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Pathogenesis of Human Diffusely Adhering *Escherichia coli* Expressing Afa/Dr Adhesins (Afa/Dr DAEC): Current Insights and Future Challenges

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This article is dedicated to the memory of Arlette Darfeuille-Michaud (UMR 1071 "Microbes, Intestine, Inflammation and Host Susceptibility," Inserm, Inra, and Université d'Auvergne, Clermont-Ferrand, France).

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SUMMARY

The pathogenicity and clinical pertinence of diffusely adhering *Escherichia coli* expressing the Afa/Dr adhesins (Afa/Dr DAEC) in urinary tract infections (UTIs) and pregnancy complications are well established. In contrast, the implication of intestinal Afa/Dr DAEC in diarrhea is still under debate. These strains are age dependently involved in diarrhea in children, are apparently not involved in diarrhea in adults, and can also be asymptomatic intestinal microbiota strains in children and adult. This comprehensive review analyzes the epidemiology and diagnosis and highlights recent progress which has improved the understanding of Afa/Dr DAEC pathogenesis. Here, I summarize the roles of Afa/Dr DAEC virulence factors, including Afa/Dr adhesins, flagella, Sat toxin, and *pks* island products, in the development of specific mechanisms of pathogenicity. In intestinal epithelial polarized cells, the Afa/Dr adhesins trigger cell membrane receptor clustering and activation of the linked cell signaling pathways, promote structural and functional cell lesions and injuries in intestinal barrier, induce proinflammatory responses, create angiogenesis, instigate epithelial-mesenchymal transition-like events, and lead to *pks*-dependent DNA damage. UTI-associated Afa/Dr DAEC strains, following adhesin-membrane receptor cell interactions and activation of associated lipid raft-dependent cell signaling pathways, internalize in a microtubule-dependent manner within urinary tract epithelial cells, develop a particular intracellular lifestyle, and trigger a toxin-dependent cell detachment. In response to Afa/Dr DAEC infection, the host epithelial cells generate antibacterial defense responses. Finally, I discuss a hypothetical role of intestinal Afa/Dr DAEC strains that can act as "silent pathogens" with the capacity to emerge as "pathobionts" for the development of inflammatory bowel disease and intestinal carcinogenesis.

INTRODUCTION

Human *Escherichia coli* strains are classified as commensal microbiota *E. coli*, enterovirulent *E. coli*, and extraintestinal pathogenic *E. coli* (ExPEC) on the basis of their genetic features and clinical outcomes (1). Their serotypes are based on virulence factors present in small or large virulence-associated plasmids or chromosomal pathogenicity islands (PAIs) (2) and the molecular and cellular mechanisms by which the intestinal disease is thought to be provoked. For the pathogenic enteric *E. coli* strains, six pathotypes, i.e., enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adhering *E. coli* (DAEC), were first defined by James P. Nataro and James B. Kaper (3). Recently (4, 5), a seventh group of enteric *E. coli* strains has been defined, the Crohn's disease-associated adherent-invasive *E. coli* pathotype (AIEC) (6), which have particular mechanisms of pathogenesis (7). It is noticeable that, distinct from enterovirulent *E. coli* in expressing particular virulence determinants and developing pathogenesis in extraintestinal tissues, ExPEC strains include uropathogenic *E. coli* (UPEC) (8), sepsis-associated *E. coli* (SEPEC) (9), and neonatal meningitis-associated *E. coli* (NEMEC) (10).

The diffusely adherent *E. coli* (DAEC) class of pathogenic *E. coli* (1, 3) was previously subdivided into two subclasses: DAEC expressing Afa/Dr adhesins (Afa/Dr DAEC) and DAEC not expressing Afa/Dr adhesins (11). The subclass of DAEC that does not express Afa/Dr adhesins has recently evolved. Indeed, the main member of this subclass, i.e., the diarrhea-associated DAEC expressing the *aidA* gene, encoding an adhesin involved in diffuse adherence (AIDA-I) (12–15), belongs to the newly defined second class of EPEC designated "atypical EPEC" (aEPEC) since it is *eae* positive. The EPEC class of enterovirulent *E. coli* has been recently subdivided into two subclasses: typical EPEC (tEPEC) and atypical EPEC (aEPEC) (4). The aEPEC subclass (16) comprises *eae*-positive strains that express a wide range of genes, such as *aida-1*, *fimA*, *ecpA*, *csgA*, *elfA*, *hcpA*, and *lda*, which code for known adhesive factors triggering localized adherence-like (LAL), DA, or aggregative (AA) patterns of adhesion, and that do not express-bundle forming pili (BFP), a type IV pilus encoded by the EPEC adherence factor (EAF) plasmid (pEAF), which allows interconnection between bacteria within the dense microcolonies that form the localized adhesion (LA) pattern of tEPEC.

Afa/Dr DAEC strains are associated with urinary tract infections (UTIs), pregnancy complications, and diarrhea in children of ages 18 months to 5 years, but they can also be asymptomatic intestinal microbiota strains in children and adults (11, 17). Five phylogenetic groups, including the main phylogenetic groups A, B1, B2, and D, have been identified in Gram-negative species using multilocus enzyme electrophoresis and sequence typing methods. Afa/Dr DAEC strains belong to the phylogenetic B2 group (18, 19). In commensal *E. coli* from humans (in Europe, the United States, Australia, and Japan), B2 group *E. coli* strains are predominant (20), and it is noteworthy that these *E. coli* strains displayed a high capacity to colonize epithelia (21–23). The name "Afa/Dr DAEC" was proposed in 2005 to define a family of human UTI- or diarrhea-associated clinical *E. coli* isolates harboring ad-

hesins encoded by the *afa* (24–28), *dra* (29, 30), and *daa* (31, 32) operons, having a similar genetic organization and displaying a similar receptor specificity for human decay-accelerating factor (hDAF) and members of the family of human carcinoembryonic antigen cell adhesion molecules (hCEACAMs) (11). It is important to note that the name "Dr family" has been used by Bogdan Nowicki and coworkers as dictated by the receptor specificity of Afa, Dr, and F1845 adhesins for the Dr blood group antigen (33, 34). In this review, I summarize recent advances in our understanding of Afa/Dr DAEC pathogenesis in the urinary and intestinal tracts by analyzing how the Afa/Dr DAEC virulence factors contribute to cause disease in humans.

EPIDEMIOLOGY

Detection

In order to detect *E. coli* bearing Afa/Dr adhesins, phenotype and genotype methods have been developed. Scaletsky et al. (35) and Nataro et al. (36), investigating the adhesion of diarrheagenic *E. coli* onto cultured, nonintestinal, undifferentiated epithelial Hep-2 and HeLa cells, were the first to observe three specific patterns of adhesion: diffuse adherence (DA), resulting in adherent bacteria being randomly distributed on all the whole cell surface; localized adherence (LA), where adherent bacteria form organized microcolonies randomly distributed on the cell surface; and aggregative adherence (AA), in which adherent bacteria form typical "stacked-brick" microcolonies randomly distributed on the cell surface. However, this cell adhesion assay is not suitable for the detection of enteric Afa/Dr DAEC, since several aEPEC strains also developed a DA pattern of adhesion (16). Moreover, DA adhesion onto Hep-2 or HeLa cells has been also observed for UPEC strains expressing Afa-I (37), Afa-III (28), and Dr (38).

Goluszko et al. (39) have proposed a HeLa cell receptor assay designated the diffuse clustering assay (DCA), which associates the cell diffuse adhesion of Afa/Dr DAEC with the property of Afa/Dr adhesins to promote hDAF receptor clustering around adhering bacteria (40–42). However, the DCA did not detect all the *E. coli* strains bearing Afa/Dr adhesins, considering that AfaE-VII and AfaE-VIII adhesins did not recognize hDAF (26). This is a particular drawback for the detection of human AfaE-VIII adhesin-positive ExPEC strains (43–45). Moreover, the DCA could give overestimated results since several aEPEC strains have been found to be *daaC* positive (13, 46–53).

To detect *daaC*, *daaE*, *afaB*, and *afaC* sequences, probes and PCR primers have been developed (32, 45, 54, 55). DNA probes included the following: *drb* (56), a 260-bp PstI fragment of the pIL14 plasmid (*afa-1* operon) coding for the AfaE-I adhesin of uropathogenic Afa/Dr DAEC KS52 strain (25); *daaC*, a 300-bp PstI fragment of the plasmid pSSS1 *daa* operon (57); a 390-bp I fragment of the pSLM852 *daa* operon (58); a DNA fragment homologous to *daaE* (59); and a probe designed from the M030 sequence found to be specifically present in wild-type, diarrhea-associated Afa/Dr DAEC strain C1845 (60, 61).

PCR approaches have been developed, including primers designed to amplify a 750-bp fragment of the *afaB* gene (62) and the 390-bp PstI fragment of the pSLM852 *daa* operon (63). Others PCR assays have been developed to detect all the Afa/Dr adhesins, including the afa1 and afa2 primers designed on the partial sequence of the *afa-1* gene operon overlapping the *afaB* and *afaC* genes (54), primers for *afaE-I*, *afaE-II*, *afaE-III*/*draE*, and *afaEV* (64); and primers afa-f and afa-r, which flanked a 672-bp DNA segment internal to the *afaC* gene of the *afa-3*, *afa-7*, and *afa-8* operons. Yamamoto et al. (65) established a multiplex PCR to detect UPEC-associated genes, including *afa* genes. Multiplex PCRs to detect UPEC-associated genes have been described, including *afa1* (65) or genes expressed by diarrheagenic *E. coli*, including *daaD* (66–68).

The specificity of the *daaC* probe for the characterization of diarrheagenic Afa/Dr DAEC has recently been questioned. Smith et al. (69) found that 80 of the 86 EAEC strains positive for the AEAC probe (1-kb EcoRI-PstI fragment from pCVD432) (70) also hybridized with the probe derived from the *daaC* gene. Gomes et al. (71) reported that 5 of the 197 *daaC*-positive *E. coli* isolates hybridized with the AAEC probe (*eaeA*). Recently, Snelling et al. (72) have revealed that the *daaC* probe cross-reacts with strains belonging to EAEC. This is due to 84% identity between the *daaC* locus and the EAEC fimbria II cluster gene *aafC* at the nucleotide level (73). Moreover, Montiero et al. (74), in a study investigating the presence of dispersin in pathogenic and nonpathogenic intestinal *E. coli* isolates, observed the presence of *agg3C*-positive *E. coli* strains despite the absence of expression of the pilin-encoding gene *agg3A* and have suggested that the *agg3C* primers may have cross-reacted with an Afa/Dr usher-encoding gene(s). Indeed, the biogenesis of the well-characterized EAEC adhesins AAF-I, -II, and -III and Hda (75–78) involved a periplasmic chaperone, an outer membrane usher protein, a major adhesin subunit, and a capping subunit (79–81). Afa/Dr DAEC and EAEC strains are cause of diarrheal illness in young children. The cross-reaction makes it difficult to establish a clear identification of Afa/Dr DAEC in relation to diarrhea, notably in regions of the world in which EAEC and Afa/Dr DAEC strains are known to be responsible for acute diarrhea in children. It is obvious that new PCR probes that are more specific for diarrhea-associated Afa/Dr DAEC are called for future epidemiological studies. Blanc-Potard et al. (60) have identified M030, S109, and S111 sequences in the diarrhea-associated, wild-type strain C1845. These sequences are highly widespread (77 to 80%) among Afa/Dr strains, but have low prevalence (12 to 23%) in non-Afa/Dr strains. Additionally, analysis shows that only the M030, S109, S111, and S164 sequences are present in diarrhea-associated Afa/Dr DAEC strains and absent from non-Afa/Dr ECOR strains and diarrhea-associated clinical isolates (60). Moreover, M030 positivity has been found in human enteric DAEC isolates belonging to phylogroups A, B2, and D (61). In contrast, M030 positivity has been found to be absent in ETEC, EAEC, EPEC, and EHEC isolates (61). Epidemiological studies associating probes designed from these sequences and associating probes specific for EAEC remain to be conducted in areas such as Latin America, where Afa/Dr DAEC and EAEC have been found to be prevalent in children with acute and persistent diarrhea illness (see below).

Urinary Tract Infections

The role of UPEC expressing Afa/Dr adhesins in recurrent UTIs has been clearly established (82, 83). *afaBC*/*daaC* positivity has been found in UPEC strains belonging to the B2 phylogroups (61). Epidemiological studies show that *E. coli* isolates expressing Afa/Dr adhesins are involved in cystitis in children (25 to 50%) and pyelonephritis in pregnant women (30%) (34, 53, 55–57, 84–93). In addition, these pathogenic *E. coli* strains cause UTIs in pregnant women (30, 90, 91, 94–97). In patients with a first UTI,

the presence of *E. coli* isolates expressing Afa/Dr adhesins leads to an elevated occurrence of a second UTI (56, 57, 64, 98, 99). In patients with pyelonephritis, there was a variable distribution of *afaE* subtypes in *afa*-positive strains (100). Zhang et al. (64) have found that UTI-associated and fecal *E. coli* isolates were *afaE1* positive (18%), *afaE2* positive (1.3%), *afaE3* positive (1.3%), *draE* positive (12%), *daaE* positive (1.3%), and *draE*-*afaE*3 hybrid positive (12%). Some human pyelonephritis *E. coli* isolates have been found to be positive for *afa1*-*afa2* and *afa*-f-*afa*-r PCR probes and in some cases to express the *afaE1* (5%), *afaE8* (39%), and *afaEX* (20%) operons (45). A UTI-associated *E. coli* strain generally expresses a multiplicity of adhesive factors. Foxman et al. (92), analyzing *E. coli* strains isolated from women with first-time UTIs, observed that *drb*-probe positive *E. coli* isolates displayed positivity with the type 1 pilus probe (80 to 100% positivity) and the P fimbria probe (50% positivity) and no positivity for the S fimbria probe. Szemiako et al. (101) have observed that combinations of genes encoding two adherence factors (P and Dr fimbriae or S and Dr fimbriae) in UTI-associated *E. coli* isolates result in an increased risk of translocation to the vascular system, leading to bacteremia. Moreover, *daaC*-positive, UTI-associated clinical isolates have been found to express aerobactin (89), hemolysin (56, 86, 89, 90, 92, 102, 103), and cytotoxic necrotizing factor (CNF) (19, 56, 89, 92). The same numbers of strains expressing the *drb* probe have been found in isolates from the urinary tract or rectum of women with UTIs (104).

Afa/Dr adhesins are frequently found in *E. coli* associated with pyelonephritis in pregnant women with gestational complications (30, 62, 90, 96, 97). In addition, Afa/Dr DAEC strains are associated with preterm labor/birth (82, 95, 105). Sledzinska et al. (97) have reported the presence of an *E. coli* strain harboring the combination of P and Dr fimbriae in a case of fatal sepsis in a pregnant woman who developed pyelonephritis.

Diarrhea

It is well established that pathogenic ETEC, tEPEC, aEPEC, and AIEC colonize the small intestine, EAEC colonizes small intestine and/or colon, EHEC colonizes the distal ileum and colon, and EIEC colonizes the colon (4, 5). In contrast, the intestinal site(s) colonized by diarrhea-associated Afa/Dr DAEC currently remains to be determined. There is an absence of a role of Afa/Dr DAEC in diarrhea in adults. Indeed, when the wild-type strain C1845 was inoculated in adult volunteers, none of the patients developed diarrhea, despite the strain being detected in duodenal cultures and stools (106). Moreover, examination of a large number of human diarrhea-associated DAEC strains has shown that only two carried the *daaE* gene, suggesting that the F1845 fimbria is rare among diarrheagenic DAEC strains (59). In addition, epidemiological studies in various areas of the world are inconclusive with regard to a role of *daaC*-positive *E. coli* strains in diarrhea in children or adults (13, 48, 50, 99, 107–115). However, the relationship between Afa/Dr DAEC and diarrhea in children as a function of age has been more convincingly demonstrated in age cross-sectional studies showing an increased incidence in children $<$1 to 5 years of age. These studies were conducted in the United States (116), Mexico (117), and different South American countries, including Chile (118), Brazil (58, 71, 119–121), Colombia (122), Peru (123–127), and Argentina (128), as well as in Thailand (50), Bangladesh (129), Japan (119), New Caledonia (45, 63, 130), various places in Africa (131–133), and European countries, including the United Kingdom (134) and France (135, 136). In Afa/Dr DAEC strains isolated from stools of children, the *sat* gene has been found in almost half of the diarrhea-associated Afa/DrDAEC strains and was not present in all the non-diarrhea-associated Afa/DrDAEC strains (137). Why and how Afa/Dr DAEC isolates are potential pathogens in children with an age-dependent occurrence remain to be determined. A possible explanation, but one that does not exclude other possible causes, is that in children in this age range, the intestinal epithelial barrier is not structurally and functionally mature, and therefore the strong host defense responses against infection by Afa/Dr DAEC, which are described below, are not yet functional.

The observation of *daaC* positivity of some aEPEC strains is indicative that Afa/Dr adhesins are expressed by enterovirulent *E. coli* strains other than Afa/Dr DAEC (13, 46–53). Moreover, *E. coli* isolates displaying *daaC* and *afaBC* positivities have been found among AIEC, inflammatory bowel disease (IBD)-associated, and intestinal cancer-associated (6, 138–140) strains.

Intestinal Asymptomatic Portage

Most of the epidemiological studies conducted in various areas of the world that were intended to identify Afa/Dr DAEC as cause of diarrhea in children over 5 years of age and in adults were inconclusive, as the same numbers of *daaC*-positive strains were found in cases and controls (13, 48, 50, 71, 99, 107–115, 141). This observation highlights the existence of asymptomatic carriers of intestinal Afa/Dr DAEC strains and suggests that these pathogenic *E. coli* strains can be tolerated or controlled if the mature intestinal epithelial barrier is in a healthy condition.

VIRULENCE FACTORS

Afa/Dr Adhesins

The processes by which epithelia are infected by pathogenic *E. coli* start by the attachment of bacteria to specific host cells. To do this, pathogenic bacteria express a wide variety of surface-exposed adhesins responsible for specific binding to structural or functional cell membrane-associated molecules (142). The attachments onto the target host cells allow enteric and urinary tract bacterial pathogens to resist clearance by peristalsis and micturition, respectively. The bacterial adhesion to target host cells can be more than a simple attachment due to pathogen-specific recognition of host cell membrane-associated molecules, since several of these molecules functioned intrinsically as signaling molecules or after recognition/activation recruited cytosolic signaling molecules (143). Attachment by fimbrial or afimbrial structures allows bacterial pathogens to interact with the host cell membrane to ensure the optimal delivery of their cytotonic or cytotoxic toxins in the vicinity of their membrane-associated receptors, triggering signaling events that affect transport/secretion functions or the cell structural organization. For other pathogenic bacteria, adhesive factors allow the intimate association of bacteria with the cell membrane that is necessary for the initiation and completion of signaling-controlled structural lesions, which in turn dramatically impair host cell functions. For invasive bacterial pathogens, attachment initiates an orderly series of signaling-controlled events that lead to host cell membrane rearrangements that are necessary for the achievement of bacterial cell entry followed by the development of sophisticated bacterial intracellular lifestyles.

Two major classes of adhesins are present on the bacterial sur-

TABLE 1 Characteristics of Afa/Dr adhesins and Afa/Dr-related adhesins

Adhesin	Type	Host	Receptors		
			Type IV collagen	hDAF	hCEACAMs
AfaE-I	Afimbrial	Human	Negative	Positive	Positive
AfaE-II	Afimbrial	Human	Unknown	Positive	Unknown
AfaE-III	Afimbrial	Human	Negative	Positive	Positive
AfaE-V	Afimbrial	Human	Unknown	Positive	Positive
AfaE-VII	Afimbrial	Bovine	Unknown	Negative	Unknown
AfaE-VIII	Afimbrial	Human/animal	Unknown	Negative	Negative
Dr	Fimbrial	Human	Positive	Positive	Positive
Dr-II	Afimbrial	Human	Negative	Positive	Negative
F1845	Fimbrial	Human	Negative	Positive	Positive
Distant members[a]					
NFA-I	Afimbrial	Human	Unknown	Positive	Unknown
AAF-I	Fimbrial	Human	Unknown	Unknown	Unknown
AAF-II	Fimbrial	Human	Unknown	Unknown	Unknown
AAF-III	Fimbrial	Human	Unknown	Unknown	Unknown
HdaA	Fimbrial	Human	Unknown	Unknown	Unknown

[a] Like Afa/Dr adhesins, AAF-I, -II, and -III and HdaA promote an MRHA phenotype in human erythrocytes (78).

face of Gram-negative pathogens: the fimbrial adhesins, consisting of linear homopolymers or heteropolymers, and the afimbrial adhesins, formed of single proteins or homotrimers (142). For the completion of fimbrial and afimbrial adhesins in Gram-negative pathogens, different secretion systems have been identified, including Sec-independent and Sec-dependent pathways (144). The major families of adhesive proteins (145) include the classical chaperone/usher pathway-dependent fimbrial adhesins (146, 147), the alternate chaperone/usher pathway-dependent *E. coli* surface pili (148), the extracellular nucleation precipitation-dependent curly or thin aggregative fimbrial adhesins (149), the type I secretion system-dependent afimbrial adhesins (150), the type III secretion system-dependent integral outer membrane proteins (151), the polymerization-assembled type IV pili (152), and the type V secretion system-dependent nonfimbrial trimeric autotransported adhesins (153).

The Afa/Dr family of adhesins contains fimbrial (29, 32, 75, 76, 154, 155) or afimbrial (25–27, 30, 37, 44, 64, 84, 156) adhesins (Table 1). These adhesins are encoded by genes present in operons containing five major genes, including highly conserved genes *A* to *D*, encoding accessory proteins, and more divergent genes *E*, encoding the adhesin subunits (Fig. 1A). Assembly via the FGS (with a short F1-G1 loop) and FGL (with a long F1-G1 loop) classes of periplasmic chaperones has been described, and the FGL chaperone/usher protein secretion system assembles Afa/Dr adhesins (146, 147) (Fig. 1B). The structural organization of long and short Afa/Dr adhesins develops by the assembly of the bacterial membrane usher, successive E adhesin subunits, and one D subunit capping the structure. It is interesting to note that the chaperone usher functions in UPEC to form adhesive structures such as the P pili, resulting in the orderly assembly of PapA, PapK, PapE, PapF, and adhesive PapG subunits, and type 1 pili, formed by assembly of FimA, FimF, FimG, and adhesive FimH subunits (146, 147). The PapG subunit, localizing at the tip of the fimbria, triggers recognition of host cell membrane-associated globoseries glycolipids, and the FimH subunit triggers the mannose-dependent recognition. Crystallographic and nuclear magnetic resonance (NMR) studies coupled or not coupled with mutagenesis have been used to define the functional domains of the DraE and AfaE-III-Dsc adhesins, which are required for binding to host receptors such as hDAF (157–160), hCEACAMs (160), and collagen type IV (161, 162), and to explain the differential sensitivity to chloramphenicol (161, 162).

