serogroups that are FAS positive and EAF positive or negative.

A great deal has been learned in the last 15 years concerning the virulence of EPEC. In particular, the use of genetic techniques has significantly improved our understanding of EPEC pathogenesis. Continuation of this work will lead to a better understanding of EPEC and how these organisms cause disease, which it is hoped will result in better control and eventually eradication of this infection.

ACKNOWLEDGMENTS

I thank David Denning and David Acheson for useful comments during the preparation of this review. I also thank Stuart Knutton for providing the photographs.

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