Afa adhesins. Agnès Labigne and Chantal Le Bouguénec have extensively described the pathogenicity mechanisms of *E. coli* strains bearing the afimbrial adhesins (Afa) encoded by the *afa* operons (Table 1). The first Afa adhesin was isolated from the wild-type prototype UPEC strain KS52 by Labigne et al. (25, 37). The 6.7-kb chromosomal DNA fragment essential for a mannose-resistant hemagglutination (MRHA) phenotype in human erythrocytes and for adhesion onto uroepithelial cells contains five genes: *afaA*, *afaE*, *afaD*, *afaB*, and *afaC* (24). Adhesins AfaE-II and AfaE-III were then isolated from two other UPEC strains, A22 and A30, by Labigne et al. (84). Le Bouguénec et al. (27) isolated from the UPEC strain A30 a 9-kb plasmid region containing the *afa-3* gene cluster. The *afa-3* gene cluster contains six genes designated *afaA* to *afaF* (163). The AfaE-III and DraE adhesin subunits displayed 98% identity (of 160 amino acids, 157 are similar) (27, 30), and the *afa-3* gene cluster and *daa* operon are closely similar (31, 32, 164, 165). The atomic resolution structure for the AfaE-III subunit has been determined (158, 159, 166, 167). Nuclear magnetic resolution and biophysical studies have revealed that the structural organization of Afa-III adhesin develops by assembly onto the bacterial membrane usher of AfaE-III adhesins subunits (158, 159) capped by the AfaD-III subunit (166–168). The AfaD-III subunit also has the ability to separate from the Dr fimbriae (42). A diffuse and not well-ordered cell surface localization of the AfaD-III subunit has been observed by immunoelectron microscopy (42, 168, 169). Using chimeras constructed from the *afa-3* and *daa* operons, a study has revealed that the afimbrial or fimbrial morphologies of the adhesins were influenced by the order in the genes coding for the afimbrial or fimbrial adhesin subunits (28). The AfaE-III adhesin subunit is involved in recognizing host cell receptors (158, 159) such as the DraE and DaaE adhesin subunits (170). As shown in the analysis of epidemiological studies below, Afa-possessing *E. coli* strains have been found to be ex-

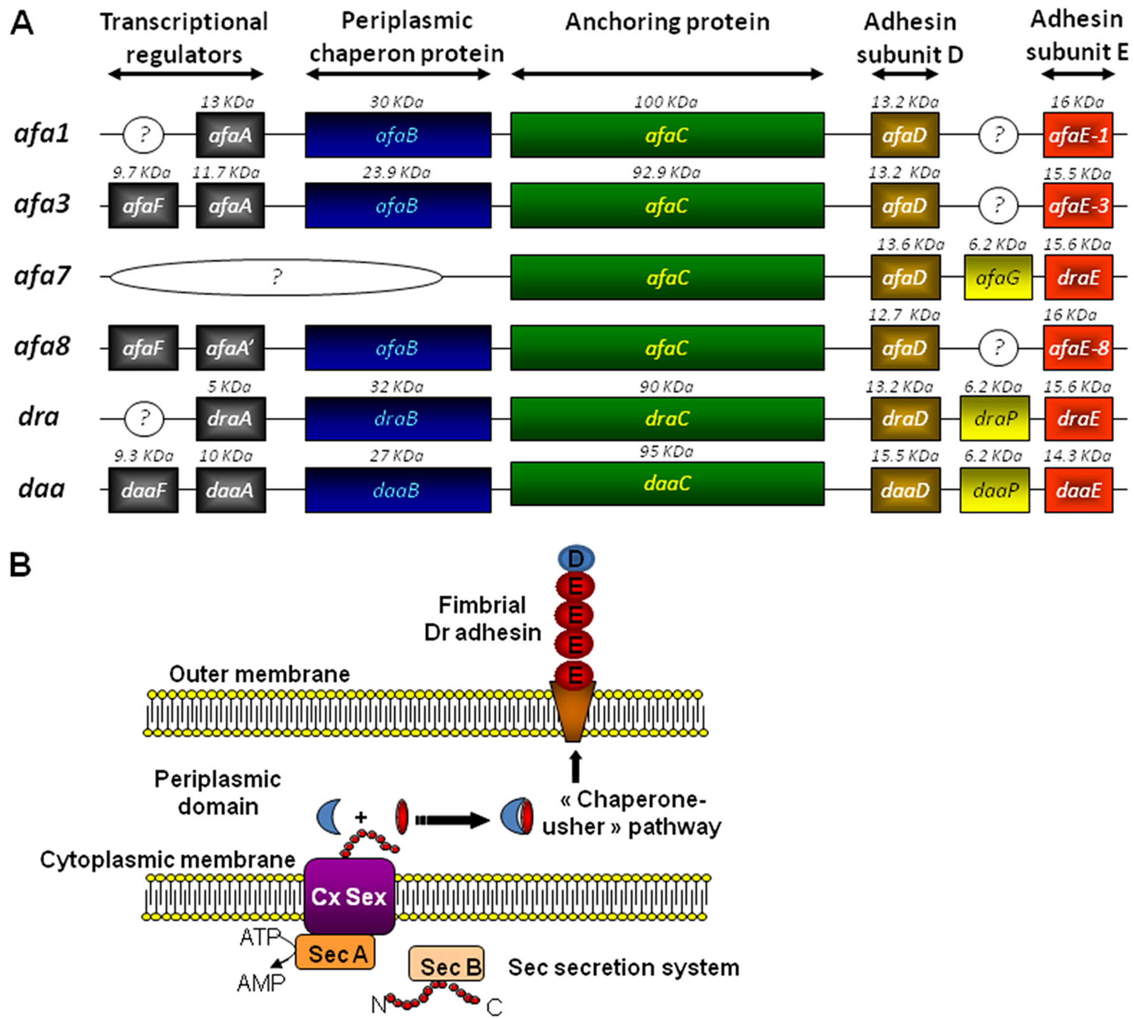

FIG 1 Genetic organization of Afa/Dr operons (A) and assembly of Dr adhesin via the chaperone-usher pathway (B).

pressed by UPEC, diarrhea-associated *E. coli*, and *E. coli* isolated from the feces of asymptomatic patients.

The *afa-7* and *afa-8* operons encode the AfaE-VII and AfaE-VIII adhesins (26, 44) (Table 1). Like the Afa-III adhesin, the Afa-VII and Afa-VIII adhesins can aggregate to form amorphous masses (26, 42). Among the four sRNA genes (171) present in the PAI_{AL862} strain and expressed by *afa-8*-positive *E. coli* (44), the AfaR small RNA, the transcription of which is temperature controlled, regulates the expression of the AfaD-VIII subunit (172). It is noticeable that despite the presence of the Afa-VIII adhesin in human intestinal *E. coli* isolates (43), these *E. coli* isolates have never been found to be responsible for diarrhea in humans (45). In contrast, in calves, pigs, and poultry with diarrhea there was the presence of *E. coli* isolates expressing sequences of the *afa-8* operon (173).

Dr adhesins. Bogdan Nowicki and coworkers have magnificently demonstrated the role of *E. coli* expressing Dr adhesins in pyelonephritis, recurrent bladder cystitis, and pregnancy complications and have also dissected their molecular and cellular mechanisms of infection. The human pyelonephritis-associated wild-type prototype UPEC strain *E. coli* IH11128 (O75) has a K5 capsule, lacks the flagellar antigen, expresses a type 1 pilus, develops MRHA, does not exert hemolytic activity, and does not produce colicin (155) (Table 1). Strain IH11128 exhibits mannose-resistant Dr fimbriae (29). Five Dr operon-associated proteins with molecular masses of 15.5, 5, 18, 32, and 90 kDa are necessary for the development of the complete MRHA (55).

The role of the FGL chaperone/usher biogenesis pathway of Dr fimbriae has been investigated in detail (174–178). To allow proper folding of the DraE adhesin subunits to occur, the DraC usher creates an assembly and secretion platform, and premature DraE subunit-subunit association is prevented by the DraB chaperone. When the DraC is lacking, there are no protein subunit secretion and fimbria assembly, since the protein complexes amass in the periplasm. Mutagenesis of the DraC N terminus shows that DraC-F4A, DraC-C64, DraC-C100A, and DraC-W142A play a pivotal role in the bioassembly of fimbriae. In the case of the E subunit, two conserved cysteine residues forming a disulfide bond are important for stabilizing elements of the immunoglobulin fold of the Dr fimbriae. With regard to the DraD subunit (179), it has been reported that when the DraE subunit assembles, the DraD subunit localizes at the tip of the fiber (158, 159, 166, 167, 180). Jedrzejczak et al. (181) have shown that the DraD subunit localizes at the tip, because it lacks a donor strand

and as a consequence functions only as an acceptor. However, expression of the DraD subunit has been found to be independent of the DraC usher, and DraD appears not to be necessary for the polymerization of DraE subunits (182). Recently, Zalewska-Piatek et al. (183) showed that the DraD subunit can be produced by a chaperone/usher-independent, type II secretion-dependent process that allows the translocation of the DraD subunit onto the cell surface of Dr-positive *E. coli*.

The major structural subunit DraE is involved in host cell receptor recognition (170), as are the AfaE-III (158, 159) and DaaE adhesin subunits (170). Calculation of the electrostatic potentials of the DraE structure shows an electronegative area around the cluster of amino acids involved in binding onto hDAF (Asp61, Asp63, and Asp75) (157). In the genes encoding Dr fimbriae, single-nucleotide polymorphisms conferring an adaptive advantage have been identified (184). Dr fimbriae are unique among Afa/Dr adhesins in expressing chloramphenicol sensitivity for binding onto host cell receptors, whereas binding of Afa-I to -III and F1845 is not affected (27, 185, 186). Korotkova et al. (187) have interestingly shown that genes encoding Dr fimbriae form eight structural groups displaying a high level of amino acid sequence diversity among them. It is noticeable that a functional analysis has revealed the presence of distinctly different binding phenotypes controlling affinity to hDAF, capability to bind collagen type IV and hCEACAMs, and sensitivity of adhesiveness capacity to chloramphenicol. Since the AfaD/DraD/DaaD subunits localize at the tip of the Afa/Dr fimbriae, their possible involvement in adhesion onto epithelial cells and, in addition, that of the AfaE/DraE/DaaE subunits has been envisaged. Conflicting results have been obtained. Recombinant DraE$^-$/DraD$^+$ or AfaE$^-$/D$^+$-III *E. coli* failed to adhere to differentiated primary bladder cells (188) and CHO-hDAF-$\alpha_5\beta_1$ cells (189), respectively. In contrast, Zalewska-Piatek et al. (190) have reported that in HeLa cells, DraE$^-$/DraD$^+$ *E. coli* displays a low level of adhesion, ~3-fold lower that of DraE$^+$/DraD$^+$ *E. coli*. In contrast to the chloramphenicol-sensitive adhesion of DraE, the DraD-induced binding is chloramphenicol insensitive (190).

The Dr-II adhesin has been isolated from the human pyelonephritis-associated strain EC7372 (Table 1). Compared to the members of the Afa/Dr adhesin family, the Dr-II adhesin displays poor sequence identity (17 to 20%) (30). Dr-II has 96% identity with the nonfimbrial adhesin I (NFA-I) expressed by UTI-associated *E. coli* (191). Interestingly, NFAs and Afa/Dr adhesins have a very similar genetic organization, and the *nfa* gene cluster encodes NfaA subunits assembled via the chaperone-usher pathway (191).

F1845 adhesin. Steve L. Moseley and coworkers discovered the diarrhea-associated *E. coli* expressing F1845 adhesin and beautifully described the structural aspects of the interaction between Afa/Dr adhesins and their epithelial cell hDAF and hCEACAM receptors. The human wild-type prototype diarrheagenic strain C1845 expresses a fimbrial adhesin, designated F1845 (Table 1). The order and regulation of the genes necessary for F1845 adhesin assembly have been identified (31, 32, 164, 165, 192–194). The F1845 and Dr adhesins display 57% identity (91 amino acids of 160 are identical) (30). Five polypeptides (10, 95, 27, 15.5, and 14.3 kDa) are encoded by *daaA*, *daaB*, *daaC*, *daaD*, and *daaE* genes, respectively. The major structural subunit, DaaE, is involved in host cell receptor recognition like the AfaE-III (158, 159) and DraE adhesin subunits (170). Bilge et al. (31) have demonstrated that the fimbrial gene expression in the *daa* operon was regulated by both phase variation and environmental regulatory mechanisms. White-Ziegler et al. (193) have reported that in response to multiple environmental signals, the histone-like H-NS acts as an overall regulator by controlling transcription of the *daa* operon.

Flagella

The biogenesis of flagella involves the coordinated structural assembly of flagellar proteins (195). A variety of flagellar structural proteins and capping proteins compose the flagellar propeller (195), and cytoplasmic membrane proteins compose the force-generating unit of the flagellar motor (196). In an aqueous environment, many bacterial species move by rotating their flagella, allowing individual bacteria to swim in three dimensions (197). Moreover, flagellar swarming coordinates the movement of bacteria across the host cell surface (198). Flagella expressed by UPEC contribute to colonization of the epithelium, dissemination to the kidney by ascending progression from the bladder, and biofilm formation (199). It has been observed that UPEC strains expressing type 1 pili or P fimbriae are less flagellated and display repressed motility, suggesting that when fimbrial expression is switched off, UPEC strains are motile (200, 201). Afa/Dr DAEC strains express or do not express flagella. The prototype pyelonephritis-associated, wild-type Afa/Dr DAEC strain IH11128, expressing a type 1 pilus, does not possess flagellar antigens (155). In contrast, the UPEC wild-type strain A30, which does express AfaE-III adhesin, is positive for flagellar antigen (unpublished data), and the animal wild-type Afa-VIII-positive strain AL511 is H8 positive (202). It is worth mentioning that that the prototype diarrheagenic wild-type Afa/Dr DAEC strain C1845 (32) does not express flagellar antigens (unpublished data). According to Arikawa et al. (203), only seven of the 19 *afaE1*-, *afaE2*, or *afaEX*-positive, diarrhea-associated *E. coli* isolates they examined are motile. In contrast, Meraz et al. (107), who examined 18 DAEC isolates, found that all nine diarrhea-associated, *afaE1*- or *afaEX*-positive *E. coli* isolates are motile. These findings indicate that UPEC and diarrhea-associated Afa/Dr DAEC display heterogeneous flagellum expression.

Secreted Autotransporter Toxin

Secreted autotransporter toxin (Sat) belongs to the type V secretion pathway-dependent subfamily of serine protease autotransporters of *Enterobacteriaceae* (SPATE) toxins (81, 153, 204). As the result of differences in the toxins structures and activities, there are two classes of SPATE toxins. Class I includes plasmid-encoded toxin (Pet) of EAEC, extracellular serine protease, plasmid encoded (EspP) of EHEC, EspC of EPEC, SigA of *Shigella flexneri* and EAEC, Sat of intestinal *E. coli* and ExPEC, and the hypothetical EspC-like SPATE toxins with EcPCN033-C1sp (NCBI accession number EGP21815.1) of ExPEC, EcNA114-C1sp (NCBI accession number AEG39156.1) of UPEC, and EcM605-C1sp (NCBI accession number ZP_08351236.1) of AIEC (204). Class II includes protein involved in intestinal colonization (Pic) of *Shigella*, EAEC, and UPEC, SepA of *Shigella*, EatA of ETEC, vacuolating autotransporter toxin (Vat)-like toxins of UPEC, SEPEC, and NEMEC, EcRN587-C2sp (NCBI accession number EFZ76879.1) of EAEC and EPEC, and EpeA of Shiga toxin-producing *E. coli* (204). Class I SPATE toxins are generally cytotoxic, whereas class II display diverse activities, including the cleavage of mucus, which provides a competitive advantage for host epithe-

lium colonization (81, 204). The *sat* gene has been characterized in the UPEC prototype strain CFT073 (205), where it resides within PAI-II$_{CFT073}$ (206–209). The *sat* gene is prevalent in UPEC strains, including those bearing Afa/Dr adhesins (8, 56, 137, 206, 208, 210–214), resident intestinal microbiota *E. coli* stains, and pathogenic strains of *E. coli*, including EAEC (137, 215–218), and *Shigella* isolates (219, 220). The *sat* gene has been found present in *daaC*-positive *E. coli* strains isolated from stools of children with diarrhea in Brazil and France (137, 218, 221). In Afa/Dr DAEC isolates, the *sat* gene has been found to be expressed equivalently by diarrheic and asymptomatic adults (222). Interestingly, the *sat* gene is prevalently expressed in Afa/Dr DAEC isolated from children in a context of diarrhea (222).

Hemolysin

The pyelonephritogenic strain EC7372, which expresses Dr-II adhesin (30), is the only Afa/Dr DAEC strain that produces a functional hemolysin. Indeed, unlike other Afa/Dr DAEC strains, strain EC7372 promotes a strong cellular lysis in epithelial cells preceded by apoptosis (102). On the basis of results reported by Blanc-Potard et al. (60), the hemolysin-positive strain EC7372 carries both the *hly* and *pap* operons and seems to have acquired a larger part of the PAIs$_{CFT073}$ (207–209) than Afa/Dr DAEC. The recombinant *E. coli* strain EC901, which carries plasmid pBJN406 and contains the *draA* to *-E* genes involved in expression of Dr fimbriae (223), has been observed to display a curious hemolytic activity. Insertion mutations in *draD* and *draE*, but not in *draA*, *draB*, and *draC*, abolish hemolytic activity, indicating that this activity is supported by the extracellular domain of Dr fimbriae. This observation is intriguing, since strain IH11128, gestational pyelonephritis Dr-positive *E. coli* isolates (94, 155), and clinical Dr-positive *E. coli* isolates (60) all lack either hemolytic activity or *hly* gene expression. In contrast, the wild-type O75X strain IH11032 does display hemolytic activity (155). Moreover, four *afaE1*-positive and one *afaEX*-positive diarrheagenic *E. coli* isolates have been found to trigger hemolysis, while 14 other *afaE1*-positive and one *afaEX*-positive isolates do not (203). Collectively, these findings show that Afa/Dr DAEC strains are heterogeneous in terms of α-hemolysin expression, suggesting a variable distribution of the part of PAI$_{CFT073}$ containing the *hly* gene among the Afa/Dr DAEC strains.

Other Factors

Blanc-Potard et al. (60) identified several short sequences (73 to 495 bp) that are prevalent in Afa/Dr adhesin-positive *E. coli* clinical isolates in comparison with *E. coli* clinical isolates not expressing Afa/Dr adhesins (GenBank accession numbers AZ935556 to AZ935604). Several sequences are homologous to virulence genes expressed in other pathotypes of *E. coli*, including genes for two siderophores (*irp2* and *iuc*), a catechol siderophore receptor (*iroN*), and two transport systems (*shu* and *modD*) (60). Interestingly, several C1845-specific sequences display no likeness with known sequences (60). Importantly, the diarrhea-associated wild-type C1845 strain does not express the genes encoding ETEC and EAEC virulence factors and is devoid of genes encoding EPEC and EHEC virulence factors, including the genes of the locus of enterocyte effacement (LEE) island involved in the type III secretion system (T3SS) or T3SS-associated effector proteins and not hybridized with *eae* probes (60). The wild-type C1845 and IH11128 strains expressed a part of PAI$_{CFT073}$ (207–209) not including the *hlyA*, *hlyD*, *hp1-hp4*, *papG*, or *papF* sequences (60). A remnant of the *pap* operon which has the F10 *papA* allele but lacks most of the central region of the *pap* operon has been detected. It is noteworthy that regions of the PAI$_{CFT073}$ complete genome sequence (207, 209, 224) have been found in *E. coli* strains of the B2 phylogenic group (208) and are prevalently expressed in ExPEC strains of group B2 involved in UTIs (8). Moreover, parts of PAI$_{CFT073}$ have recently been found in intestinal commensal *E. coli* strains, particularly those of phylogenic group B2 (225–227), and in an AIEC strain (228).

The PAI$_{AL862}$ expressed by *afa-8*-positive *E. coli* strains (44) includes the *deoK* gene, which confers metabolic adaptability and increases the competitive advantage with regard to host infectivity (229). The locus designated *vpe* (virulence-associated phosphotransferase) contains the *vpeA*, *vpeB*, and *vpeC* genes, which encode, respectively, the EIIA, EIIB, and EIIC constituents of a putative carbohydrate-specific permease of the SgaTBA family (230). This locus is present in the pyelonephritis-associated strain AL511, which expresses the *afa-8* operon (43), which confers an ability to adapt for kidney and intestinal colonization (231). The presence of the *vpe* locus in other UTI- and diarrhea-associated Afa/Dr DAEC strains has not been documented.

The capacity to form filamentous forms results from a plasticity capacity developed by a bacterial pathogen in order to escape host defenses when in an intracellular location or to assemble to form a biofilm-like structure that leads to resistance to anti-infective treatments, such as antibiotics (199). Some excellent experiments have demonstrated that type 1 pilus-positive UPEC strains, after internalization into superficial epithelial cells known as "umbrella cells" lining the luminal surface of the bladder, form biofilm-like bacterial assemblages designated "intracellular bacterial communities" (IBCs) that function as transient protective structures for UPEC intracellular growth (199, 232). UPEC cells in IBCs constitute reservoirs of UPEC, which, after switching to filamentous forms, become detached from the bacterial community and may be flushed out of the host cells. Zalewska-Piatek et al. (233) were the first to observe that that Dr-positive *E. coli* formed biofilms. This phenomenon means that Dr-positive *E. coli* strains form live filamentous bacteria, depending on their nutritional environment (190, 233). It has been observed that adhering Dr-positive *E. coli* forms filamentous forms at the cell surface of CHO-hDAF-α5β1 (189) or CHO-hDAF (234) cells. Filamentous bacteria residing within the phagosome escaped phagosomal killing as the bacteria manipulated the phagosome compartment by blocking the acquisition of hydrolytic components (235, 236). Even though the intracellular vacuole-containing Dr-positive *E. coli* in HeLa cells lack the characteristics of a degradative compartment (189), no filamentous forms of Afa- or Dr-positive *E. coli* residing intracellularly have ever been observed. This aspect of Afa/Dr pathogenesis remains to be explored in the appropriate model of bladder epithelial cells. Bacterial biofilm formed by UPEC after aggregation of three-dimensional structured cells connected by self-produced exopolysaccharide matrix plays a major role in persistent and chronic UTIs (199). Exopolysaccharide production, which plays a pivotal role in biofilm completion, has been found in UTI-associated *E. coli* strains expressing Dr (190, 233) or Afa-VIII (231) adhesins. Interestingly, exopolysaccharide production is controlled by the *vpeBC* gene (231), which is present in the *vpe* locus of *afa-8*-positive *E. coli* (43). DraE^{+}/DraD^{+} *E. coli* strains

form dense biofilms, and DraD, whether associated with fimbriae or not, plays a role in biofilm formation (190, 233).

A large variety of bacteria have been found to produce toxins, named cyclomodulins, that dramatically interfere with the cell cycle (237). Cyclomodulins produced by pathogenic *E. coli* included colibactin, cycle-inhibiting factor (Cif), cytotoxic necrotizing factor (CNF), and cytolethal distending toxin (CDT) (238). Currently, the two known genotoxins are colibactin and CDT (238). The cluster of genes known as the "*pks* island" (239) encodes a multienzymatic machinery for synthesizing the hybrid, nonribosomal, peptide-polyketide genotoxin colibactin (240). It has been suggested that the *pks* island may affect the host immune response and could be involved in chronic inflammation, in the accumulation of genomic instability, and in tumor progression (241). Whether the *pks* island contains other genes encoding additional bacterial factors and whether the *pks*-related colibactin is a prototype of a family of molecules or not remain to be investigated. The *pks* genomic island is present in the prototype Afa/Dr DAEC wild-type IH11128 and C1845 strains (J. P. Nougayrede and E. Oswald, unpublished result) and in colonic *afa-I*-positive *E coli* strains isolated from patients with IBD and colorectal cancer (140). The *pks* island has been also found in ExPEC strains of phylogenetic group B2 (242), in fecal *E. coli* strains isolated from healthy patients but not in pathogenic EPEC and EHEC isolates (243), in group B2 *E. coli* strains that are long-term colonizers of the intestine (22), in *E. coli* isolated from the mucosa of patients with IBD (244), in mucosa-associated or internalized *E. coli* of tumors and mucosa of colorectal cancer patients (244–246), and in urosepsis *E. coli* strains (247). It was noted that the intestinal microbiota *E. coli* strain Nissle 1917 expresses the *pks* genomic island and displays similarities with the prototype Afa/Dr DAEC wild-type C1845 and IH11128 strains, since it harbors parts of PAI_{CFT073} that lack the expression of α-hemolysin and P fimbriae but includes iron uptake systems (225, 227). This probiotic *E. coli* strain with diverse activities (248) is intriguing since its promotion of gut homeostasis activity in response to mucosal injury cannot be dissociated from the presence of the *pks* island (249). Whether the presence of the *pks* island in intestinal *E. coli* and ExPEC strains is deleterious for the host or without pathological consequences remains to be investigated.

MECHANISMS OF PATHOGENICITY

Host Cell Receptors for Afa/Dr Adhesins

On the basis of the differential recognition of human epithelial cell membrane-associated receptors by Afa/Dr adhesins (Table 1), Afa/Dr DAEC strains have been subdivided into two subclasses (11). The first subclass includes *E. coli* strains harboring the Afa-I (25, 37), Afa-II (27), Afa-III (27), Afa-V (64), Dr (29, 155), Dr-II (30), and F1845 (32) adhesins recognizing hDAF, which also may or may not recognize members of the hCEACAM family. The second subclass includes strains that express Afa-VII (26, 44) and Afa-VIII (26, 44) adhesins that do not recognize hDAF. In addition, the NFA-I adhesin of UPEC (191) belongs to the Afa/Dr family of adhesins (Table 1). Moreover, despite a similar genetic organization with the gene clusters triggering the biogenesis of Afa/Dr adhesins, the EAEC adhesins AAF-I (77), AAF-II (76), AAF-III (75), and Hda (78) are distant pathogenic factors of the Afa/Dr family of adhesins (Table 1). The four major characteristics of EAEC pathogenesis (79–81) are as follows: (i) adherence to the intestinal mucosa via adhesins (18, 75–78), (ii) the formation of typical "stacked-brick" microcolonies as each bacterium interacts with others, (iii) production of enterotoxins and cytotoxins, and (iv) the development of a severe mucosal inflammation. Boisen et al. (78), analyzing this superfamily of adhesins, have proposed a pertinent phylogram composed of three distinct clusters. The first cluster comprises Afa-I, Afa-II, Afa-III, Afa-V, Dr, Dr-II, and F1845, the second comprises AAF-I, AAF-II, and AAF-III, and the third comprises Afa-VII, Afa-VIII, and Hda. It is worth mentioning that cluster 3 (78) also includes the nonfimbrial M-agglutinin encoded by the *bma* gene cluster of UPEC (250).

hDAF. Nowicki et al. (33) were the first to report that human decay-accelerating factor (hDAF) (CD55) expressing the antigens of the Cromer blood group system (251) acts as an epithelial cell receptor for *E. coli* expressing Afa/Dr adhesins (Fig. 2) (Table 1).

(i) Structure and functions. DAF is a complement-regulating protein with an M_r of 55,000 to 70,000 (251). The physiological function of DAF is to control the amplification of the complement cascade by a direct interaction with membrane-bound C3b or C4b, which in turn impedes the ulterior uptake of C2 and factor B. Membrane-bound DAF is formed by a membrane glycosylphosphatidylinositol (GPI) anchor followed by a serine/threonine/proline (STP)-rich region and by four complement control protein repeat (CCP) domains, previously named short consensus repeats (SCRs) (Fig. 2). Modeling of the extracellular domain of DAF reveals that CCPs are organized in a helical manner. While CCP-1 had no effect on hDAF regulatory activity, deletion of CCP-2, CCP-3, or CCP-4 entirely abolished the regulatory activity. Interaction of DAF with the convertases is mediated predominantly by two patches approximately 13 Å apart, one centered around Arg69 and Arg96 on CCP-2 and the other around Phe148 and Leu171 on CCP-3 (252). Phe123 and Phe148, localizing at the interface between CCP-2 and CCP-3, and also Phe154, which is present in the CCP-3 cavity, are pivotal for the regulatory activity (253). The GPI anchor increases the lateral mobility of DAF within the cell membrane in relation to its localization into membrane-associated lipid rafts, and the O-glycosylated STP serves as a spacer for the projection of the hDAF functional domains at the cell membrane (253).

(ii) Receptor for Afa/Dr adhesins. hDAF is one of the receptors recognized by Afa/Dr adhesins in epithelial cells (Fig. 2) (Table 1). It is noteworthy that Afa/Dr adhesins bind specifically to hDAF but not to rodent or pig DAF (254). Dr fimbria binding develops in the digestive, urinary, genital, and respiratory epithelia and in skin (255), consistent with the hDAF expression (251). Only uropathogenic and diarrhea-associated *E. coli* strains bearing the F1845, AfaE-I, AfaE-III, AfaE-V, Dr, and Dr-II adhesins recognized hDAF as a receptor (62, 160, 256). In contrast, the Afa-VIII adhesin expressed by human ExPEC does not recognize hDAF (26, 43, 44). It has been established from functional studies and atomic resolution models that Afa/Dr adhesins recognize the CCP-2 and CCP-3 on hDAF (41, 158, 159, 257–259) (Fig. 2). In contrast, gestational pyelonephritis-associated *E. coli* expressing *dra*-related X adhesins recognized the CCP-3 and CCP-4 domains of hDAF (62). In the CCP-3, a single point substitution (Ser155-Ala and Ser165-Leu, mimicking the Dra-to-Drb allelic polymorphisms) results in a complete loss of Dr fimbria binding to hDAF (257, 260). The amino acids (148 to 171), in particular Ser155, present at the surface of CCP-3 controlled the Dr adhesin binding (260). A surface plasmon resonance study of Afa-III adhesin bind-

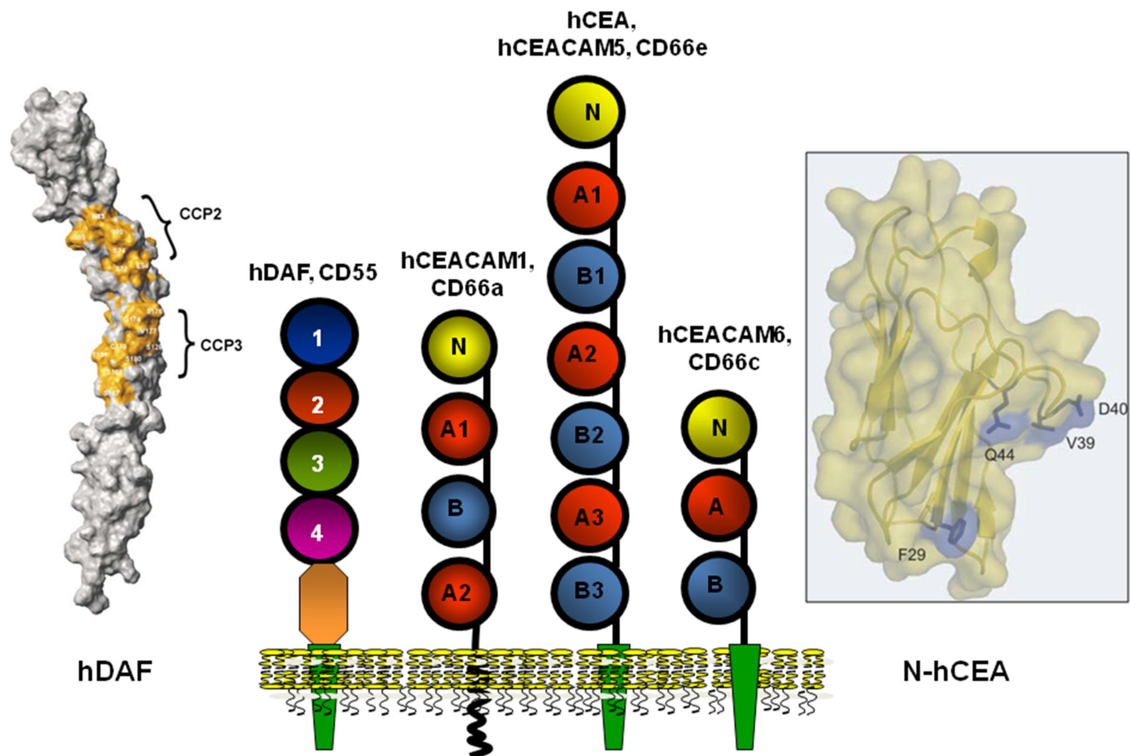

FIG 2 Membrane-associated proteins expressed by human epithelial cells that function as receptors for Afa/Dr adhesins. Center, representations of the structures of hDAF and hCEACAMs. Left, surface representation of hDAF. Right, homology model of human N-CEA. (Representations of hDAF and N-hCEA reprinted from reference 158 with permission of Elsevier and from reference 160 with permission of the publisher, respectively.)

ing onto CCP domains of hDAF has revealed that a construct formed of CCP-1 and -2 did not show any measurable binding to the AfaE-III adhesin subunit, while constructs formed of CCP-2, -3, and -4, CCP-2 and -3, or CCP-3 and -4 allowed AfaE-III binding with affinities comparable to that for the entire hDAF, confirming the previously observed importance of the combined CCP-2 and CCP-3 domains for the recognition of hDAF by Afa/Dr adhesins (158, 159). The Dr adhesin-binding and complement-regulating epitopes of hDAF have been found to be distinguishable and are approximately 20 Å apart (260). However, Anderson et al. (158) observed that the binding of AfaE-III to hDAF antagonized the hDAF regulatory activity.

The AfaE-I, AfaE-III, AfaE-V, DraE, and DaaE subunits function as receptor ligands for hDAF (27, 160, 170) (Fig. 3A to D). In DraE/AfaE-III subunits, the hDAF-binding site forms a large convex surface involving seven β strands (158, 159). The residues Asp61, Ile73, and Asn77 have been found to be important for binding to hDAF (158, 159). Mutagenesis and crystallographic studies of DaaE have been conducted in order to define the detailed molecular interactions between Afa/Dr adhesins and hDAF (157). Five *daaE* mutants (T8N, A60V, D61A, D63V, and T133S) showed a 30 to 50% reduced ability to bind onto CHO cells transfected for hDAF expression (157). Mapping the sites of DaaE reveals that positions Asp61 and Asp63 are necessary for binding to hDAF, and calculation of the electrostatic potentials of the DaaE structure has revealed an electronegative region around the cluster of amino acids involved in hDAF recognition (Asp61, Asp63, and Glu126) (157). Moreover, the ability of the DraE adhesin to bind hDAF has been found influenced by individual amino acid changes at positions 10, 63, 65, 75, 77, 79, and 131 of the mature DraE sequence (261).

Binding of the DraE adhesin subunit onto hDAF is sensitive to chloramphenicol, which also inhibits the hDAF-dependent MRHA of human erythrocytes (chloramphenicol-sensitive hemagglutination [CSHA]) (33, 256). In HeLa cells, the presence of chloramphenicol diminished the adhesion of $DraE^+/DraD^+$ *E. coli* by ~3-fold and totally abolished the adhesion of $DraE^+/DraD^-$ *E. coli* but did not change the adhesiveness capacity of $DraE^-/DraD^+$ *E. coli* (190). According to Swanson et al. (262), the domains involved in the CSHA are present within the N-terminal domain of the DraE subunit. According to Pettigrew et al. (161, 162), a hydrophobic pocket including Gly113, Gly42, Pro40, Pro43, Ile111, Tyr115, and Ile114 plays a pivotal role in the chloramphenicol-binding site in the DraE subunit. The inhibition of the binding of the DraE subunit onto hDAF by chloramphenicol has received a structural explanation, since by covering the functional portion of the adhesin subunit, chloramphenicol disrupts the recognition of hDAF (161, 162). In contrast to the case for the Dr adhesin, chloramphenicol does not affect the hDAF-dependent MRHA exerted by the AfaE-I, AfaE-III, and F1845 adhesins (27, 256). This is a result of a difference in expression of amino acids between the adhesin subunits (161, 162). Moreover, it has been established that binding of chloramphenicol onto the DraE subunit develops via the interaction of its chlorine "tail" rather than its benzene ring (161, 162). Analyzing structural chloramphenicol modifications, Pettigrew et al. (162) have demonstrated that acylating the 3-hydroxyl group has no effect on the binding onto hDAF.

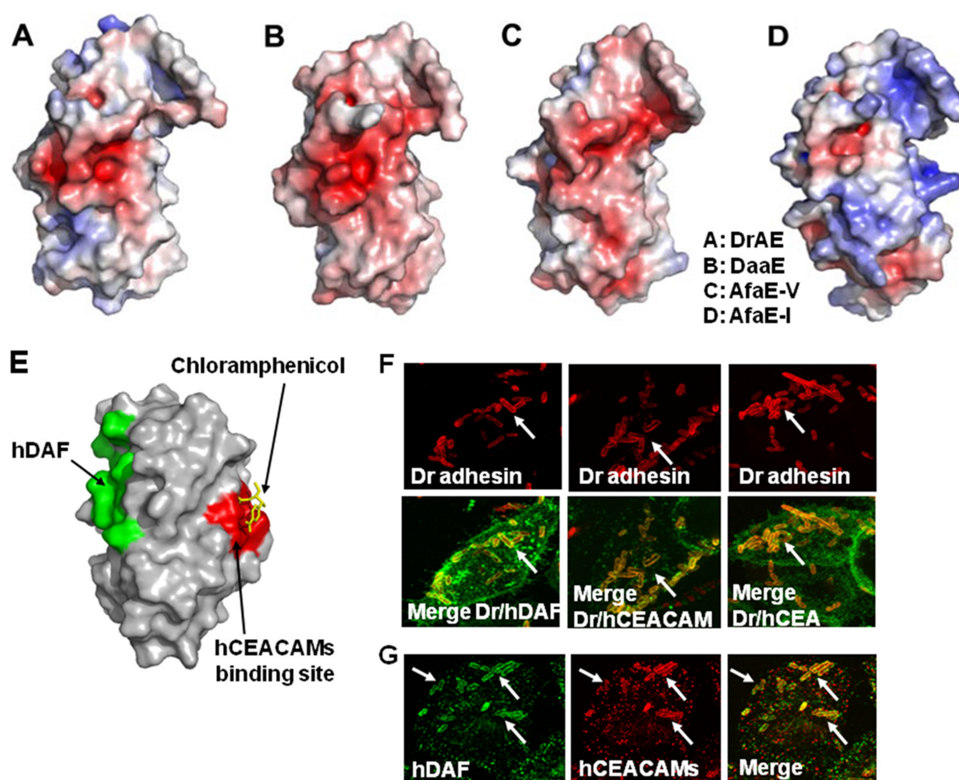

FIG 3 Receptor clustering by Afa/Dr DAEC. (A to D) Representations of the DraE, DaaE, AfaE-V, and AfaE-I adhesin subunits, respectively. Surface electrostatic potentials of the DraE, DaaE, AfaE-V, and AfaE-I adhesins (red indicates the negative charges and blue the positive charges) are shown. (Reprinted from reference 157 with permission of the publisher.) (E) Representation of DraE adhesin subunit-associated surfaces allowing the specific recognition of hDAF or N-hCEA. Green, surface recognition of hDAF. Red, surface recognition of N-hCEA. Yellow, chloramphenicol bound onto the domain of AfaE-III that recognizes N-hCEA. (Reprinted from reference 160 with permission of the publisher.) (F) Micrographs showing the observation by confocal laser scanning microscopy (CLSM) of hDAF, hCEACAM1, and hCEA receptor clustering around Dr adhesin-positive *E. coli* adhering to untransfected HeLa cells constitutively expressing hDAF and to transfected HeLa cells expressing hCEACAM1 and hCEA. Yellow shows colocalization of immunolabeling of Dr adhesin (red) and hDAF, hCEACAM1, or hCEA (green). (Reprinted from reference 274 with permission of the publisher. Copyright 2004 Blackwell Publishing Ltd.) (G) Receptor clustering of hDAF (green) and hCEACAM1 (red) around Dr adhesin-positive *E. coli* adhering onto transfected HeLa cells expressing hCEACAM1. Yellow, colocalization of immunolabelings of hDAF and hCEACAM1. Arrows show immunolabelings of interest around adhering bacteria. (Reprinted from reference 274 with permission of the publisher. Copyright 2004 Blackwell Publishing Ltd.)

(iii) Receptor for microbial pathogens and viruses. The cell membrane-bound hDAF is also hijacked by viruses, including coxsackievirus serotypes B1, B3, and B5 (263, 264) and coxsackievirus A21 (265), enteroviruses (266), and echoviruses (267, 268). Various different hDAF sites are recognized by echoviruses (269). It is worth underlining that like Afa/Dr DAEC (254), echoviruses and coxsackieviruses (270) express high specificity for hDAF. In addition, hDAF acts as a receptor for hantavirus (271). Moreover, epithelial hDAF has been identified as a gastric epithelial receptor for *Helicobacter pylori* and has been found to be upregulated by the pathogen in relation to inflammatory responses (272, 273).

hCEACAMs. Guignot et al. (41) were the first to show that hCEA (CEACAM5, CD66e) is recruited around the prototype Dr adhesin- or F1845 adhesin-positive wild-type Afa/Dr DAEC strains IH11128 and C1845, respectively, adhering to cultured human enterocyte-like Caco-2 cells and that an anti-CD66 antibody inhibits this bacterial adhesion (Fig. 2) (Table 1). Berger et al. (274), using Chinese hamster ovary (CHO) cells and human cervical cancer HeLa cell lines transfected for the expression of each of the human carcinoembryonic antigen-related cellular adhesion molecules (hCEACAMs) (CEACAM1 to 8), found that the Dr, F1845, and AfaE-III adhesins bound only to cells expressing epithelial hCEACAM1, hCEA, or hCEACAM6, whereas the AfaE-I and Dr-II adhesins did not (Fig. 2) (Table 1). Korotkava et al. (160) demonstrated Afa-V adhesin binding to hCEA (Table 1). In addition, the Dr, F1845, and AfaE-III adhesins recognize the nonepithelial CEACAM3 as a receptor (188, 189). In contrast, the murine CEACAM1 is not recognized by Afa/Dr adhesins (274).

Twelve members, i.e., CEACAM1 (biliary glycoprotein [BGP], CD66a), CEACAM3 (CEA gene family member 1 [CGM1], CD66d), CEACAM4 (CGM7), CEA (carcinoembryonic antigen, CD66e), CEACAM6 (nonspecific cross-reacting antigen [NCA], CD66c), CEACAM7 (CGM2), CEACAM8 (CGM6, CD66b), CEACAM16, and CEACAM18 to -21, compose the family of CEACAMs (275). CEACAM proteins generally have one variable (V)-like Ig domain, identified as the N domain (except CEACAM16, which has two N domains), but they differ in the number of constant C2-like Ig domains as well as in their membrane anchorage (Fig. 2). CEACAM5, CEACAM6, CEACAM7, and CEACAM8 are anchored within the cell membrane through a GPI linkage, whereas six other CEACAM family members (CEACAM1, CEACAM3, CEACAM4, CEACAM19, CEACAM20, and CEACAM21) are anchored via *bona fide* transmembrane domains (275) (Fig. 2). CEACAM16 is devoid of any membrane an-

chorage and is the only known secreted CEACAM. The CEACAM1 cytoplasmic domain has immunoreceptor tyrosine-based inhibitory motifs (ITIMs), whereas CEACAM3, CEACAM4, CEACAM19, and CEACAM20 carry immunoreceptor tyrosine-based activation motifs (ITAMs) (Fig. 2). All family members are highly glycosylated on their extracellular domains, and as a function of the cell type and differentiation state of the cells, the level of glycosylation of each CEACAM may vary, since multiple glycoforms of the same protein have been characterized. Epithelial, endothelial, and hematopoietic cells variously expressed CEACAMs (275, 276). CEACAMs function mainly as adhesion molecules engaged in homotypic and/or heterotypic intercellular adhesion, and several CEACAMs exert regulating cell signaling activities (275, 276). CEACAMs are engaged during complex biological processes such as cancer progression, inflammation, immune responses, angiogenesis, and apoptosis (275, 276).

(i) hCEACAM1 structure and functions. CEACAM1 was present in leukocytes, including granulocytes, activated T cells, B cells, and $CD16^{-}/CD56^{+}$ natural killer cells (275). It was present in endothelial cells (275). CEACAM1 was also expressed in epithelial of the stomach, intestine, bile ducts, kidney, prostate, endometrium, and mammary ducts (275). The gene encoding CEACAM1 contains 9 exons that, after alternatively splicing, generate 11 different isoforms with long or short cytoplasmic tails and long or short cytoplasmic domains (275). The long cytoplasmic domain contains two ITIMs (Fig. 2). ITIMs after tyrosine phosphorylation associate with diverse cytoplasmic signaling molecules, including the tyrosine kinases of the Src family, the tyrosine phosphatase Src homology 2 (SH2) domain-containing protein tyrosine phosphatase 1 (SHP-1) or 2 (SHP-2), and Shc (275). In contrast, the cytoplasmic S domain lacks the presence of tyrosine residues (275). The two major isoforms CEACAM1-L and CEACAM-1S can be cell coexpressed, but CEACAM1-L isoforms predominate in most cell types (275). However, the CEACAM1-L/CEACAM-1S ratio can vary as a function of the cell types and the cell differentiation states. In polarized epithelial cells, the CEACAM1-L and CEACAM-1S isoforms are expressed at both the apical domain and cell-cell contact areas (277, 278). CEACAM1 acts as a cell-cell adhesion molecule by hemophilic interaction (275). Down-expression of CEACAM1 occurred in several tumor types, such as breast, prostate, and colorectal cancer, and high levels of CEACAM1 expression are related to poor prognosis and tumor metastasis (275).

(ii) hCEA structure and functions. hCEA is a GPI-anchored protein (275) (Fig. 2). hCEA was initially defined as a tumor-associated marker, since it is overproduced in an elevated number of carcinomas. Overexpression is often associated with enhanced metastatic potential and thus with poor prognosis (279, 280). However, it is important to remember that despite its name, hCEA is normally expressed in tissues, including intestinal M cells, enterocytes, and colonic cells, in which it is abundantly expressed at the brush border (281). hCEA is localized within cell membrane-associated lipid rafts via its GPI anchor and can act as a cell membrane-bound cell signaling receptor (275). In addition, hCEA is present in the intestinal apical glycocalyx. The physiological role played by hCEA remains unknown, but it has been shown to mediate cell-to-cell Ca^{2+}-independent, homotypic interactions.

(iii) hCEACAM6 structure and functions. Like CEA, CEACAM6 is a GPI-anchored protein (275) (Fig. 2). In colorectal cancers, the deregulation of CEACAM6 expression suggests a role in tumor onset (275). CEACAM6 is brush border expressed in polarized epithelial intestinal cells. CEACAM6 has the capacity to signal in cells. For example, following CEACAM6 cross-linking, there was subsequent activation of Src that led to the tyrosine phosphorylation of focal adhesion kinase (FAK), in turn triggering cross talk with $\alpha_v\beta_3$ integrin, and cell interaction with extracellular matrix molecules (ECMs) (282, 283).

(iv) Receptors for Afa/Dr adhesins. Human UPEC and diarrhea-associated Afa/Dr DAEC strains expressing the Dr, Afa-III, Afa-V, and F1845 adhesins recognize the epithelial hCEACAM1, hCEA, and hCEACAM6 and the nonepithelial hCEACAM3 as host cell receptors (41, 160, 187–189, 274, 284) (Fig. 2) (Table 1). The Afa-I adhesin does not recognize hCEACAMs well, and the Dr-II adhesin fails to recognize hCEACAMs (274). It is not known whether hCEACAMs are recognized by the Afa-VIII adhesin. By surface plasmon resonance (SPR) binding analysis, the N-terminal domain of hCEA has been identified as being recognized by the DraE, AfaE-I, AfaE-III, AfaE-V, and DaaE adhesin subunits (160) (Fig. 2). The recognition of the N-terminal domain of nonepithelial hCEACAM3 observed by SPR analysis (187) has been confirmed in transfected cells expressing hCEACAM3 (188). The AfaE-I subunit displays lower-affinity binding to hCEA than the DraE, AfaE-III, AfaE-V, and DaaE adhesin subunits (160), which is consistent with a previous observation in transfected hCEACAM-expressing epithelial cells (274). For the hCEA/DraE interaction, the N-terminal 58 amino acids of hCEA are necessary, since the N-terminal F29I, I91A, and L95A hCEA mutants showed a decreased affinity for the DraE adhesin subunit (160, 284). Coupled mutagenesis analysis has identified residues F29, Q44, and D40, localizing in the exposed loops of the GFCC′C″ face of the N-terminal domain of hCEA, as being involved in DraE adhesin subunit binding. In contrast, the hCEACAM8 N-terminal domain is not recognized by Afa/Dr adhesins (160), which is consistent with previous results in transfected CHO cells expressing hCEACAM8 (274). A nuclear magnetic resonance (NMR) analysis of the hCEA binding site of DraE and AfaE-III-*dsc* adhesin subunits has revealed a site that overlaps a surface area of approximately 1,446 Å, localizing primarily in the A, B, E, and D strands (160) at the opposite end of the β sheet including the binding site for hDAF (158) (Fig. 3E). Korotkova et al. (188) analyzed the receptor clustering induced by *E. coli* expressing DraE, the DraE D61A adhesin subunit mutant deficient in hDAF binding, or the NfaE adhesin subunit, which binds only to hDAF, and showed that the DraE-expressing *E. coli* recruited both hDAF and hCEACAMs, that the DraE-D61A adhesin subunit-expressing *E. coli* recruited only hCEACAMs, and that the NfaE adhesin subunit-expressing *E. coli* recruited only hDAF when adhering to primary epithelial bladder cells expressing hDAF and hCEACAMs.

The P40S, P43V, R86G, G113A, and Y115A mutations in the DraE adhesin subunit severely affect binding to hCEA (160). As for hDAF (161, 162, 170, 185, 187, 261), the binding of DraE adhesin subunit-expressing *E. coli* to hCEA was inhibited by chloramphenicol, whereas the binding of the AfaE adhesin subunit III-expressing *E. coli* to hCEA was resistant to chloramphenicol (160).

Some hCEACAMs can form homophilic (hCEACAM1/hCEACAM1, hCEA/hCEA, and hCEACAM6/hCEACAM6) and heterophilic (hCEA/hCEACAM1, hCEA/hCEACAM6, and hCEA/hCEACAM8) complexes which form strong intercellular adhesion bonds that are involved in cell-to-cell interactions (275). Al-

though no relationship has been established with microbial pathogenesis, the recognition of the N-terminal domain of hCEA by the DraE adhesin subunit leads to an unexpected structural consequence (284). The binding of the DraE adhesin subunit to the N-terminal domains of the hCEA/hCEA dimer is followed by the dimerization of the complex. It is noteworthy that in the physiological situation of the epithelia, the homophilic and heterophilic dimers of hCEACAM are not accessible to Afa/Dr DAEC, since they are located at the junctional domain of polarized epithelial cells.

(v) Receptors for microbial pathogens. hCEACAM1, hCEA, and hCEACAM6 have been shown to be recognized by some strains of *E. coli* and some *Salmonella* species, probably at extramembrane glycosylated domains (285–287). Moreover, hCEACAM6 functions as a cell receptor for the AIEC strain LF82 (288). hCEACAMs are important for the pathogenicity of *Neisseria*, since after they are recognized by opacity proteins (Opa), these membrane-bound molecules triggered cell signaling, allowing the bacteria to penetrate into human tissues (289). As described above for Afa/Dr adhesins, Opa interactions with hCEACAM1, hCEACAM3, hCEA, and hCEACAM6 have been identified, whereas Opa do not interact with hCEACAM4, hCEACAM7, and hCEACAM8. It is worth underlining that Opa_{52} binds hCEACAM1, hCEACAM3, hCEA, and hCEACAM6, Opa_{53} specifically recognizes hCEACAM1, Opa_{54} binds to hCEACAM1 and hCEA, and Opa_{55} is hCEA specific. It is important to note that CEACAM recognition by *Neisseria* Opa (290) and Afa/Dr adhesins (274) is highly human specific. hCEACAM1 is also recognized as a receptor by the outer membrane protein P5 of typeable and nontypeable *Haemophilus influenzae* which can cause diseases including otitis media, conjunctivitis, sinusitis, pneumonia, and chronic bronchitis and the progression of chronic obstructive pulmonary disease (COPD) (291). Moreover, a major outer membrane protein of *Moraxella catarrhalis* strains associated with sinusitis, exacerbations of asthmatic conditions, and otitis and a cause of lower respiratory tract infections in adults, especially in patients with COPD, also interacts with CEACAM1 (292). It is interesting to note that like Afa/Dr DAEC (160, 284), the N-terminal domains of CEACAMs are targeted by the adhesive factors of *Neisseria* (293), *H. influenzae* (291), *M. catarrhalis* (292), and mouse hepatitis virus strain A59 virus (294, 295). In contrast, the FimH variant of the AIEC prototype wild-type strain LF82 probably recognizes glycosylated epitopes at the IgAs domain of CEACAM6 (288). In addition, CEACAM1-4L acts as a receptor for lipopolysaccharide (LPS) and lipooligosaccharide (296), in turn promoting Toll-like receptor 4 (TLR4)-dependent cell signaling responses (296, 297).

Basement membrane type IV collagen. Basement membrane-associated proteins include fibronectin, laminin, tenascin, and heparin sulfate proteoglycans and type IV collagen (298). Type IV collagen interacts with integrins expressed at the membrane basal domain of polarized intestinal cells to structurally form the epithelium (299). Dr adhesin is unique in the Afa/Dr family of adhesins as recognizing the 7s domain of the type IV collagen (186, 300) (Table 1). By SPR analysis, the resonance signal indicates that the DraE adhesin subunit and type IV collagen form a stable complex (187). Carnoy and Moseley (185) have shown that mutations at positions 32, 40, 54, 88, 90, and 113 of the DraE adhesin subunit affect the type IV collagen binding and chloramphenicol sensitivity of binding, without affecting the hDAF-binding capability. The amino acids Pro40, Pro43, Ile114, and Tyr115 are also important for DraE adhesin subunit/type IV collagen interactions, since mutations P40A, P43V, I114A, and Y115A lead to a complete loss of recognition (187). Moreover, mutations in the two conserved cysteine residues forming a disulfide bond, which is necessary for stabilizing elements of the immunoglobulin fold of the Dr adhesin (174), abolish both MRHA and binding to type IV collagen (185). The role of type IV collagen in the pathogenesis of Afa/Dr DAEC remains largely elusive. It has been reported that the type IV collagen-binding phenotype is necessary for Dr adhesin-positive *E. coli* to induce pyelonephritis in a mouse model (301). However, the basolateral localization of type IV collagen in the epithelium prevents it from functioning as a receptor for Dr adhesin during Afa/Dr DAEC intestinal and urinary tract infections, since epithelial colonization strikingly develops at the cell domain facing the luminal compartments, which are devoid of type IV collagen expression. In the context of diseases in which the epithelia are structurally deregulated, the basement membrane domain becomes available for pathogenic bacteria that recognize basement membrane-associated molecules as receptors.

β1 Integrin. AfaED-*dsc*, but not AfaE-*dsc*, interacts with two integrins: α5β1 and αvβ3 (167). However, there was a low-affinity interaction between DraD subunit and β1 integrin as observed by SPR analysis (167, 188). Considering the presence of DGR tripeptides and an RDG sequence in the AfaD-III subunit, Cota et al. (167) have proposed that the recognition of β1 integrin at a low level of affinity results in binding by these two nonsequential motifs. Intriguingly, when Korotkova et al. (188) used SPR analysis to test the binding to β1 integrin of the whole Dr adhesin, they found no detectable association, suggesting that the association detected with the DraD adhesin subunit cannot reflect the normal bacterial situation in which the Dr adhesin is well formed and expressed at the *E. coli* cell surface.

Receptor Clustering and Cell Signaling

As recently reviewed by Schmick and Bastiaens (302), the signaling activity at cellular membranes depends on constant membrane reshaping plus interactions with the dynamic cytoskeleton, thereby regulating the potency of molecular reactions between membrane-associated structural components and signaling molecules. The epithelial membrane-bound proteins that function as receptors for Afa/Dr adhesins are known to trigger cell signaling after antibody ligation (303, 304) or activation by chemical molecules (305–307). It is noteworthy that the physiological ligands that produce cell signaling by hDAF and hCEACAMs are not known. Adhesive factors of bacterial pathogens and viruses have been reported to trigger cell signaling in epithelial cells expressing hDAF and/or hCEACAM1 and CEACAM6 (308, 309). Whether cell signaling is induced following the recognition of hCEA by adhesive factors of bacterial pathogens remains in debate. Afa/Dr adhesins have been found to trigger various different cell signaling pathways after recognition of hDAF and hCEACAM1 in epithelial cells, some of which are involved in a wide variety of cellular injuries or cell responses.

Mobilization of adhesin receptors and constituents of cell membrane-associated lipid rafts. Membrane lipid rafts are heterogeneous sterol-sphingolipid-enriched domains that can dynamically associate and dissociate (310, 311). How lipids and proteins assemble for the structural and functional organization of the membrane lipid rafts remains not entirely understood. In the

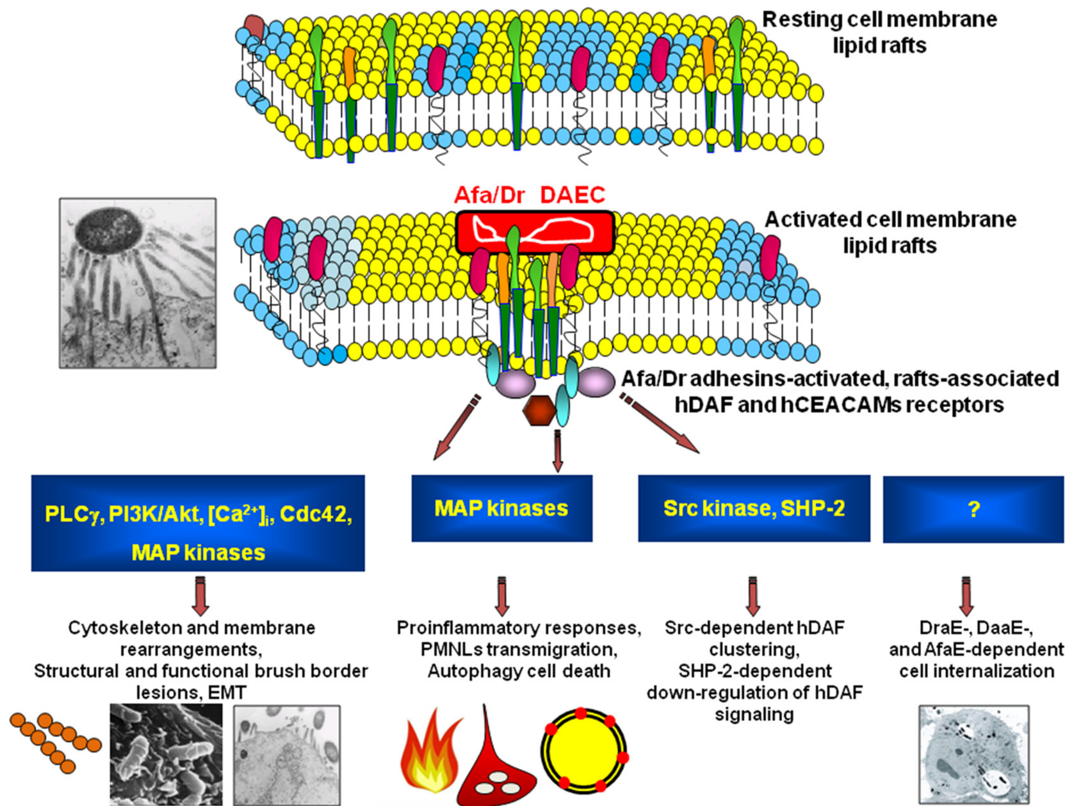

FIG 4 Summary of lipid raft-associated signaling pathways involved in Afa/Dr DAEC pathogenesis. A high-magnification micrograph shows a bacterium interacting with a large number of microvilli at an early time postinfection. Afa/Dr adhesins recognize as receptors the GPI-anchored hDAF, hCEA, and hCEACAM6 and the transmembrane hCEACAM1 proteins. hDAF, hCEA, and hCEACAM6 are endogenously associated with lipid rafts, and a part of hCEACAM1 is translocated within membrane lipid rafts after Afa/Dr DAEC infection. hDAF-dependent signaling involving protein tyrosine kinase(s), phospholipase Cγ, phosphatidylinositol 3-kinase (PI3K), protein kinase C, and an increase in $[Ca^{2+}]_i$ leads to structural and functional lesions at the brush border of enterocyte-like cells. hDAF-, hCEA-, and hCEACAM6-dependent signaling involving the Rho GTPase Cdc42 and ERM proteins leads to membrane elongation. hDAF-dependent signaling involving MAPKs and PI3K/Akt lead to HIF-α-dependent VEGF production and epithelial-mesenchymal transition (EMT). hDAF-dependent signaling involving MAPKs leads to proinflammatory cytokines responses, PMNL transmigration, and autophagy followed by cell detachment. Src kinase is necessary for hDAF clustering around adhering bacteria. Phosphorylation of hCEACAM1-4L at ITIMs and recruitment of SHP-2 lead to a negative regulation of phosphorylation of Src associated with hDAF signaling. The DraE-, DaaE-, and AfaE-triggered dynamic microtubule-dependent internalization of bacteria is a lipid raft-dependent phenomenon involving hDAF, hCEACAM1, hCEA, and hCEACAM6.

cell membrane, these dynamic entities assemble molecules expressing the GPI anchor, acylation, or certain transmembrane domains which, following the recruitment and connection with cytoplasmic effectors, function as platforms of signal transduction. Different models of lipid raft organization have been proposed, including the model for the apical membrane of epithelial cells proposed by Kay Simons and coworkers (312) consisting of a continuous lipid raft phase within which isolated non-lipid raft-phase domains are randomly distributed. It has been proposed that the family of integral membrane flotillin/reggie proteins facilitates the physical organization of lipid raft macrodomains (313–315). Moreover, a particular lattice network of filaments named the cortical actin cytoskeleton underlies the plasma membrane and allows a connection with lipid rafts (316). In addition, other proteins, including supervillin, myosin-IIA, myosin IG, and ezrin-radixin-moesin (ERM), associate with lipid rafts to establish a connection with the cortical actin cytoskeleton (316). By their adhesins that recognize signaling molecules associated with lipid membrane rafts, Afa/Dr DAEC activates diverse signaling pathways that produce deleterious effects on the host cells but also various cellular defense responses against infection (Fig. 4). Wild-type Afa/Dr DAEC and recombinant strains of *E. coli* that express the AfaE-I, AfaE-II, and AfaE-III Dr adhesin subunits, the Dr-II adhesin, or the F1845 adhesin promote the hDAF receptor clustering around bacteria adhering to the epithelial cell surface (39–41, 45, 102, 188, 189, 258, 317, 318) (Fig. 3F and G). Using *draE* mutants with impaired type IV collagen and chloramphenicol binding sensitivity but retaining hDAF-binding capability (185), it has been observed that D54V, D54Y, T90M, and I113T DraE adhesin subunit mutants conserved the property of inducing hDAF receptor clustering around adhering bacteria, while the D54G mutant (Asp54 was replaced with glycine) and the D54C mutant (Asp54 was replaced with cysteine) lose the receptor-clustering activity (41). Das et al. (319) observed that hDAF receptor clustering around adhering bacteria was lower in HeLa and CHO-hDAF cells infected with *E. coli* mutants in which mutations at the T31A and Q34A amino acids of the DraE adhesin subunit hydrophobic II domain had been induced. Guignot et al. (41) determined the roles of hDAF epitopes in hDAF receptor mobilization around adhering Dr-positive bacteria. Using hDAF mutants ex-

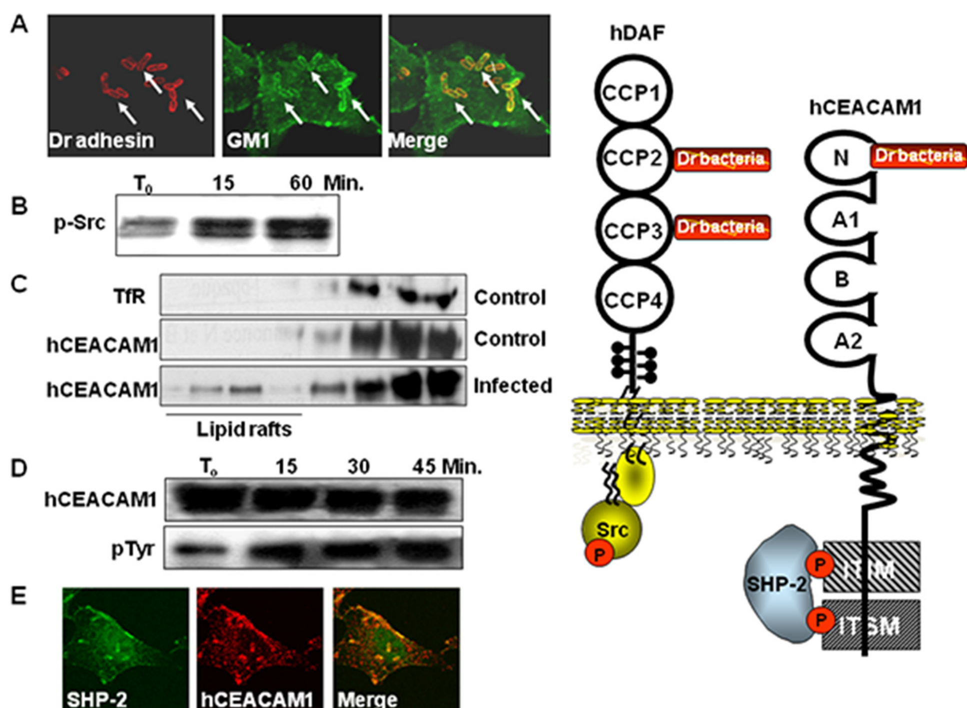

FIG 5 hDAF- and hCEACAM1-4L-associated lipid raft signaling pathways involved in Afa/Dr DAEC pathogenesis. (A) Micrographs show the observation by CLSM of the mobilization of the lipid raft marker ganglioside GM1 around Dr adhesin-positive *E. coli* adhering to HeLa cells. Yellow, colocalization of Dr adhesin and GM1 immunolabelings. (Reprinted from reference 317.) (B) Dr adhesin-induced phosphorylation of Src in infected HeLa cells. (C) Passage of hCEACAM1 into lipid rafts in transfected hCEACAM1-HeLa cells infected with Dr adhesin-positive *E. coli*. TfR, non-lipid raft transferrin receptor. (D) Recruitment of SHP-2 around Dr adhesin-positive *E. coli* adhering to transfected HeLa cells expressing hCEACAM1. (E) Time-dependent association of SHP-2 with hCEACAM1 in transfected HeLa cells expressing hCEACAM1 and infected with Dr adhesin-positive *E. coli*. (In panels B to E, data and micrographs reprinted from reference 318 with permission.) The drawing on the right indicates the sites of phosphorylation observed in hDAF and hCEACAM1-4L after Afa/Dr DAEC infection.

pressed by stably transfected CHO cells, it has been found that the absence of CCP2 or CCP3 entirely abolished the receptor-clustering activity. Absence of the CCP-4 domain did not affect receptor clustering, whereas the role of the CCP-1 domain remains uncertain (41, 258). Moreover, the lack of the heavily O-glycosylated STP region abolished the receptor-clustering activity (41).

Guignot et al. (41), when they first identified hCEA as a receptor for Dr and F1845 adhesins in stably transfected HeLa cells expressing hCEA, observed the hCEA receptor clustering around Dr adhesin-positive *E. coli* infecting HeLa cells (Fig. 3F and G). Berger et al. (274), when identifying the epithelial hCEACAM1 and hCEACAM6 as additional receptors for the Dr adhesin, F1845 adhesin, and AfaE-III adhesion subunit, observed hCEACAM receptor clustering around adhering Dr adhesin-positive *E. coli* (Fig. 3F and G). Like hDAF receptor clustering, the hCEA receptor clustering is not promoted by the DraE adhesion subunit mutant D54C (41). Consistent with the presence of distinct hDAF and hCEA binding domains in the DraE adhesin subunit (160) (Fig. 3E), colocalization of hDAF and hCEACAM immunolabeling develops around Dr adhesin-positive *E. coli* adhering to HeLa cells constitutively expressing hDAF and transfected for the expression of hCEA (274) (Fig. 3G).

Membrane-associated lipid rafts are currently defined as dynamic sterol-sphingolipid-enriched nanoscale domains of different sizes containing GPI-anchored proteins (310–312). Interestingly, quite large and highly stably organized "super lipid rafts" are present at the membrane of the brush border of enterocytes (320, 321). Adherence of Dr adhesin-positive bacteria to epithelial HeLa cells constitutively expressing hDAF, to stably transfected CHO cells expressing hDAF, hCEA, and/or hCEACAM6, or to HeLa cells transfected for stable expression of hCEA or hCEACAM6 results in recruitment of raft markers GM1 and VIP21/caveolin (188, 317) (Fig. 5A). A similar recruitment of VPI21/caveolin has been observed around Dr adhesin-positive *E. coli* infecting primary bladder epithelial cells (188). The mobilization of lipid rafts has also been observed for viruses recognizing hDAF (322, 323) and Opa-expressing *Neisseria* recognizing hCEACAMs (324, 325). The cortical actin cytoskeleton provides a structural organization of lipid rafts, and a well-organized actin cytoskeleton is required for the completion of regulating raft-associated signaling events (316). In stably transfected cells expressing hDAF, hCEA, or hCEACAM6, the receptor clustering around adhering Dr adhesin-positive *E. coli* is associated with the clustering of fine rings of cytoskeleton-associated proteins, such as F-actin, α-actinin, and phosphorylated ezrin (40, 188, 317). In contrast and surprisingly, there was an absence of recruitment of F-actin around Dr adhesin-positive *E. coli* infecting primary bladder epithelial cells constitutively expressing hDAF (188). The Afa/Dr adhesin-induced F-actin mobilization differed markedly from the dramatic F-actin mobilization induced by EPEC (326) and *Salmonella* (327). Fine rings of F-actin have also been observed to be associated with Opa-expressing *Neisseria* infecting hCEACAM1-, hCEA-, and hCEACAM6-expressing cells, whereas dense rings of F-actin ringed the Opa-expressing *Neisseria* adher-

ing to hCEACAM3-expressing cells (328). The fine rings accompanied the low level of F-actin-independent cell entry into hCEACAM1-, hCEA-, and hCEACAM6-expressing cells, and the dense rings accompanied the high level of F-actin-dependent, small Rac1- and Cdc42 GTPase-triggered cell entry into hCEACAM3-expressing cells (329, 330). It is noticeable that the low level of Afa/Dr DAEC internalization into hCEA- and hCEACAM6-expressing HeLa and CHO cells is not affected by cytoskeleton blockers (189, 284, 317, 331). Moreover, the recruitment of F-actin around adhering Dr adhesin-positive bacteria plays no role in bacterial internalization, since although the DraE adhesion subunit mutant D54G has impaired F-actin mobilization, it displays an unchanged level of cell entry (317). Collectively, the results obtained with Afa/Dr DAEC indicated that the recruitment of F-actin forming a fine ring around adhering bacteria probably reflects the physical mobilization of F-actin-containing lipid rafts by the adhering bacteria rather than the recruitment of F-actin for subsequent bacterium-triggered cellular events.

During the adhesion step preceding cell entry, the α5β1 integrin has been observed to be mobilized so as to form fine rings around adhering Dr adhesin- or AfaE-III adhesion subunit-positive bacteria (188, 189, 317, 332). In addition, AfaE/D adhesin subunit-coated beads adhering onto HeLa cells are decorated by rings of positive immunofluorescence for α5β1 integrin (167). Moreover, it was noted that the clustering of β_1 integrin around *E. coli* adhering to CHO-hDAF-$\alpha_5\beta_1$ cells occurred for bacteria expressing DraE or AfaE-III adhesin subunits alone, regardless of the presence or absence of DraD or AfaD-III adhesin subunits (189). The mobilization of β1 integrin by Afa/Dr DAEC adhering to the cell surface of undifferentiated epithelial cells could result from the mobilization of lipid rafts containing integrins. Indeed, integrins have been found in lipid rafts engaged in cell adhesion (333–335), cell migration (336, 337), and contractile forces for cell invasion (337–339). As discussed above for F-actin mobilization, the recruitment of β1 integrin by adhering Afa/Dr DAEC can reflect the physical mobilization of β1 integrin-containing lipid rafts by the adhering bacteria.

hDAF-dependent signaling. hDAF has a signal transduction capacity associating Src tyrosine kinases p56lck and p59fyn (251). Phosphorylation of Src develops in epithelial HeLa cells infected with Dr-positive *E. coli* (318) (Fig. 5B). When examining the involvement of Src kinases in Dr adhesin-induced hDAF signaling, Queval et al. (258) observed the recruitment of phosphorylated Src kinases together with hDAF around adhering recombinant Dr adhesin-positive *E. coli* in infected hDAF-transfected CHO cells and in constitutively hDAF-expressing HeLa and human embryonic kidney HEK293 cells. CCP-4 of hDAF plays a crucial role in the recruitment of phosphorylated Src kinases and Src kinase activation, while deletion of CCP-1 had no effect. Moreover, small interfering RNA (siRNA) silencing of c-Src in HeLa cells abolishes hDAF clustering around adhering Dr-positive bacteria, while siRNA silencing of the Src kinases Yes, Fyn, and Lyn does not (258). Finally, the observation that the D54C DraE adhesion subunit mutant fails to induce hDAF clustering and Src recruitment confirms the predominant role of this domain of the DraE adhesin subunit in triggering cell responses.

A variety of cell signaling pathways are activated after the recognition of hDAF by Afa/Dr adhesins. Phosphatidylinositol 3-kinase (PI3K) is activated in undifferentiated intestinal INT407 cells (340), and phosphorylated PI3K is recruited around Dr adhesin-positive *E. coli* adhering to human differentiated primary bladder cells expressing hDAF and hCEACAMs (188). Triggered by the Dr and F1845 adhesins, an hDAF-dependent activation of mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated kinase 1/2 (Erk1/2), p38 mitogen-activated protein kinase (p38), and stress-activated protein kinase/c-Jun N-terminal kinase SAPK/JNK (JNK), occurred in enterocyte-like Caco-2 cells and colonic T84 cells (341–345). Src kinase and NF-κB and AKT signaling pathways are hDAF-dependently activated in C1845-infected T84 cells (345). Moreover, hDAF-dependent activation of tyrosine kinases and protein kinase C (PKC) develops in Afa/Dr DAEC-infected polymorphonuclear leukocyte (PMNL)-like cells (346). In addition, MAPK and NF-κB activation occurred in PMNL-like cells triggered by type 1 pili and independently of hDAF recognition (347).

hCEACAM-dependent signaling. Like the Opa52 protein of *Neisseria*, which recognizes the N-terminal domain of CEACAM1-4L and promotes its association with the tyrosine phosphatases SHP-1 and SHP-2 in the cytoplasmic ITIM domains (348), Dr adhesin-expressing *E. coli* binding to HeLa cells transfected for the expression of hCEACAM1-4L leads to the translocation of a part of CEACAM1-4L into the membrane lipid rafts and phosphorylation of its ITIM and SHP-2 recruitment (318) (Fig. 5C to E). In turn, hCEACAM1-4L has been found to play a key role in down-regulating the activity of the protein tyrosine Src kinase associated with hDAF signaling (318).

Urinary Tract Infections and Pregnancy Complications

Recurrent cystitis in the bladder and acute pyelonephritis in the kidney, corresponding to 80% of all UTIs, result from infection by UPEC (199, 349). Bacteriuria is the clinical sign of UTI. Recurrent cystitis is a major health problem. Indeed, a recurrence of cystitis within 3 to 4 months develops in 20 to 30% of women who had developed a first acute infection. Lower UTIs affect the urethra and bladder, whereas upper UTIs affect the ureters and kidneys, and both can be either uncomplicated or complicated. Cell insults together with an intense mucosal inflammatory response, including the recruitment of neutrophils, lead to cystitis in bladder and pyelonephritis in kidney. It should be noted that asymptomatic bacteriuria (ABU) has been observed in 2 to 20% of the population, depending on age and gender (199). The infecting *E. coli* strain involved in ABU colonizes the urothelium and may remain present for months or years, resulting in a low-level deleterious tissue attack effect and an innate immune response that is too weak to cause symptoms. The observation that UPEC virulence gene sequences remained present in ABU strains has led to the suggestion that the lower virulence of ABU results from a shift from UPEC as a result of genome reduction caused by inactivation of virulence genes as well as by deletions or by the accumulation of point mutations (350, 351), although other causes can also be involved (352).

UPEC strains have an elevated organ tropism and ascend the urinary tract from the urethra to the bladder and kidneys (1, 199, 232). To do this, UPEC expresses a wide variety of adhesive factors, including type 1 pili, type IV pili, and P, S, F1C, Auf, Yad, Ygi, and F9 fimbriae (199, 353). Moreover, UPEC has developed a reciprocal regulation of adhesive factors and motility (199). UPEC strains have developed sophisticated strategies to avoid clearance by micturition involving colonization of the urothelium and cell

internalization, which thus allow them to survive and evade host innate immune defenses. For pathogenesis, UPEC expresses PAIs of different sizes containing assemblages of genes encoding virulence factors such as adhesins, invasins, capsule, proteases, and multiple siderophore systems, including aerobactin, IroN, and IreA (199). Moreover, UPEC secretes cytotoxic toxins, including the repeat-in-toxin α-hemolysin (HlyA), cytotoxic necrotizing factor (CNF-1), and diverse SPATE toxins, such as Sat, Pic, PicU, Tsh, and Vat. In addition, UPEC adapts to survive in the urine by expressing factors involved in scavenging of nutrients.

Internalization. Cell entry of *E. coli* expressing the Dr, F1845, or Afa-III adhesin has been examined in cervical epithelial HeLa cells and endometrial cells constitutively expressing hDAF but not hCEACAMs, in undifferentiated intestinal cells and differentiated primary epithelial bladder cells constitutively expressing hDAF and hCEACAMs, and in CHO or HeLa cells transfected for stable expression of hDAF, hCEACAM1, hCEA, or hCEACAM6 (38, 42, 188, 189, 234, 259, 317, 319, 331, 332, 354). Afa/Dr DAEC displays a rate of cell entry similar to that observed with UPEC expressing type 1 pili (199, 232). Dr and Afa-III adhesin-positive *E. coli* used a membrane zipper-like mechanism to enter the HeLa cells (42, 317), and Afa-III adhesin-positive *E. coli* entered the cells via a single internalization vacuole (42). The cell membrane lipid rafts play a pivotal role in the internalization of Dr- and Afa-III adhesin-positive *E. coli* into cultured cervical HeLa cells, primary cultured human bladder cells, and epithelial cells transfected to express hDAF, hCEACAM1, hCEA, or hCEACAM6 by a mechanism involving cell microtubules but not microfilaments (38, 188, 189, 259, 317, 331). A particular network of microtubules, i.e., the dynamically unstable microtubule network, has been found to be involved in the cell entry of Dr adhesin-positive *E. coli* (331). Rana et al. (234), using a PCR-based proximity ligation assay to detect protein-protein interactions, observed a strong fluorescent signal of hDAF/tubulin in Dr-positive *E. coli*-infected HeLa cells resulting from the proximity between the two molecules, suggesting that hDAF and microtubules can be physically associated after infection. The Afa/Dr DAEC cell entry resembles the lipid raft- and microtubule-dependent zipper-like uptake of Opa-expressing *Neisseria* into transfected CHO and HeLa cells expressing hCEA or hCEACAM6 (330). It was noted that a few invasive bacteria, including, for example, *Haemophilus influenzae*, *Klebsiella pneumonia*e, and pilus type 1-expressing UPEC, also require a functional host microtubule network for the invasion of host epithelial cells (355). Consistent with the F-actin microfilament-independent cell entry of Dr-positive *E. coli*, the DraE adhesin subunit mutant strains D54G and D54C, showing absence and presence of changes in F-actin mobilization around adhering bacteria, respectively, displayed unchanged levels of cell internalization (317). Microtubule-dependent internalization of Dr adhesin-positive *E. coli* within primary bladder epithelial cells has been found to result from the engagement of the actin-binding proteins ezrin/radixin/moesin (ERM) (188). Observations that phosphorylation of ERM accompanied the Dr-positive *E. coli* cell entry (188) agree well with the know role of ERM, which together with Rho GTPases act in the remodeling of host cell cytoskeleton (356). It has been noticed that the efficiency of bacterial cell entry resulting from the action of T3SS-dependent effectors seems to be higher than that resulting from microtubule-dependent processes triggered by bacteria that do not express the T3SS.

Selvaragan et al. (259) have determined the roles of hDAF epitopes in the cell entry of Dr adhesin-positive bacteria using hDAF deletion mutants expressed by stably transfected CHO cells. The absence of CCP-2 or CCP-3 entirely abolished the cell entry of Dr-positive bacteria, whereas the lack of CCP-1 or CCP-4 had no effect. Deletion of the heavily O-glycosylated STP region abolished the cell entry. In contrast, replacing the GPI anchor with the transmembrane anchor of HLA-B44 or membrane cofactor protein did not modify the cell entry. Rana et al. (234) have examined for adhesion/invasion five hDAF mutants previously used to map the extracellular CCP-2/CCP-3 domains of hDAF involved in Dr adhesin-positive *E. coli* adhesion (260). Compared to hDAF, the Phe123-Ala mutant conserves both normal binding activity and invasion rate, the Ser165-Ala mutant conserves normal binding activity but displays a lower invasion rate, and the Gly159-Ala mutant shows both reduced binding capacity and a lower invasion rate. In contrast, the Phe148-Ala and Phe154-Ala mutants display normal binding capacity combined with an increased rate of invasion.

In terms of cell signaling, protein tyrosine kinases (PTKs), phospholipase Cγ (PLC-γ), and protein kinase C (PKC) blockers have no effect on the cell internalization of Dr adhesin-positive *E. coli* (317). In contrast, the PI3K-dependent signaling pathway is engaged in the internalization of Dr adhesin-positive *E. coli* into hCEACAM1-, hCEA-, and hCEACAM6-expressing epithelial cells but not hDAF-expressing cells (188). While Src-dependent signaling does not play any role in internalization (188), Src and phosphorylated Src have been observed to be recruited around adhering Dr-positive *E. coli* (258).

The β1 integrin is the host cell receptor triggering the zipper-like internalization of type 1 pilus-expressing UPEC (199, 232). A polyclonal antibody directed against anti-α5β1 integrin abolishes the basolateral entry of Dr adhesin-positive *E. coli* within undifferentiated intestinal Caco-2 cells (331). Comparison of the cell entry of beads coated with rAfaD-III adhesin subunit into mouse endodermal carcinoma F9-TKO cells stably transfected or not to express β1 integrin shows that the presence of the integrin increased the bead internalization level 2.3-fold (332). In addition, in HeLa cells subjected to incubation with AfaD-III or AfaD-VIII adhesin subunits, membrane immunoprecipitation showed that AfaD-III and -VIII adhesin subunits coprecipitated with β_1 integrin (332). The role of $\alpha_5\beta_1$ integrin as a receptor for internalization of *E. coli* expressing Dr- or Afa-III adhesin subunits into epithelial cells has been recently revisited (189). The overexpression of $\alpha_5\beta_1$ integrin as a result of transfection in the CHO B2 clone, which does not express $\alpha_5\beta_1$ integrin, does not increase the levels of adhering and internalized Dr- or Afa-III adhesin-positive *E. coli* compared to that in untransfected cells. To test whether the coexpression of $\alpha_5\beta_1$ integrin together with hDAF, hCEACAM1, hCEA, or hCEACAM6 influences the adhesion and/or cell entry of Dr adhesin-positive *E. coli*, CHO cells transfected for the stable expression of hDAF, hCEAM1, hCEA, or hCEACAM6 were transfected again with the gene coding for the α_5 or β_1 integrin subunit (189). In all cases, the presence of $\alpha_5\beta_1$ integrin together with hDAF or CEACAMs did not increase the levels of adhering and internalized Dr adhesin-positive *E. coli*. In addition, the knockdown of the gene coding for β1 integrin by siRNAs in HeLa cells constitutively expressing $\alpha_5\beta_1$ integrin did not affect the level of adhering and internalized Dr- or Afa-III adhesin-expressing *E. coli* compared to that in untreated cells (189). It is important to note

that the fact that integrins are located exclusively at the basolateral domain and never at the brush border of the polarized intestinal epithelial cells renders the integrins inaccessible for intestinal epithelium infection by enterovirulent Afa/Dr DAEC residing within the luminal compartment.

The identity of the virulence factor(s) of Afa/Dr DAEC involved in the invasion of epithelial cells has long been debated. It has been proposed by Le Bouguénec and coworkers (357) that the D adhesin subunits encoded by the *dra*, *daa*, and *afa-1* to *-3* operons function as an invasin for the entry of Afa/Dr DAEC into urothelial and bladder cells. DraD and AfaD adhesin subunits have been identified at the tips of Afa/Dr fibrils (167, 180). AfaD protein, unlike the AfaE protein, was able to detach from the bacterium (28, 168, 169). Using recombinant *E. coli* producing the AfaD or AfaE-III adhesin subunit, it has been observed that the AfaE-III adhesin subunit triggers the binding of the recombinant *E. coli* to cells and that the AfaD adhesin subunit mediates the cell entry within epithelial HeLa cells (42, 168). When HeLa cells are infected with an Afa-III adhesin-positive *E. coli* strain, the AfaE-III protein remains localized at the membranes of infected cells, and only the AfaD-III protein coats the internalized bacteria residing within an internalization vacuole (169). In addition, coating polycarbonate beads with AfaD protein allows the beads to penetrate the epithelial cells (28). Moreover, using colloidal gold-tagged AfaE-III and AfaD proteins, it has been shown that AfaE-III–gold complexes associate with the cell surface, whereas AfaD-gold complexes are internalized within the cells (42). In human undifferentiated Caco-2, T84, and INT407 cells, urothelial T24 cells, and cervical HeLa cells, rAfaD-III- and rAFaD-VIII adhesin subunit-coated beads invaded the cells, whereas rAfaE-III adhesin subunit-coated beads were not internalized (332). In contrast, according to Nowicki and coworkers, the DraE adhesin subunit alone is sufficient to allow the entry of Dr adhesin-positive bacteria into epithelial cells. Indeed, both the purified Dr fimbriae and latex beads coated with Dr adhesin are internalized into HeLa cells (259, 319). Moreover, the insertional *draE*, *draC*, and *draB* and adherent *draD* mutants were unable to enter epithelial cells, and complementation of the *draE* mutation restored the invasion property (38). Mapping of the DraE adhesin subunit shows that amino acids localized on hydrophilic domain II, and in particular the V28, T31, G33, Q34, L35, T36, and P40 amino acids, reduced or abolished bacterial cell entry into transfected CHO-hDAF cells and HeLa cells constitutively expressing hDAF but did not affect attachment (319). The role of E and/or D adhesin subunits in the internalization of Afa/Dr DAEC has been recently reexamined using appropriate *E. coli* mutant strains and cultured unpolarized epithelial cells and bladder cells (188, 189). Korotkova et al. (188) found that expressing combinations of the $DraE^+/DraD^+$ and $DraE^+/DraD^-$ adhesin subunits allowed *E. coli* to enter differentiated primary bladder cells, while the combination of $DraE^-/DraD^+$ adhesin subunits did not. Guignot et al. (189) have found that in CHO cells transfected to express hDAF and $\alpha_5\beta_1$ integrin, recombinant *E. coli* expressing combinations of $DraE^+/DraD^+$ or $AfaE^+/AfaD^+$-III adhesin subunits adhered and were internalized and that deletion of the DraD adhesin subunit did not modify the level of internalized bacteria. Collectively, these last findings fit in well with the previous results reported by Nowicki and coworkers (38, 319), and taken together they clearly establish that the DraE and AfaE-III adhesin subunits are necessary and sufficient to promote the cell entry of Dr and Afa-III adhesin-expressing *E. coli* into epithelial cells. However, one question remains unresolved concerning the functions of the DraD, AfaE, and DaaD adhesin subunits in the pathogenesis of Afa/Dr DAEC when the proteins are detached from the fimbriae (42) or when each of the proteins are present alone at the bacterial cell surface (42, 168, 169). It is noteworthy that the DraD adhesin subunit, whether fimbria associated or not, seems to function in the formation of biofilm by Dr adhesin-expressing *E. coli* (190, 233).

Intracellular lifestyle. When internalized within nonphagocytic epithelial cells, enteric bacterial pathogens reside and survive in the cell cytoplasm in small or large vacuoles, where they may or may not replicate (358); only a few invasive enteric bacterial pathogens have developed strategies to escape from the vacuole to gain access to and proliferate within the host cell cytosol (359). After entering HeLa cells inside a single internalization vacuole, Afa/Dr DAEC survived within large late vacuoles (38, 40, 42, 94, 189, 234, 259, 319, 331, 332) (Fig. 6A). As revealed by transmission electron microscopy examination, the late large vacuole containing internalized Dr or Afa-III adhesin-expressing bacteria appears to result from the fusion of early vacuoles formed during the initial step of the internalization process, each containing a single bacterium (42, 234, 259, 319). Recent data have shown that the internalization vacuoles containing Dr adhesin-positive bacteria interacted with the cell endocytic pathway. This pathway includes a complex and multifunctional set of vesicular compartments essentially derived from internalization of the plasma membrane, resulting in the formation and maturation of well-formed endocytic compartments (360). These compartments are sequentially modified by acquiring diverse vesicular elements to form the early endosomes and subsequently the late endosomes. In the last step, the intraluminal vesicles are taken for delivery of their contents to the lysosome, which is a stable organelle that avoids self-degradation (361). The intracellular vacuoles containing Dr adhesin-positive *E. coli* display some of the characteristics of late endosomes, such as the membrane expression of the Lamp-1, Lamp-2, and CD63 proteins but not of cathepsin D, and are acidic (189) (Fig. 6B). This resembles the characteristics of the UPEC UTI89-containing vacuoles, which are positive for Lamp-1 and CD63, cathepsin D negative, and acidic (362). Surprisingly, despite the facts that Dr adhesin-positive bacteria bind to membrane-associated hDAF during the first step of cell association (41, 257–260) and that hDAF has been observed associated with the membrane invagination during the first step of adhesion/internalization (45), the membranes of the large late vacuoles containing the internalized Dr-positive bacteria do not contain hDAF (189).

Cellular autophagy (363) is an evolutionarily conserved process by which cellular cytosolic structures and cell constituents are degraded and recycled (364). Some intracellular bacteria are detected and eliminated via the autophagic pathway, and several intracellular bacteria have developed sophisticated avoidance strategies, but this pathway may also serve as a protected niche providing a source of nutrients for intracellular bacteria (365). Infecting UPEC was rapidly cleared in the presence of a deficiency of the key autophagy protein Atg16L1, suggesting that UPEC may subvert autophagy proteins to establish latency (366). However, the autophagy pathway can favor the pathogen, since internalized bacteria present within intracellular bacterial communities in superficial bladder cells colocalized with Atg16L1 and LC3 puncta (366). No interaction has been observed between the Dr adhesin-positive *E. coli*-containing vacuoles and the autophagic pathway

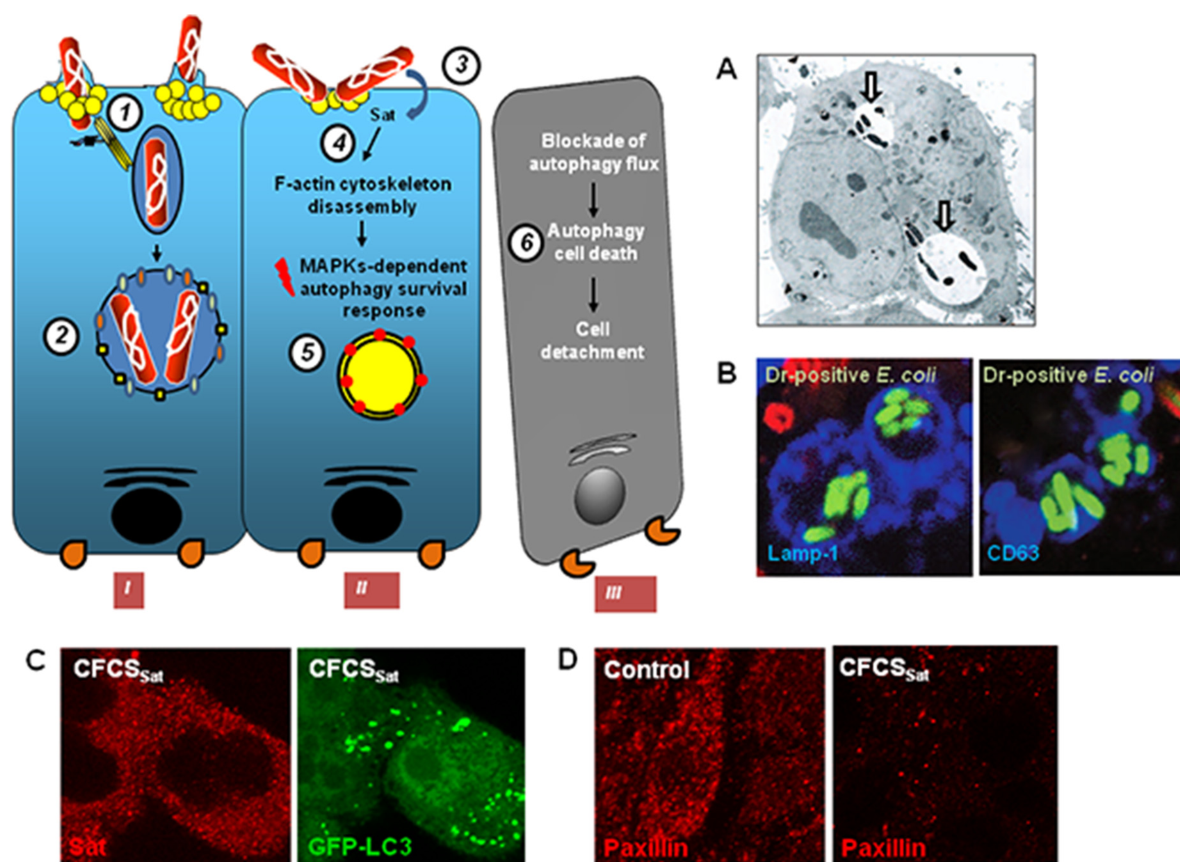

FIG 6 Cellular events observed in cervical epithelial HeLa cells infected with uropathogenic Afa/Dr DAEC or subjected to Sat toxin treatment. The drawing on the left summarizes the observed cellular events. Internalization of bacteria occurs in nonpolarized epithelial cells via the recognition of membrane-associated hDAF, or hCEACAM1, hCEA, and hCEACAM6 by a mechanism involving lipid rafts and dynamic microtubules (1). Internalized Afa/Dr DAEC cells have survived within large vacuoles, which seems to result from the fusion of early vacuoles, each containing one bacterium formed during the initial step of internalization, that tested positive for early and late endosome markers but not for the autophagy LC3 marker (2). The secreted and cell internalized Sat toxin (3) promotes a dramatic loss of F-actin stress fibers (4), and in turn the intoxicated cell engage an autophagy cell survival response (5). The massive appearance of autophagosomes is followed by the blockade of autophagy flux, leading to the lack of maturation of autophagosomes into autophagolysosomes (5). As the result of autophagic cell death, the cells lose the focal adhesion contacts and become detached from the substratum (6). (A) Transmission electron micrograph showing vacuoles containing internalized Afa-III adhesin-positive *E. coli* in HeLa cells. Arrows indicate the vacuole-containing internalized bacteria. (Reprinted from reference 332 with the permission of the publisher. Copyright 2003 Blackwell Publishing Ltd.) (B) High-magnification micrographs show the observation by CLSM of Lamp1 or CD63 immunolabeling (blue) in membranes of vacuoles containing the internalized Dr adhesin-positive *E. coli* in infected HeLa cells (green). Red, extracellular adhering bacteria. (Reprinted from reference 189.) (C) High-magnification micrographs show the observation by CLSM of Sat immunolabeling present in the cytoplasm of cells treated with cell-free culture supernatant of AAEC185$_{pSatIH11128}$ containing the secreted Sat toxin (CFCS$_{Sat}$) (red) and the appearance of green fluorescent protein (GFP)-LC3 autophagic vacuoles in CFCS$_{Sat}$-treated cells (green). (Reprinted from reference 374 with the permission of the publisher. Copyright 2011 Blackwell Publishing Ltd.) (D) High-magnification micrographs show the observation by CLSM of paxillin immunolabeling (red). Note the disappearance of paxillin in in CFCS$_{Sat}$-treated cells. (Reprinted from reference 374 with the permission of the publisher. Copyright 2011 Blackwell Publishing Ltd.)

(189, 234). Moreover, induction of autophagy in Dr adhesin-positive *E. coli*-infected cells has no effect on the survival of internalized bacteria (137). It is noteworthy that autophagy clears intracellular AIEC and that induction of autophagy accelerates this phenomenon (367–369). These results indicate that by blocking the association of vacuole-containing bacteria with the autophagosome, Dr adhesin-positive *E. coli* has developed a strategy to escape the host defense autophagy. The same phenomenon has been observed for several invading pathogens (370).

Internalized Dr adhesin-positive *E. coli* survived up to 72 h postinfection in HeLa cells transfected to express hDAF and to a lesser extent in cells expressing hCEACAM1, hCEA, or hCEACAM6 (189). Dr adhesin-positive bacteria intracellularly present within a vesicular compartment in the host cell have no impact on the cell survival (189, 331) and do not affect functional epithelial intestinal cell differentiation (331). It has been recently observed that a Ser165-Ala mutation in the CCP-3 extracellular domain of hDAF promotes the dormant vacuolar persistence of internalized Dr adhesin-positive *E. coli*, whereas a Phe154-Ala mutation promotes the multiplication of vacuolar bacteria (234). Whether these mutations in the extracellular domain of cell membrane-associated hDAF can impact the lifestyle of internalized Dr adhesin-positive *E. coli* remains unclear and needs to be explained, considering that hDAF is not present at the membrane of intracellular vesicles containing the internalized bacteria (189). On the basis of these results, it looks as if internalized Dr adhesin-positive *E. coli* cells enter the endocytic pathway (364, 371) in order to reach a protective niche with a low-pH environment where they

can live and remain metabolically active in order to replicate or enter dormancy. The observation that the Dr adhesin-positive *E. coli*-containing large vacuoles are phenotypically quite similar to lysosomes indicates that internalized bacteria have developed a capacity to actively modify this compartment so as to create a distinct compartment. The mechanism(s) by which internalized uropathogenic Afa/Dr DAEC cells modify the vacuolar compartment and lead it to engage in dormancy or intracellular replication remains to be elucidated. A process designated "nutritional virulence" (372) has recently been defined as used by several invasive bacteria which, to allow themselves to grow, subvert the host cell machinery so as to create a vesicular developmental niche where they receive components from the secretory pathway as a source of nutrients. It will be interesting to investigate whether internalized Afa/Dr DAEC cells do or do not develop a "nutritional virulence" mechanism in order to permit their intracellular residence and survival. The observation of an intracellular lifestyle of Dr adhesin-positive *E. coli* with intracellular survival is interesting in term of persistence and recurrence of infection. Indeed, it is worth mentioning that type 1 pilus-positive UPEC after cell entry resided in endocytic vesicles in which its replication is restricted and which form an intracellular niche that is protected from host immunity responses and killing of intracellular bacteria by antibiotics (232). This intracellular lifestyle permits the long-term maintenance of quiescent UPEC in the bladder cells (232).

Cell detachment. To protect the epithelia against bacterial colonization and invasion and to maintain tissue homeostasis, the host has developed defense systems including exfoliation and cell death of bacterially infected cells (370). The eviction of cells from the epithelium can occur by a process known as extrusion following detachment from the extracellular matrix and loss of cell-to-cell contact or toward the basal surface by delamination after disruption of cell-to-cell contacts (373). For example, exfoliation of fully differentiated umbrella epithelial cells infected by FimH-positive *E. coli* is an innate host defense mechanism that has the effect of removing adhering and invaded bacteria from the bladder (199, 232). However, some invasive bacterial pathogens have developed countermeasures intended to antagonize the host epithelial turnover to exploit infected epithelial cells as a survival and replicative niche (370). As an adverse effect for the host, the detachment of cells containing intracellular UPEC promotes the reinitiation of the UPEC infectious cycle (199, 232).

A cell detachment effect triggered by the Sat toxin expressed by Afa/Dr DAEC has been recently described (137, 206, 210–212). Sat promotes a profound disorganization of the F-actin cytoskeleton in HeLa cells that is followed by a host cell survival response involving the noncanonical autophagy pathway (374). The autophagy pathway induced is not intended to destroy the intracellular toxin, since the toxin is never present in autophagic vesicles. The Sat-intoxicated cells displayed a massive intracellular appearance of autophagic vacuoles which, at the autophagosome step, fail to mature into autophagolysomes. In turn, the Sat-intoxicated cells overexpressing autophagosomes die as a result of autophagy cell death. A similar effect has been reported for α-hemolysin of *Streptococcus aureus* (375). The Sat-induced autophagy cell death is accompanied by a dramatic disassembly of the focal adhesion-associated vinculin and paxillin, which finally promotes cell detachment (374). It is noteworthy that Capello et al. (376) have observed that the class I SPATE toxin Pet of EAEC induces the cleavage of FAK and a deterioration of focal adhesion complexes resulting from the redistribution of paxillin and vinculin and the depletion of phosphotyrosines. Together with a loss of F-actin stress fibers and α-actinin and spectrin network disassembly, these dramatic rearrangements of structural and functional proteins result in cell rounding and detachment of Pet-intoxicated cells from the substratum (376–378). Interestingly, the UPEC pore-forming HylA triggering cell exfoliation of bladder epithelial cells induces cell rounding and a complete loss of the microtubule network and F-actin stress fibers, accompanied by the degradation of paxillin (379).

Inflammatory responses. New information on immune responses accompanying UPEC infection have recently been obtained in mice models (380, 381). UPEC infection is accompanied by a strong inflammatory response, including the production of cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), IL-8, IL-17a, and granulocyte colony-stimulating factor (199, 382). Moreover, neutrophils and macrophages play a pivotal role in the defense against UTI (383). Although not observed in urinary cell models, it is important to note that the UTI-associated wild-type Afa/Dr DAEC strain IH11128 promotes an hDAF- and MAPK-dependent production of IL-8 (341) followed by transepithelial migration of PMNLs (341), triggering the production of TNF-α (342).

Animal models of UTIs. Dr adhesin-positive *E. coli* leads to chronic pyelonephritis in experimental mice (301, 384). In contrast, wild-type strain IH11128, an isogenic mutant that is devoid of Dr adhesin, does not cause kidney infection or cellular lesions and is gradually eliminated compared to the wild-type strain. In the kidney tissue of Dr adhesin-positive *E. coli*-infected mice, Dr antigen was present in the injured parenchymal regions characterized by histological changes indicating tubulointerstitial nephritis (384). It is notable that mouse DAF (mDAF) probably plays no role in this Dr adhesin-induced chronic ascending pyelonephritis in mice, since Hudault et al. (254) have demonstrated that Dr adhesin binds specifically to hDAF but not to rodent DAF. Importantly, Selvarangan et al. (301) have demonstrated the crucial role of the type IV collagen-binding capability of Dr adhesin for pathogenesis in mouse kidney. Indeed, an isogenic DraE adhesin subunit mutant lacking binding to type IV collagen fails to persist within the mouse renal tissues, and the transcomplementation of the mutant strain, restoring the type IV collagen-binding activity, allows the reestablishment of a long-term renal infection. It was noticed that immunization of LPS-nonresponder C3H/HeJ mice with purified Dr protein resulted in delayed mortality when the immunized mice were infected by instilling wild-type Dr adhesin-positive strain IH11128 into the bladder, and incubation of IH11128 with sera of immunized mice resulted in a marked decrease in bacterial adhesion to *ex vivo* specimens of mouse bladders and kidneys (385). However, additional bacterial factors may also be involved, since Meitinen et al. (386) observed that after injection of O75X fimbriae in mice, there was an absence of histological damage despite persistent O75X deposits in glomeruli. Interestingly, the Sat toxin of the prototype UPEC wild-type strain CFT073 has been found to promote renal histological lesions in mice (387). It remains to be determined whether Sat expressed by strain IH11128 plays a role in the IH11128-induced mice kidney lesions.

Pregnancy Complications

Bogdan Nowicki and coworkers have illuminated the risk generated by UTIs caused by UPEC harboring the Dr adhesin in women who have undergone pregnancy termination or preterm labor (105), and this is related to the levels of tissue expression of hDAF (82, 388–390) and TLR4 (390, 391). Dr adhesin-positive *E. coli* strains have been found to be often present in pyelonephritis-associated *E. coli* strains isolated from pregnant females (30, 62, 90, 91, 96, 97). Nowicki and coworkers (82, 105) have convincingly demonstrated the role of Dr-positive UPEC in pregnancy complications. In the uterus, hDAF is present in the endometrial glands, spiral arterioles, and myometrial arteries. In these tissues, the density of hDAF expression correlates with a high level of regulation of the complement-induced cell damage (392, 393). Moreover, it has been demonstrated that interindividual differences in hDAF density in the endometrium influence the tissue colonization by Dr adhesin-expressing *E. coli* (393, 394).

A set of elegant studies have provided a clear demonstration of the role of UPEC expressing the Dr and Afa adhesins in the lethal outcome of gestational infection. Nowicki et al. (395) have shown that the level of uterine Dr adhesin-positive *E. coli* infection in pregnant rats was higher than that in nonpregnant rats. In an experimental model of nonpregnant lipopolysaccharide-hyporesponder C3H/HeJ mice, Kaul et al. (396) observed that Dr adhesin-positive *E. coli*-induced chronic pyelonephritis resulted in a high level of preterm deliveries compared to that in mice infected with an *E. coli* isogenic Dr mutant. A high rate of maternal mortality has been observed in rats infected with an $AfaE^{+}/AfaD^{+}$ strain that causes high rates of maternal mortality (397). Mice infected with the $AfaE^{+}/AfaD^{-}$ strain show a 2-fold-decreased level of maternal mortality and infection with $AfaE^{-}/AfaD^{+}$ strain results in a 5-fold decrease in death, while in contrast, the $AfaE^{-}/AfaD^{-}$ double mutant fails to promote death, even though all the mutants showed an equal infection level in uteri. The observation that the decrease in the mortality rate paralleled the decrease in the invasiveness capacity of a Dr adhesin-positive *E. coli* strain (397) correlates well with the *in vitro* observation by Goluszko et al. of a high rate of invasiveness capability in Dr adhesin-positive *E. coli* isolated from pregnant women with UTIs (94).

It has been proposed that infectious complications of pregnancy such as host gestation-dependent sensitivity to UPEC are related to the host nitric oxide (NO) status. When Dr-positive *E. coli* invades human endometrial adenocarcinoma Ishikawa cells, there is a decrease of cell entry after an induction of NO production and an increase of cell entry after NO inhibition (354). Interestingly, elevation of NO production is accompanied by a significantly reduced expression of hDAF protein and mRNA (354). Mechanistically, NO triggers a displacement of hDAF from the membrane lipid rafts coupled to its internalization within human endometrial cells (398). In rodents, an increase in rat uterine NO synthase (NOS) activity has been observed during pregnancy, and one function of NO generated in the uterus, which declines at term (399), is to inhibit uterine contractility (400). It has been observed, interestingly, that urogenital tract colonization by Dr adhesin-positive *E. coli* is followed by a defense mechanism involving the production of NO (401). Moreover, there is a localized increase in type II NOS expression and NO production after intrauterine Dr-positive *E. coli* infection in pregnant versus nonpregnant rats (402). Comparing LPS responder (C3H/HeN) and nonresponder (C3H/HeJ) mice and Dr adhesin-positive *E. coli* and P fimbria-positive *E. coli* infections, Nowicki et al. (403) observed that the infection level in the Dr-positive *E. coli*-infected C3H/HeN group treated with an inhibitor of NO, nitro-L-arginine methyl ester (L-NAME), was about 100-fold higher than that in the P adhesin-positive *E. coli*-infected, L-NAME-treated group. Dr adhesin-positive *E. coli* infection in mice (403) and AfaE/AfaD adhesin subunit-positive *E. coli* infection in rats (404) are followed by complications in pregnancy and death. Moreover, the death rate was increased by treatment with the NO blocker L-NAME in both mice and rat (403, 404). As indicated above for Dr adhesin-induced pyelonephritis in the mouse model, the role of the mouse DAF in Dr adhesin-induced complications during pregnancy in the rat model is intriguing, considering that the rodent DAF is clearly not recognized by Afa/Dr adhesins (254). Banadakoppa et al. (405) recently demonstrated an NO-independent regulation of hDAF expression in endometrial Ishikawa and cervical HeLa cells involving the PI3K/Akt pathway engaging the PI3K/Akt regulatory protein PTEN. Interestingly, the PI3K/Akt pathway negatively regulated the membrane expression of hDAF and consequently downregulated the adhesion of Dr adhesin-positive *E. coli*. As underlined by Nowicki and coworkers, two independent host cell systems, NO and PI3K/Akt, by the downregulation of hDAF expression at cell membrane level represent a functional host pathogen strategy to achieve a well-controlled limited level of infection by Dr adhesin-positive *E. coli*.

Intestinal Tract Infection

Facing the luminal compartment, the intestinal epithelium is lined by a monolayer of highly polarized epithelial cells, including four extremely specialized cell phenotypes, each with specialized functions: enterocytes, neuroendocrine cells, goblet cells, and Paneth cells. Enterocytes, neuroendocrine cells, and goblet cells are constantly renewed by detachment from the villus tip (373) via a specific type of apoptosis known as "anoikis" (406) and are replaced by a cell cycle renewal characterized by a differentiation/migration process occurring along the crypt-villus axis and starting from stem cells localizing at the crypt of the villus (407). Polarized intestinal epithelial cells express an apical domain facing the luminal compartment. In the cell lateral domain, the cell-to-cell junctional domain, including the tight junction (TJ), the adherens junctions (AJs), and the desmosome, establish tight contacts with neighboring cells and seal the intestinal cell barrier (299, 408). In addition, the basal domain establishes a connection with the basement membrane. Structural or functional breaches of the intestinal epithelial barrier by enterovirulent bacteria lead to diseases (409).

Structural and functional injuries at the intestinal epithelial barrier. Afa/Dr DAEC strains infecting cultured enterocyte-like Caco-2 (410–412) and colonic T84 (342) cells attach at the brush border. Adhesion of F1845 adhesin-positive *E. coli* strain C1845 parallels the cell differentiation-dependent appearance of the brush border (413). As the result of the recognition of brush border-associated hDAF, recombinant Dr adhesin- and Afa-I adhesin-positive *E. coli* strains adhere to cultured human enterocyte-like HT-29 and Caco-2 cells (414). Similarly, Adlerberth et al. (415) have found that Dr adhesin-positive *E. coli* cells adhere at the brush border of freshly isolated ileal enterocytes or colonic cells. The wild-type strain C1845 displays a low level of cell entry when infecting the permissive epithelial Hep-2 cell line, 20-fold lower

than that of the prototype invasive AIEC wild-type strain LF82 but equaling the rate of internalization of noninvasive EHEC and EAEC strains (416). Moreover, Afa/Dr DAEC strains are noninvasive when they infect cultured enterocyte-like cells via the natural apical infection route. Indeed, Guignot et al. (331) have shown that after apical infection of cultured human enterocyte-like Caco-2/TC7 cells, the level of internalized Afa/Dr DAEC was very low, indicating that these bacteria are noninvasive when in contact with the brush border of human enterocytes lining the intestinal epithelial barrier. In contrast, when Afa/Dr DAEC infected the Caco-2/TC7 cells basolaterally, it was internalized by a mechanism involving the basolaterally expressed β1 integrin. It should be noted, however, that Afa/Dr DAEC never uses the basolateral domain of intestinal epithelial barrier as its natural way of infection. However, treating Caco-2/TC7 cells with Ca^{2+}-free medium containing EGTA, disrupting intercellular junctions and exposing the junctional domain of the cells, results in an increased level of internalized Afa/Dr DAEC infecting the brush border. This indicates that when Afa/Dr DAEC infects a diseased intestinal epithelial barrier in which the closure at the junctional domain is impaired, it can enter the cells.

(i) Structural lesions at the brush border. The highly differentiated apical pole structure of enterocytes is composed of a dense array of microvilli, considerably increasing the intestinal surface area (299). The formation of the brush border in enterocytes during the establishment of the apical-basal polarity results from a complex and highly regulated epithelial polarity program (408). Microvilli are formed by assembly of parallel arrays of actin filaments that create the actin bundle, and myosin-1a links the actin network to the plasma membrane (417, 418). In addition, zonula occludens (ZO) proteins required for TJ assembly also regulate the organization of the apical cytoskeleton, particularly the perijunctional antimyosin ring, and in turn function in the polarized organization of the cells (419).

The molecular and cellular mechanisms of the intestinal pathogenesis of Afa/Dr DAEC have been investigated using cultured fully differentiated human intestinal Caco-2 cells and T84 cells, which structurally and functionally mimic enterocytes and colonic cells of the intestinal barrier (420, 421). Unlike that of other enteric pathogenic *E. coli* strains (4, 5, 422), the intestinal pathogenesis of Afa/Dr DAEC is triggered predominantly via interactions between Afa/Dr adhesin and brush border membrane-associated proteins functioning as signaling receptors (410–412). Adhering diarrhea-associated wild-type strain C1845 and recombinant F1845 adhesin-positive *E. coli* induced hDAF-dependent injuries in microvilli in fully differentiated Caco-2 and T84 cells (341, 410) (Fig. 7A to F). Bacteria at the sites of attachment were trapped by an elevated number of microvilli as a result of contact with the tips of microvilli (Fig. 4). Elongation of microvilli occurs, and finally, the lesion results in a loss of microvilli (Fig. 7C to F). This brush border injury results in rearrangements of structural brush border proteins, including the disappearance of the F-actin cytoskeleton and clumping of villin, fimbrin, and α-actinin within cytoplasmic aggregates (410, 412). Loss of the brush border has been also observed after apical infection of human enterocyte and cultured enterocyte-like cells by EPEC and EHEC (326, 423). The EPEC- and EHEC-triggered attaching and effacing (A/E) lesions at the microvilli of the intestinal brush border follow a common mechanism of virulence resulting from the expression of the PAI locus of enterocyte effacement (LEE) controlling via a type III secretion system (T3SS) the completion of a bacterial syringe allowing the translocation of virulence factors into target intestinal host cells (4, 5, 422). Even though the disappearance of brush border microvilli induced by Afa/Dr DAEC, EPEC, and EHEC infection is morphologically similar, the mechanism controlling the deleterious effect of Afa/Dr DAEC at the brush border is very different from those for EPEC and EHEC, since Afa/Dr DAEC does not express any T3SS and is devoid of the EPEC and EHEC virulence factors involved in A/E lesions (60).

Mechanistically, the lesions induced by diarrhea-associated wild-type strain C1845 in the F-actin cytoskeleton result from F1845 adhesin-triggered, hDAF-dependent signaling events. In permanently undifferentiated human embryonic intestinal INT407 and undifferentiated intestinal Caco-2 cells, both expressing hDAF, infection by the wild-type strain C1845 and by an F1845 adhesin-expressing recombinant *E. coli* strain triggers F-actin cytoskeleton disassembly accompanied by the appearance of cytoplasmic phosphotyrosylated proteins and the activation of signaling molecules, including PTKs, phospholipase Cγ, PI3K, PKC, and Ca^{2+} (340). In enterocyte-like Caco-2 cells, the F1845 adhesin-triggered disassembly of brush border cytoskeleton-associated proteins is Ca^{2+} signaling dependent (412).

Elongation of microvilli precedes the disappearance of brush border in diarrhea-associated wild-type strain C1845-infected enterocyte-like Caco-2 cells (410). Cell membrane extensions attached or entrapping adhering Afa/Dr DAEC have been observed in infected epithelial cells. Yamamoto et al. (51) observed that cell membrane extensions are connected to the *daaC*-positive strain D2 adhering to Hep-2 and HeLa cells. Cookson and Nataro (424) observed cell membrane extensions in close contact with adhering bacteria in C1845-infected Hep-2 cells. Berger et al. (274) have dissected the mechanism by which Afa/Dr DAEC promotes cell membrane extensions. In epithelial cells infected with a Dr adhesin-expressing *E. coli* strain, the phenomenon results from the recognition by Dr adhesin of membrane-associated GPI-anchored receptor hDAF, hCEA, or hCEACAM6. In contrast, the phenomenon does not occur after recognition of the transmembrane receptor hCEACAM1. Mechanistically, the Dr adhesin-induced cell membrane extension is microfilament dependent and follows the activation of the small GTPase Cdc42 and the phosphorylation of ERM (274). The observation that phosphorylation of ERM accompanied the Dr adhesin-induced cell membrane extensions (274) is consistent with the known roles of Rho GTPases and ERM in the completion of the actin cytoskeleton (356). Indeed, the low-molecular-weight GTPases RhoA, Rac, and Cdc42 are enzymes that in the host cells control a wide range of physiological processes, including membrane trafficking, cytoskeletal dynamics, and nuclear importation and signal transduction pathways (425). The endogenous activators of the Rho family GTPases are guanine nucleotide exchange factors and GTPase-activating proteins (426). Bacterial pathogens hijacking the low-molecular-weight GTPases are critical targets of bacterial effector proteins (427).

After infection of cultured human enterocyte-like Caco-2 cells with the diarrhea-associated wild-type strain C1845, there was an original cell lesion characterized by the release of the tips of microvilli that were in close contact with the infecting bacteria (410). Indeed, disrupted tips of microvilli in contact with adhering bacteria vesiculated and remained attached to the bacteria. The released C1845 bacteria are newly positive for the brush border-

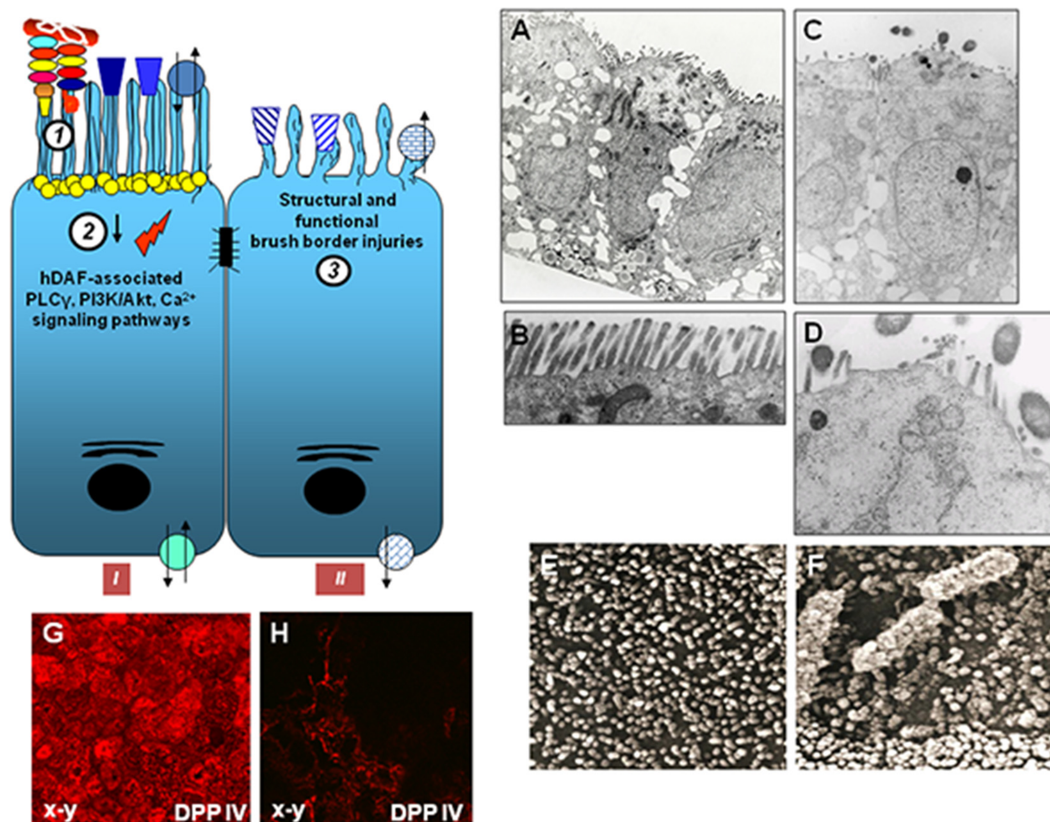

FIG 7 Structural and functional brush border lesions caused by diarrhea-associated Afa/Dr DAEC in enterocyte-like cells. The drawing on the left summarizes the observed cellular events. Afa/Dr DAEC interact with the brush border-associated hDAF and hCEACAM1, hCEA, and hCEACAM6 receptors (1). In turn, hDAF-associated signaling pathways, including protein tyrosine kinase(s), phospholipase Cγ, phosphatidylinositol 3-kinase, protein kinase C, and Ca^{2+}, are activated (2). Loss of the brush border results in the disassembly of the microvillus cytoskeleton and induces defective expression of functional proteins, such as SI, DPP IV, SGLT1, and GLUT5 (3). (A and B) Low and high magnifications of transmission electron micrographs showing the well-ordered brush border microvilli of uninfected cells. (C to F) Low and high magnifications of transmission electron and scanning electron micrographs show the disappearance of the brush border at a late time postinfection. (Micrographs in panels A to F reprinted from reference 410.) (G and H) Micrographs show the observation by CLSM of immunofluorescence labeling of brush border-associated DPP IV (*x-y* section). (Reprinted from reference 412.)

associated functional dipeptidylpeptidase IV (DPP IV), indicating a decoration by detached membrane microvilli (410). Under physiological conditions, vesicles expressing membrane-bound sucrase-isomaltase (SI) and maltase-glucoamylase aminopeptidase N (APN) are spontaneously formed at the microvillar tips by a mechanism involving the membrane-binding actin-based motor Myo1a, but not Myo2, and are subsequently shed into the luminal compartment (428, 429). Interestingly, the released vesicles contain particularly high levels of proteins that are preferentially partitioned into lipid rafts (429). Several vesicle cargoes containing brush border hydrolases and several proteins that have immunological function or are involved in inflammatory responses have been identified (429). This Afa/Dr DAEC-induced sacrificial cellular effect, which has as a direct consequence the extrusion of brush border-attached bacteria, probably has a limited and/or temporary impact on intestinal function, since the intestinal epithelial cells are entirely renewed at between 3 and 5 days (299, 373, 406).

(ii) Functional lesions at the brush border. In enterocyte-like Caco-2 cells (430–434), the asymmetric presence of membrane-associated functional proteins is monitored during the epithelial polarity program by biosynthetic and recycling routes mediating the apical or basolateral delivery of proteins possessing these specific apical or basolateral sorting signals (408). Functional proteins, including hydrolases, transporters, exchangers, some members of the aquaporin (AQP) family, and glycophosphatidylinositol (GPI)-anchored proteins, are expressed at the enterocyte brush border (299). It is interesting to note that large and highly well-organized "super lipid rafts" have been detected at the membrane of the brush border of enterocytes (320, 321, 435) containing some of the major brush border-associated functional proteins (436), including maltase-glucoamylase aminopeptidase N (APN) and sucrase-isomaltase (SI) (437), Na^+/H^+ exchanger (NHE) isoforms 1 to 3 (438), downregulated in adenoma Cl^-/base exchanger (DRA) (439), $Cl^-/HCO_3^-/OH^-$ exchanger (440), and peptide transporter 1 (PEPT1), which is responsible for the uptake of di- and tripeptides (441). Sophisticated strategies including the hijacking of the cellular machinery have been developed by enteric pathogenic *E. coli*, which result in the alteration of activities of membrane-associated transporters, ion channels, and/or exchangers and water channels, in turn modifying the normal transports of nutrients and the water balance (4, 5, 409, 422, 442). Accompanying the C1845-induced brush border structural injuries in enterocyte-like Caco-2 cells (410, 412), the

distribution at the brush border of functional intestinal proteins such as SI and DPP IV hydrolases, sodium/glucose cotransporter 1 (SGLT1), and the fructose GLUT5 transporter was profoundly impaired (412) (Fig. 7H and I). The default of SI and DPP IV expression at the brush border induced by strain C1845 relates directly to the promoted disassembly of the brush border cytoskeleton, since stabilizing the F-actin cytoskeleton by jaspakinoline treatment abolishes the disappearance of SI and DPP IV (411). In C1845-infected cells, there is a strong decrease of enzyme activity of SI and DPP IV (411, 412). It was logical to assume that this decrease in enzyme activity was related to the disorganization of the microvillus cytoskeleton and the disappearance of microvilli. However, it was not. Indeed, unlike C1845-induced, signaling-dependent cytoskeleton disassembly (340, 412), the loss of SI and DPP IV enzyme activity is independent of PTK, phospholipase Cγ, PI3K, PKC, and Ca^{2+} signaling pathways and also of C1845-induced cytoskeleton disassembly (411). This indicates that when SI and DPP IV are delocalized from the brush border in infected enterocyte-like cells, the hydrolases relocated into the cytoplasm lose their enzyme activity. In addition, the biosynthesis of the two hydrolases is severely affected without any change in mRNA levels and protein stability (411).

(iii) Structural and functional lesions at the junctional domain. The junctional domain in epithelia plays a critical role in health, and barrier dysfunction at the junctional domain can lead to disease (409). The adherence junctions (AJs) and desmosomes act as adhesive domains between intestinal epithelial cells. Indeed, cadherin-based cell-cell junctions are mechanical connections creating contractile force which, after interaction with the contractile antimyosin cortex, actively couples neighboring cells in the intestinal epithelial barrier (443, 444). The most apical junctional complex is TJs, which are highly regulated and formed by the assembly of specialized proteins such as the cytoplasmic ZO-1, ZO-2, and ZO-3 proteins connected with the F-actin cytoskeleton, the transmembrane occludin connected both with ZO proteins, cytoskeleton, and junctional adhesion molecules, and claudins connected with ZO proteins (445, 446). Functionally, TJs act as a "fence" separating the apical and basolateral membrane domains of polarized intestinal cells. This function results in the segregation in each membrane domain of cell proteins and lipids. Moreover, TJs, by the sealing of intercellular space, act as a "gate" regulating the paracellular passage of small particles and solutes. On the basis of functional studies (447), it has been recently evidenced that the gate activity includes two functional paracellular pathways: the first one, named the leaky pathway, engaging occludin that controls the paracellular passage of larger molecules, and the second, named the high-capacity pore pathway, engaging the claudin family of proteins functioning as cation-selective and anion-selective protein-forming channels and as protein-forming channels without a clear established selectivity. Enterovirulent bacteria have developed sophisticated strategies to breach the intestinal barrier by means of effectors that by signaling-dependent mechanisms target the structural molecules that compose the TJs (448). Pathogen-induced intestinal barrier deficiencies have been linked to the onset of inflammation and diarrhea (409).

The infection of fully differentiated Caco-2/TC7 cell monolayers by strain C1845, which expresses F1845 adhesin, is followed by an elevated level of paracellular permeability without affecting the transepithelial resistance (449). At an early stage of infection, the distributions of TJ-associated occludin and ZO-1 proteins are profoundly modified, showing the disappearance of the proteins at the TJs, whereas there was no change in expression of the AJ-associated E-cadherin. The TJ lesions develop from an F1845 adhesin-induced, hDAF-dependent mechanism. Guignot et al. (137) identified the toxin Sat of wild-type strain IH11128 as the virulence factor that triggers the TJ lesions (Fig. 8A). Sat induces rearrangements of the TJ-associated proteins ZO-1, ZO-3, and occludin without modifying significantly the claudin-1 distribution. In Sat-treated cells, there was a dramatic decrease of the membrane expression of ZO-1 and ZO-3 and a slight decrease in the membrane expression of phosphorylated and nonphosphorylated forms of occludin. In turn, Sat increases the paracellular permeability, since there is elevation the paracellular passage of mannitol without affecting the passage of nonionic molecules with higher molecular masses (137, 449). In addition, Sat induces the formation of fluid domes by increasing the transcellular passage (137) (Fig. 8B). These fluid-filled, blister-like structures known as fluid domes are randomly distributed areas that evolve permanently into the cell monolayers as the result of transcellular passage of fluids accumulating basolaterally (450, 451). This result is interesting since Taddei et al. (221) have reported that Sat can induce secretory activity, resembling the enterotoxic activity of ETEC, in rabbit ileum tissues mounted in an Ussing chamber, with a dramatic fluid accumulation in rabbit ileum loops.

Inflammatory responses. Diverse host cell proinflammatory responses followed the infection of enterocyte-like and colonic cell lines by Afa/Dr DAEC. Flagella expressed by EPEC, EAEC, and EHEC contribute to favor epithelium colonization and proinflammatory responses, such as the induction of proinflammatory IL-8 production (4, 5). Motile, AfaE1-, AfaE2-, or afaEX-positive diarrhea-associated strains induced the production of high levels of IL-8 in T84 and Caco-2 cells (203, 452). Meraz et al. (107) have reported that nine motile diarrhea-associated AfaE1- or AfaEX-positive *E. coli* strains induced the production of IL-8 in T84 cells. Paul Hofman and coworkers (341, 342, 344, 453) have nicely dissected the Afa/Dr adhesin-triggered, hDAF-dependent signaling pathways controlling the production of proinflammatory cytokines and related cellular events in colonic T84 cells. The nonflagellated, wild-type Afa/Dr DAEC C1845 and IH11128 strains were able to promote the basolateral secretion of IL-8 in monolayers of polarized intestinal T84 cells (341). The production is *daa* or *dra* operon dependent, involves binding onto brush border-associated hDAF, and develops by activation of the extracellular signal-regulated protein kinase (Erk1/2), p38, and c-Jun NH_2-terminal kinase signaling pathways. Arikawa et al. (203) observed that 12 nonmotile, AfaE1-, AfaE2-. or AfaEX-positive, diarrhea-associated strains also induced the production of IL-8, but at a level nine times lower than that found in the motile strain. Flagella isolated from motile Afa/Dr DAEC to produce IL-8 recognize the basolateral Toll-like receptor 5 (TLR5), and in addition, TJ function is observed to be reduced (454). To explain this phenomenon, it has been proposed that an unknown additional virulence factor causing the structural opening of TJs allows flagellin to reach basolaterally expressed TLR5 (454).

PMNLs play a pivotal role in maintaining intestinal homeostasis and are critical actors in the innate immune response that protects the host against microbial pathogens by generating a diversified arsenal of antimicrobial molecules, including reactive oxygen species, antimicrobial peptides, myeloperoxidase, hydrolytic enzymes, proteases, cationic phospholipase, and metal

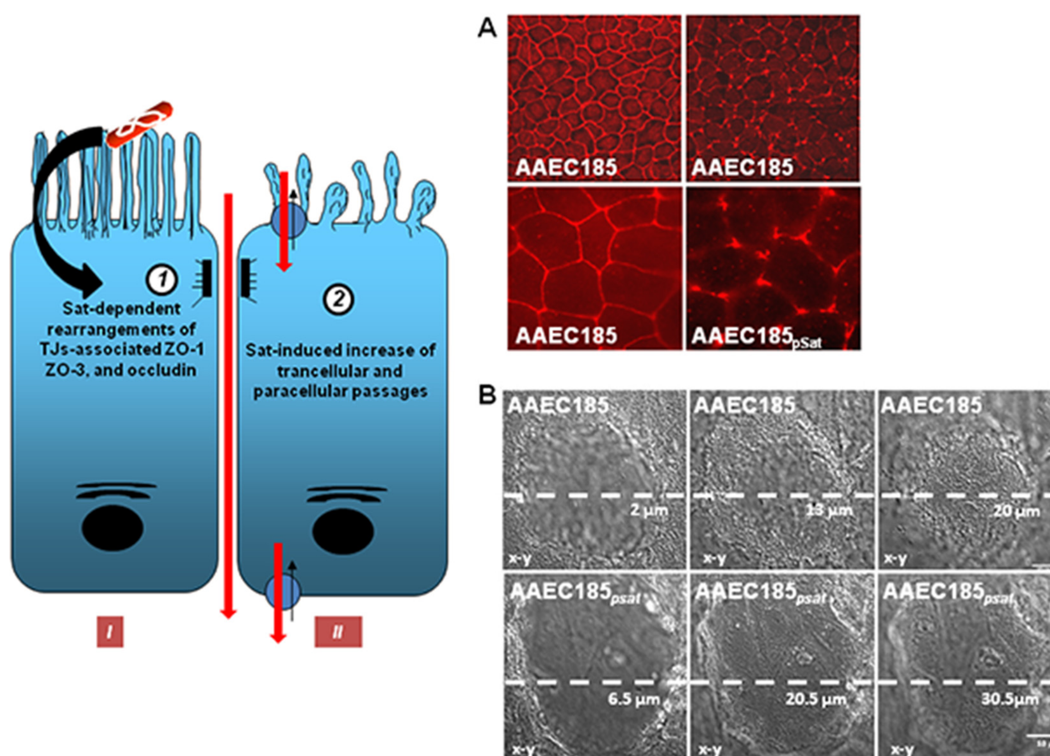

FIG 8 Structural and functional injuries at the tight junction caused by Afa/Dr DAEC in enterocyte-like cells. The drawing on the left summarizes the observed cellular events. The secreted and internalized Sat toxin induces the reorganization of the TJ-associated proteins ZO-1 and occludin without affecting E-cadherin expression (1). Sat induces an increase in transcellular and paracellular passages (2). (A) Low- and high-magnification micrographs show the immunofluorescence labeling of structural TJ-associated ZO-1 protein observed by CLMS in control and AAEC185$_{psat}$-infected cells. Note the Sat-induced disappearance of the protein from the cell-to-cell contacts. (Reprinted from reference 137 with permission.) (B) Fluid domes observed by phase-contrast CLSM in control and AAEC185$_{psat}$-infected cells. Note the Sat-induced increase of the fluid dome height and surface.

chelators, and by forming cell extensions known as neutrophil extracellular traps (NETs) (455). PMNL infiltration resulting from recruitment by chemokines produced by macrophages or epithelial cells at the site of insult is a hallmark of the host inflammatory response to infection with various different enteric bacterial pathogens. The effects of Afa/Dr DAEC strains on the transepithelial migration of PMNLs have been demonstrated in polarized monolayers of human colonic T84 cells cocultured with freshly isolated PMNLs (341). The transepithelial migration of PMNLs induced by Afa/Dr DAEC follows F1845 and Dr adhesin-induced hDAF-dependent basolateral production of IL-8 after activation of Erk1/2, p38, and JNK MAPKs. Afa/Dr DAEC-induced PMNL transmigration triggers cell synthesis of TNF-α, and IL-1β, in turn inducing the up-expression of hDAF at the brush border of the cells and increasing the adhesion of Afa/Dr DAEC bacteria (342). In addition, there was an abnormal appearance of hDAF at the basolateral domain of cells (342). The Afa/Dr DAEC-induced transmigration of PMNLs leading to a cytokine-triggered up-expression of hDAF was consistent with previously observed IL-1β-induced up-expression of hDAF (456–459). The up-expression of hDAF at the brush border is probably a cell defense response, since it has been demonstrated that hDAF functions as an antiadhesive molecule accelerating the release from the luminal surface of PMNLs that have undergone transepithelial migration (460, 461). It remains to be determined whether the abnormal basolateral expression of hDAF in infected colonic T84 cells is also a cell defense response that in turn can block the basolateral recruitment and transmigration of PMNLs.

The major histocompatibility complex (MHC) class I-related molecules A and B (MICA and MICB) are distant homologues of MHC class I molecules (462). Below the epithelium, resident lymphocytes can be activated by these nonclassical MHC class I molecules to display a diverse array of immune responses (463). Together with UL-16-binding proteins, MICA and MICB are ligands of human NKG2D (464), an activating natural killer (NK) receptor expressed on both tumor-infiltrating lymphocytes and tumors cells. NKG2D exerts cytolytic destruction of cells through recognition of its cognate ligands. NK cells circulate through the blood, lymphatics, and tissues, patrolling for the presence of pathogen-infected cells (465). MICA and MICB are more highly expressed at the surface of epithelial cells in colonic biopsy specimens from Crohn's disease (CD)-affected patients than in those from controls (466). Tieng et al. (467) have observed that the upregulation of MICA in enterocyte-like Caco-2 cells infected by AfaE-III adhesin subunit-positive *E. coli* is mediated by the specific interaction between the adhesin subunit and hDAF.

Pathogenic bacteria use quorum sensing (QS) to regulate several traits that allow them to establish and maintain infection in their host; these include motility, biofilm formation, and virulence-specific genes (468). In *E. coli*, QS involves autoinducers AI-2 and AI-3, depending on the function encoded by the *luxS* gene. It has been observed that the Dr adhesin-positive IH11128

strain produces high levels of AI-2 at the end of the exponential phase of growth (unpublished result). Sperandio et al. (469), observing that an EHEC *luxS* mutant responds to a eukaryotic cell signal activating the expression of its virulence genes, have identified epinephrine as promoting a "language" by which bacteria and host cells communicate. Diard et al. (343, 470) have demonstrated that norepinephrine is involved in Afa/Dr DAEC pathogenesis. In the wild-type strain IH11128, the thermoregulated production of Dr adhesin, which is optimal during the logarithmic phase of anaerobic growth, has been found to be accompanied by a release of Dr fimbriae without cell lysis (470). Norepinephrine increases the Dr adhesin release by affecting the production of the fimbrial subunits. Indeed, norepinephrine promotes the differential induction of genes *draC* and *draE* by a regulatory mechanism; i.e., the level of the *draE* transcript is highly increased, while expression of *draC* transcript is increased to a lesser extent, relative to basal expression (343). Like with the wild-type IH11128 (341), the released Dr adhesin induces the phosphorylation of Erk1/2, in turn promoting the production of IL-8 in enterocyte-like Caco-2/TC7 cells (343).

Proinflammatory effects of Afa/Dr DAEC after direct contact with PMNL-like PLB-985 cells have been described by Sylvie Chollet-Martin and coworkers (346, 347, 471). A rapid and massive release of reactive oxygen species and preformed intragranular mediators (myeloperoxidase and IL-8) develops in PMNL-like PLB-985 cells infected with the wild-type IH11128 and C1845 strains (347). The phenomenon is triggered not by Afa/Dr adhesins but by the type 1 pili (347) expressed by wild-type Afa/Dr DAEC strains (155).

Adherence to hDAF expressed at the membrane of freshly isolated PMNLs mediated by Dr adhesin did not lead to a significant increase of bacterial killing, indicating that the Afa/Dr DAEC could overcome the microbicidal activity of PMNLs (472). In contrast with the fact that PMNLs that have transmigrated display greater phagocytic activity against *E. coli* (473), PMNLs that have not transmigrated or transmigrated across T84 cell monolayers after Afa/Dr DAEC infection display low phagocytic activity, suggesting that Afa/Dr DAEC can block this activity (453).

Interaction of the wild-type Afa/Dr DAEC C1845 and IH11128 strains or recombinant Dr adhesin-positive *E. coli* with freshly isolated human PMNLs results in an increased rate of cell apoptosis evidenced by morphological nuclear changes, DNA fragmentation, cleavage of pro-caspase 3 and stimulation of the caspase activity, and up-expression of annexin V (453, 472). Since this phenomenon is not blocked by anti-hDAF and -hCEA antibodies, it seems to be linked to a leukocyte agglutination process rather than to the recognition of these signaling receptors. Intriguingly, phosphatidylserine appears at the cell membrane of PMNL-like PLB-985 cells, resulting from an Afa/Dr adhesin-triggered, hDAF-dependent activation of tyrosine kinase, and protein kinase C signaling occurs, but without apoptosis (346). Low levels of phagocytosis of Afa/Dr DAEC bacteria were observed in both nontransmigrated and transmigrated PMNLs, suggesting a bacterium-triggered diminished leukocyte phagocytic capacity to escape host defenses (453).

Angiogenesis. Angiogenesis is a new component of IBD pathogenesis. Cane et al. (345) have reported that infection of cultured human colonic T84 cells by the diarrhea-associated *E. coli* strain C1845 is followed by an increase in vascular endothelial growth factor (VEGF) mRNA expression and production of VEGF protein. Members of the VEGF family are secreted, dimeric glycoproteins of ~40 kDa consisting of five members, VEGF-A, -B, -C, and -D and a placenta growth factor, which function as regulators of vasculogenesis, i.e., the vascular development that occurs during embryogenesis, and the angiogenesis process that forms blood vessels (474). Importantly, the VEGF secreted during C1845-infection of T84 cells is bioactive, since the cell-free spent culture medium is able to induce tubulogenesis. This phenomenon results from the recognition of hDAF by F1845 adhesin followed by activation of a Src protein kinase upstream of the activation of the Ras/Raf/MAPK and PI3K/Akt signaling pathways (345). Upregulation of VEGF has also been observed in epithelial cells infected with an Afa-I adhesin-positive *E. coli* strain (140). Moreover, the F1845 adhesin-triggered, DAF-dependent up-production of VEGF and also IL-8 in T84 cells has been found to be controlled by an MAPK- and PI3K-dependent induction of the hypoxia-induced factor 1-α (HIF-1α), revealing a connection between hDAF-associated signaling and a translational mechanism(s) (344). It is noteworthy that HIF-1 consists of two subunits, HIF-1α and HIF-1β, and that HIF-1α, which is degraded via an oxygen-dependent process involving prolyl hydroxylases, functions as an oxygen sensor (475). It is interesting to note that the CD-associated AIEC strain LF82 also induces, by an unknown factor, the production of HIF-1 α protein and the activation of VEGF/VEGR receptor (VEGFR) signaling in T84 cells (476). All the above-reported cell responses to Afa/Dr DAEC infection are related mainly to recognition of intestinal epithelial cell-expressed hDAF by Afa/Dr adhesins. Considering that hCEACAM1, hCEA, and hCEACAM6 are known to be involved in immune responses (477), it remains to be shown whether these hCEACAMs also trigger host cell immune responses after recognition by Afa/Dr adhesins.

EMT events. Under physiological conditions, the intestinal epithelium undergoes controlled constant cell shedding. In epithelia, a programmed cell death process known as "anoikis," a Greek word meaning "homelessness," occurs when polarized epithelial cells forming an epithelial barrier are detached from the appropriate lateral domain and extracellular matrix (406). The control of anoikis in differentiated epithelial intestinal cells is a differentiation-dependent process involving the engagement of β1 and β4 integrins and activation of FAK, Src kinase, and the Erk1/2 and Pi3K/Akt signaling pathways (406). The diarrhea-associated C1845 affects the AJs of intestinal epithelial polarized colonic T84 cells. Indeed, an F1845 adhesin-triggered, hDAF-dependent loss of AJ-associated E-cadherin protein and cytokeratin 18 (CK18) has been observed, resulting from activation of HIF-1α (344). Moreover, CK18 plays a cytoskeletal function as a component of the intermediate filaments and acts as a target for caspase-mediated cleavage during cell apoptosis (478). In parallel, C1845 infection promotes a rise in fibronectin expression, accompanied by the up-expression of Twist1 mRNA (344), a helix-loop-helix transcriptional factor belonging to a small group of core transcription factors that also includes Snail, Slug, and Sip1 and which are involved in controlling the epithelial-mesenchymal transition (EMT) (479). The EMT is a transcriptional and morphological program observed during the progression of diseases, including cancers (480, 481). Three distinct subtypes of epithelial cells that transition into mesenchymal cells have been defined, depending on the physiological tissue context (481). Type 1 occurs in embryogenesis and organ development, type 2 develops in tissue re-

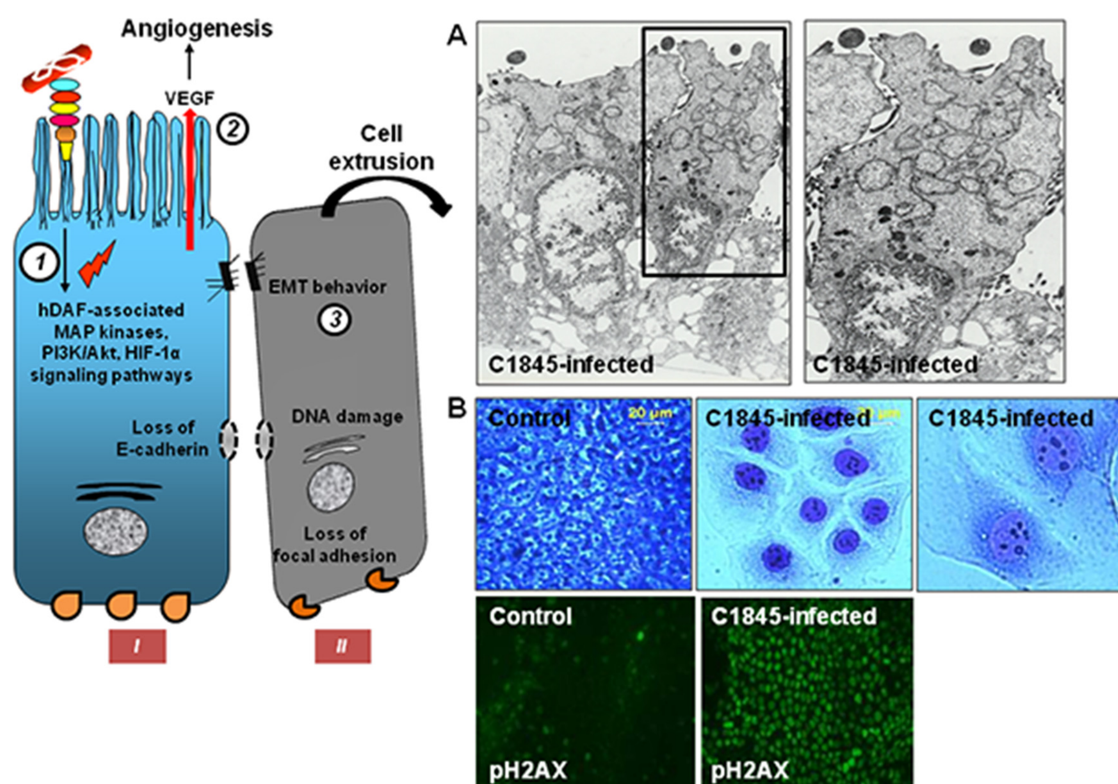

FIG 9 Diarrheagenic Afa/Dr DAEC strain C1845 promotes epithelial-mesenchymal transition (EMT)-like behavior, cell extrusion, and *pks*-dependent damage in human intestinal cells. The drawing on the left summarizes the observed EMT-related cellular events. An F1845-triggered hDAF-, MAPKs-, PI3K-, and HIF-1α-dependent production of VEGF develops in colonic-like T84 cells (1 and 2). An F1845-triggered hDAF-, MAPKs-, PI3K-, and HIF-1α-dependent EMT-like behavior develops, characterized by the typical changes in expression of mesenchymal markers, such as the upregulation of fibronectin, the downregulation of CK18, and the disappearance of AJ-associated E-cadherin (1 and 3). Finally, depolarized intestinal cells lose the lateral cell-to-cell and basal contacts and become detached from the cell monolayer. (A) Low-magnification transmission electron micrographs show the dedifferentiation of Caco-2/TC7 cells infected with the diarrhea-associated strain C1845, characterized by a disorganization of the apical domain and by the appearance of nucleus fragmentation and loss of nucleus dense electron material, indicating cell death (left micrograph). The boxed area indicates a cell with a funnel form engaging in shedding from the cell monolayer as the result of the wide opening of the lateral cell-to-cell junctional domain and detachment at the basal domain. The micrograph on the right shows a high magnification of the cell engaging in detachment and containing a high number of vesicles in the cytoplasm. (B) Low- and high-magnification micrographs show the morphological changes in Giemsa-stained C1845-infected undifferentiated Caco-2/TC7, cells characterized by an enlargement of the cell body and nucleus. Low-magnification micrographs show the increase of the phosphorylation of nuclear H2AX, the most sensitive marker of DNA damage. (Courtesy of J. P. Nougayrede and E. Oswald, reproduced with permission.)

generation and organ fibrosis, and type 3 is associated with cancer progression. However, partial EMT can develop as a function of the tissue and signaling contexts, resulting in a loss of only some of the polarized characteristics such as cell contact dissolution and actin cytoskeleton reorganization. The two major hallmarks of EMT are the dissolution of epithelial cell-cell adhesion contacts and the massive actin cytoskeleton reorganization followed by the loss of cell polarization. Transcription factors drive EMT by downregulating genes, including those encoding proteins maintaining epithelial cell-cell adhesion domains. After a long time of infection, C1845 infection promotes depolarization of the enterocyte-like Caco-2 cells, which is characterized by the complete disorganization of the apical domain and the loss of cell-to-cell contacts accompanied by the appearance of undifferentiated cells that become detached from the cell monolayers (Fig. 9A) (unpublished results). The C1845-induced cell shedding resembles the cascapase-1-dependent cell shedding observed in T84 cells (482) and the TNF-α-induced cell shedding observed in mouse intestinal and Madin-Darby canine kidney cells (482, 483). It has been mentioned above that in C1845-infected T84 cells, the induced transepithelial migration of PMNLs is followed by the production of TNF-α (342). Collectively, these results show that diarrhea-associated Afa/Dr DAEC promotes cell depolarization and injuries at the two major sites of the junctional domain of polarized epithelial cells forming the intestinal epithelial cell barrier, i.e., TJs and AJs, and in turn induces EMT-like behavior.

***pks*-related cell injuries.** The presence of the *pks* genomic island coding for the genotoxin colibactin in the prototype wild-type Afa/Dr DAEC strains leads to the appearance of known *pks*-related cell changes (240). Indeed, in C1845-infected undifferentiated Caco-2/TC7 cells there was an enlargement of the cell body and nucleus and an increase of the nuclear phosphorylation of histone H2A variant H2AX (Fig. 9B) (Nougayrede and Oswald, unpublished result). These cell injuries were abrogated when the cells were infected with a mutant colibactin strain (*clbA*::*frt*) deleted for the *clbA* gene, encoding the phosphopantetheinyl transferase ClbA within the *pks* island (484), and were restored by the complementation of the mutant strain with p-*clbA* (Nougayrede and Oswald, unpublished result). It is noteworthy that the *pks*-triggered enlarged cells cell have been recently characterized as

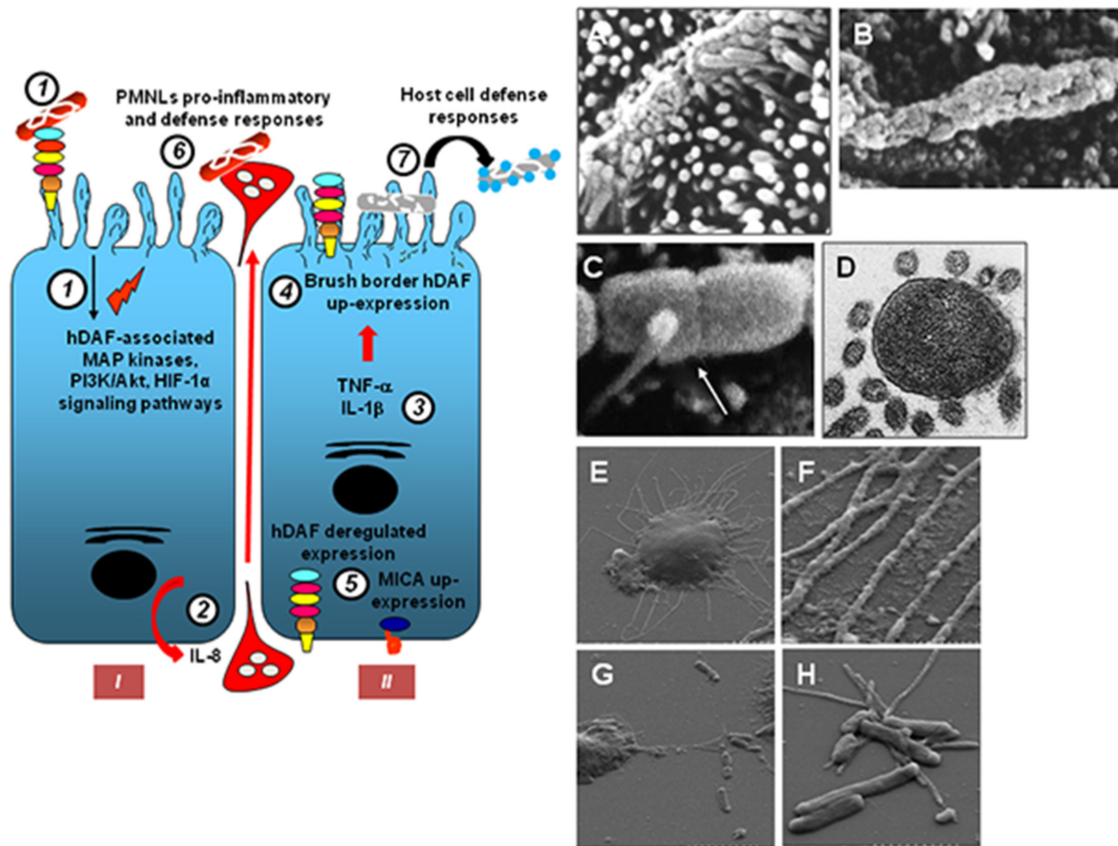

FIG 10 Proinflammatory and defense responses in enterocyte- and colonic-like cells and PMNLs after infection with the diarrhea-associated strain C1845. The drawing on the left summarizes the observed cellular events. An hDAF-dependent MAPK-, PI3K/Akt-, and HIF-1α-triggered signaling (1) leads to the production of the proinflammatory cytokine IL-8, which promotes the transepithelial migration of PMNLs (2), which in turn promotes the production of proinflammatory cytokines TNF-α and IL-1β (3) and leads to the up-expression of brush border-associated hDAF (4) and basolateral MICA (5) and the abnormal expression of hDAF at the basolateral domain (5). Afa/Dr DAEC interacting with transmigrated PMNLs induces cytotoxic proinflammatory responses and a defense response involving NET-triggered bacterial killing (6). As the result of a host intestinal cell defense response, bacteria attached at the brush border are entrapped by microvilli and killed (7). In addition, bacteria attached at the brush border are extruded from the intestinal cells when the tips of microvilli are shed (7). (A) A high-magnification scanning electron micrograph shows an altered bacterium entrapped by numerous microvilli in Caco-2 cells. (Reprinted from reference 487 with permission of Elsevier.) (B) A high-magnification scanning electron micrograph shows a highly damaged adhering bacterium at a late time of infection. (Reprinted from reference 487 with permission of Elsevier.) (C) A high-magnification scanning electron micrograph shows a bulbous membrane protrusion at the tip of elongated microvilli in close contact with an adhering bacterium. (Reprinted from reference 410.) (D) A high-magnification transmission electron micrograph shows a high number of cell membrane vesicles in close contact with a bacterium that is detached from the brush border. (Reprinted from reference 410.) (E and F) Low- and high-magnification field emission scanning electron micrographs show the NETs expressed by phorbol myristate acetate (PMA)-stimulated PMNL-like PLB-985 cells. (Reprinted from reference 471.) (G and H) Low- and high-magnification field emission scanning electron micrographs show NETs expressed by infected PMNL-like PLB-985 cells and in contact with diarrhea-associated C1845 bacteria. (Reprinted from reference 471.)

senescent cells bearing massive and irreparable damage and secreting protumorigenic factors that damage adjacent cells (485, 486).

Host cell defense responses. Infection of enterocyte-like Caco-2 cells by wild-type strain C1845 or recombinant *E. coli* expressing F1845 adhesin is followed by a strong antibacterial cell response at the brush border, since at a late time of infection the adhering bacteria are dramatically damaged (487) (Fig. 10A and B). This defense response seems to be correlated with the fact that Caco-2 cells express intestinal antimicrobial peptides in a differentiation-dependent manner (487, 488).

As described above, the intestinal cells after infection by Afa/Dr DAEC seem to react so as to resolve brush border colonization by releasing the tips of microvilli that were in close contact with the infecting bacteria (410) (Fig. 10C and D). Bacteria detached from the brush border express vesicles formed of microvillus membranes, indicating extrusion of brush border-colonizing bacteria. An increase in the microvillar vesicles shed after tEPEC infection of Caco-2_{BBe} clone cells has been reported (489). Interestingly, the interaction of these vesicles with the luminal pathogen blocks the tEPEC intimate attachment onto the microvilli of enterocyte-like HT-29 cells (489). Collectively these results indicate that the accelerated formation and shedding of microvillar vesicles constitute an efficient host intestinal cell defense response. Hammarstrom and Baranov (281) have interestingly postulated that the vesiculation of the microvillus membrane to form vesicles is an effective host defense mechanism intended to extrude adhering enterovirulent bacteria from the luminal surface of the intestinal epithelium and/or to limit the deleterious impact of enterovirulent bacteria.

NETs are formed by a nuclear DNA skeleton and transport antibacterial molecules, including antimicrobial peptides, his-

tones, and proteases (490, 491). In the human myeloid cell line PLB-985, which has the capability to differentiate into fully mature neutrophil-like cells, wild-type strain C1845 infection is followed by the projection of NETs, which by entrapment of bacterial cells trigger the killing of bacteria (471) (Fig. 10E to H). However, this defense response has an unexpected cell cytotoxic consequence, since the induced NETs promote a profound disorganization of the F-actin cytoskeleton by contact with the Caco-2/TC7 cells (471). It is worth mentioning that NETs induce cell death in endothelial cells (492, 493).

CLINICAL CONSIDERATIONS

Reservoir and Transmission

There has been no report of the isolation of *E. coli* expressing the major human AfaE-I to -III, Dr, or F1845 adhesins in a large variety of animal species. In contrast, the afimbrial Afa-VII and Afa-VIII adhesins are present in diarrhea- or septicemia-associated *E. coli* strains in calves (26, 44). Moreover, the AfaE-VIII adhesin is expressed in ExPEC infecting calves (43, 54, 494). The gene *afa-8* and *afa/dr*-related genes have been also been found in porcine, poultry, and cattle *E. coli* isolates (45, 173, 494–496). Examination of *E. coli* isolates from dogs with UTIs has revealed the presence of P- and S-positive isolates and the absence of Afa-positive isolates (497). There is an absence of evidence of direct animal-to-human transmission.

Treatments

Historically, antibiotics have provided a very effective way of resolving UTIs. The current routine treatment for UTIs is antibiotic therapy, most commonly trimethoprim-sulfamethoxazole (TMP-SMX) or ciprofloxacin. However, patients with chronic or recurrent cystitis require long-term treatment with antibiotics, and as UPEC strains have an unusual intracellular lifestyle, conventional antibiotics are not very effective when the bacteria reside within intracellular vacuoles or are present in biofilm-like structures (498–505).

Afa/Dr DAEC-associated diarrhea in children has been described as being generally limited in duration. Diarrhea, as defined by the World Health Organization (WHO), is characterized by the presence of three or more stools or watery stools within a 1-day period. Diarrhea is defined as being acute when it lasts less than 14 days and as persistent when the episode has lasted 14 days or more. In general, to prevent dehydration and nutritional default in children with infectious acute diarrhea, it has been recommended to provide an oral rehydration solution (ORS) and to continue feeding (506). Since ORS administration has no effect on the duration, severity, or frequency of infectious diarrheal episodes, ORS administration has been associated with adjuvant therapies such as luminally acting antisecretory drugs and antibiotics (507). However, these treatments can cause adverse effects outside or inside the gastrointestinal tract; one such effect that occurs if broad-spectrum antibiotics are used is the emergence of *Clostridium difficile*-associated diarrhea resulting from an antibiotic-induced imbalance of the intestinal microbiota (508, 509). It should be noted that cost and availability are limiting factors for the use of antisecretory drugs and antibiotics, particularly in developing countries. Another therapeutic strategy used to accompany ORS (which is low cost but for which availability may be a problem, particularly in developing countries) is the use of human intestinal microbiota probiotic *Lactobacillus* strains secreting antibiotic-like molecules that have demonstrated experimental *in vitro* and *in vivo* effects against the major diarrhea-associated bacterial pathogens (510).

Antibiotic Resistance

The increasing prevalence of antibiotic resistance of bacterial pathogens is a major health problem (511, 512). There has been a steady and rapid increase of UPEC resistance to antibiotics accompanied by the problematic occurrence of multidrug-resistant UPEC strains over the last decade (199, 349, 498). For examples, a recent international study revealed that more than 10% of *E. coli* cystitis isolates are resistant to at least three classes of antibiotics (513). Moreover, a recent survey of more than 12 million clinical isolates across the United States from 2000 to 2011 revealed an increase of resistance to 9 antibiotics, including TMP-SMX, ciprofloxacin, nitrofurantoin, and ceftriaxone (514). Not surprisingly, resistance to antibiotics has been reported for Afa/Dr DAEC, including resistance to ampicillin (90, 124, 515, 516), TMP-SMX (515–518), fosfomycin (517), piperacillin (515), tetracycline (124, 515), ciprofloxacin (518), co-trimoxazole (124), nitrofurantoin (518), fosfomycin (517), tetracycline (519), penicillin (516), oxacillin (516), bactericin (516), cloxacillin (516), chloramphenicol (519), and nalidixic acid (124, 516). In addition, the fact that Dr adhesin-positive *E. coli* has the capability to form biofilm (190, 233) suggested that like FimH-positive UPEC (199, 232), uropathogenic Afa/Dr DAEC strains have developed strategies to escape antimicrobial host defense systems and to reduce their sensitivity to antibiotics.

Vaccines and Pilicides

A therapeutic strategy has been developed to disarm pathogens in the host by the use of substances that mimic bacterial virulence factors such as adhesive factors, bacterial effectors, and toxins or that display antagonistic activities against the bacterial production of these factors (520, 521). Touchon et al. (522) underlined that this could make it difficult to develop a vaccine against ExPEC infections. It is noteworthy that an emerging therapeutic strategy consists of developing antagonists of QS systems in order to inhibit the bacterium-bacterium and bacterium-host cell communications involved in virulence (523). Brumbaugh and Mobley (520) have recently summarized the advances in vaccine strategy against UPEC and enteric virulent *E. coli*. These include compounds targeting surface polysaccharides and formulations composed of several virulence factors such as hemolysin, type 1, P, Dr, and S fimbrial adhesins, CNF-1, siderophores, and markers of $\text{PAIs}_{\text{CFT073}}$ and which display serotypes O1, -4, -6, -17, -75, and -77, K1, -3, -5, -13, and -95, and flagellar H:1, H:5, H:7, and H:33. Other strategies are the targeting of membrane proteins from UPEC strains or the use of mixtures of inactivated UPEC strains or genetically engineered vaccines. Although promising, the use of antagonists of adhesive factors to combat bacterial colonization of epithelia is complicated by the fact that a single pathogenic *E. coli* strain expresses a multiplicity of adhesive factors, each of which displays specific host cell receptor recognition. Consequently, it appears evident that it may be necessary to target more than one virulence factor for successful vaccine or pilicide therapies. Another subunit vaccine strategy against ExPEC has been designed to target immunodominant epitopes of the virulence-associated ExPEC proteins FyuA, IroN, ChuA, IreA, Iha, and Usp (524). For Afa/Dr DAEC infection, a vaccine strategy has been experimen-

tally tested using the Dr adhesin in the mouse pyelonephritis model (385). High titers of serum anti-Dr antibodies develop after vaccinating mice with purified Dr adhesin, and this is accompanied by a significant reduction in mortality but, surprisingly, without affecting the levels of bladder or kidney colonization by Dr adhesin-positive *E. coli*. *Ex vivo*, preincubating Dr adhesin-positive *E. coli* with Dr adhesin-immunized mouse serum reduces the bacterial adherence to mouse bladders and kidneys, whereas preincubating urine from Dr adhesin-immunized mice fails to produce this inhibitory activity.

Therapeutic-strategy-based pilicides have been developed in order to inhibit the completion of fimbrial and nonfimbrial adhesins at the cell surface of bacterial pathogens, including UPEC and enteric pathogenic *E. coli* (525, 526). Pilicides are a class of low-molecular-weight compounds which, by blocking chaperone and usher functions, inhibit pilus/fimbria completion without killing or affecting the growth of bacteria. Consequently, pilicides can prevent the first critical adhesion step required for the successful urinary tract colonization by UPEC strains involved in cystitis and pyelonephritis. On the bases that AfaC/DraC/DaaC ushers are pivotal for Afa/Dr adhesins biogenesis to occur and that the *draC* and *afaC-III* genes encoding the Afa/Dr ushers display 100% identity, it has been postulated that 2-pyridone pilicide compounds could inhibit the biogenesis of Afa/Dr adhesins (83, 176, 177). This pilicide strategy has been found to be effective against Dr adhesin biogenesis, where it produces a marked reduction of the expression of Dr adhesin and the disappearance of its properties of adherence to hDAF (175, 527).

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The importance of human Afa/Dr DAEC in UTIs and pregnancy complications has been convincingly demonstrated both experimentally and clinically. Moreover, increasing epidemiological evidences has been reported during the last decade on the role of human intestinal Afa/Dr DAEC in triggering established acute diarrhea in infants <5 years. In contrast, epidemiological studies indicate that human intestinal Afa/Dr DAEC strains are probably not responsible for established acute diarrhea in adults. A robust PCR method for detecting and identifying diarrhea-associated Afa/Dr DAEC strains remains to be developed, as the PCR methods currently used in a clinical setting are problematic with regard to the recently observed cross-reaction between the most commonly used Afa/Dr DAEC *daaC* primer and EAEC primers.

In spite of the remarkable results obtained over the past decade, the cellular and molecular mechanisms of Afa/Dr DAEC pathogenesis remain incompletely elucidated. Several aspects deserve particular attention in the future. Whole-genome sequencing is called for to identify more potential virulence factors in the wild-type Afa/Dr DAEC prototype strains in order to complete our understanding of the pathogenesis of these pathogenic *E. coli* strains. Much of our current knowledge about Afa/Dr DAEC intestinal pathogenesis has been obtained from *in vitro* observations using cultured human epithelial colon cancer cells, which, despite mimicking the situation *in vivo*, do not entirely reflect the healthy human situation and have several drawbacks (421). The high specificity of Afa/Dr adhesins for human epithelium-associated molecules acting as receptors and triggering cell signalization and cellular effects complicates the experimental examination of the cellular and molecular mechanisms of Afa/Dr DAEC pathogenesis. In comparison with the *in vitro* situation, the use of transgenic mouse models constructed for the epithelial expression of hDAF and hCEACAMs has not given convincing results in terms of cellular lesions even in the presence of Afa/Dr DAEC intestinal colonization (unpublished results). This is probably due to the endogenous presence of mouse DAF and CEACAMs and to the observation of a less-than-optimal expression of the membrane-bound human receptors (unpublished results) in the mouse epithelial tissue and/or defective connection to the mouse endogenous signaling pathways that have been observed *in vitro* to be involved in Afa/Dr DAEC pathogenesis. To overcome these problems, human intestinal *in vitro* organ culture (IVOC) models (528) can offer valuable tools for the experimental investigation of the mechanisms of virulence of intestinal Afa/Dr DAEC. Various IVOC systems corresponding to an *ex vivo* human situation have been described, including those constituting polarized IVOCs established from pediatric duodenum biopsy specimens or specimens of human colon taken at the vicinity of tumors. These IVOC systems have been used mainly to study the mechanisms of virulence of enterovirulent *E. coli*, including ETEC, EPEC, EHEC, EAEC, and AIEC (528).

Repeated observations in epidemiological studies of the intestinal presence of *daaC*-positive DAEC strains in adults in the absence of acute diarrhea are intriguing. This asymptomatic carriage deserves further exploration. Indeed, the Afa/Dr DAEC strains are clearly pathogenic with regard to the deleterious effects observed *in vitro* in human intestinal cell models, some of which resemble the pathogenic effects of EPEC and EHEC even though they result from different cellular and molecular pathogenic mechanisms. This reinforces our previous hypothesis that intestinal Afa/Dr DAEC strains act as "silent pathogens" that are well controlled under healthy conditions by the intestinal antimicrobial molecules generated by dedicated cells of the host intestinal barrier and by the barrier effect exerted by the intestinal microbiota (11). The above-described deleterious cellular effects of intestinal Afa/Dr DAEC make us think that these microbiota-resident *E. coli* in healthy humans could be good candidates for belonging to the recently defined class of "pathobionts" (529). Indeed, recent evidence suggests that the observed deleterious cellular effects may be caused by specific bacterial species with pathogenic potential that were present as symbionts in the healthy host intestinal microbiota. Although the pathophysiological mechanisms of pathobionts remain largely unknown, it appears that genetic factors, environmental factors, and/or changes in host defense systems may expose the host to developing diseases triggered by these potentially pathogenic microbiotal bacteria. Observations that some strong cell defense responses, including the extrusion of infecting bacteria from the brush border, cell detachment of intoxicated cells, and killing by NETs of activated PMNLs developed after Afa/Dr DAEC infection, all support the existence of efficient control by the host of these pathogenic *E. coli* strains in healthy situations. This leads to the question of how a resident intestinal microbiota Afa/Dr DAEC strain can emerge as an enterovirulent pathogen. Several circumstances allow the occurrence of diseases triggered by pathobionts. For example, dysbiosis, a shift in the makeup of the intestinal microbiota community and/or its abundance, leads to a change in the equilibrium between putative protective species and harmful pathogens (530). Another *E. coli* pathobiont candidate is the *E. coli* strain C25, isolated from the feces of a healthy human (531, 532). This strain expresses the SPATE toxin Pic (533), translocates across colonic T84 cell monolayers, promotes

alterations in localization of claudin-1, activates NF-κB signaling, and induces the production of IL-6, IL-8, and TNF-α (533–535), all effects that are exacerbated in a proinflammatory situation (536).

The causes of IBD have not been entirely identified but are known to be related to a susceptible human genome (537), a dysfunction of the immune system (537), a massive and/or selective modification in the composition of the intestinal microbiota (538), and the intervention of opportunistic enterovirulent bacteria (529, 539). With regard to the observed asymptomatic carriage of intestinal *daaC*-positive *E. coli* strains, some of the above-reported deleterious cellular effects of intestinal Afa/DrDAEC, including marked proinflammatory responses, indicated that these strains could play a role in intestinal inflammation. Afa/Dr DAEC belongs to phylogenic group B2, which has been observed to be prevalently associated with IBD (138, 217). It is difficult to address this question experimentally because of the lack of experimental animal models mimicking the human situation. Furthermore, epidemiological clinical studies are difficult to conduct because this would require isolating the mucosa-associated intestinal Afa/Dr DAEC in patients with chronic inflammatory diseases. Indeed, the *in vitro* results show that these strains are very effectively excluded from the brush border by the host defenses. Moreover, the sampling techniques used to isolate the mucosa-associated bacteria, which are localized exclusively on the luminal surface of the intestinal barrier, are also problematic, since these bacteria can be easily lost during the technical process. It has been found to be easier to isolate AIEC from patients with chronic inflammatory diseases simply because it is intracellularly localized in cells lining the epithelial barrier. Future progress can be expected to provide answers to these questions.

There are emerging links between microbial and parasite infections and oncogenesis (540). Moreover, the relationships and mechanisms through which disturbance of intestinal microbiota, infection, and inflammation increase cancer risks and promote tumor development were recently reviewed (541, 542). A small number of microbial pathogens have been identified as critical causes of specific chronic inflammatory conditions or malignancies. The best-characterized example of a bacterial contribution to cancer is *H. pylori* infection, which is well established as a major risk factor for gastritis leading to gastric cancer as the result of the deleterious activity of its cytotoxin-associated gene A protein (543). However, the simple observation that some bacteria are present at the site of cancerous intestinal tumors does not constitute a proof of causality. A link of causality is difficult to demonstrate here because there is a long time lag between initiation of a cellular process of carcinogenesis and the onset of overt disease. Indeed, the bacterium-triggered event can occur long before any sign of a cancer tumor manifests itself, and indeed the pathogen may no longer be present. It is becoming clear that some proinflammatory bacterial pathogens can contribute to initiating, promoting, and/or progressing cancer by their virulence factors, including toxins, which directly disrupt cellular signaling pathways that control inflammatory processes and damage DNA. Some demonstrated aspects of the mechanisms of pathogenicity of intestinal and uropathogenic Afa/Dr DAEC strains suggest a possible connection with the initiation or development of intestinal and urinary tract cancers. Some of the deleterious cellular responses triggered by Afa/Dr DAEC described above suggest that they can be connected to the initiation of cellular events in relation to intestinal cancer. The *pks* island (238, 540) is present in Afa/Dr DAEC, and it has been found that *pks*-positive *E. coli* strains belonging to the phylogenic B2 group are prevalently present in biopsy specimens of patients with intestinal cancers and induce DNA double-strand breaks in intestinal cells as well as trigger chromosomal instability, gene mutations, and cell transformation (22, 240, 246, 544). Afa/Dr DAEC infection is accompanied by the transepithelial migration of PMNLs, and in the intestinal tract migration of PMNLs across the epithelial lining is a hallmark of IBD and can create a deleterious inflammatory situation (455) and preneoplastic conditions, since transmigrated PMNLs exert a direct cytotoxic effect by releasing products such as oxidative reagents and elastase (545). Moreover, after Afa/Dr DAEC-induced transmigration of PMNLs across a monolayer of intestinal cells, the epithelial cells displayed deregulated expression of membrane-associated hDAF with altered functions, some of which are implicated in carcinogenesis (546). On the other hand, it should be noted that hCEACAMs, the receptors of Afa/Dr adhesins, are known to be implicated in carcinogenesis (275). In addition, the EMT and angiogenesis observed to be triggered by intestinal Afa/Dr DAEC are indicative that these bacteria play a role in carcinogenesis (480). An important function of autophagy in cancer is the limitation of inflammation, tissue damage, and genome instability, and when the autophagy process is blocked at the degradative autophagosome step, cells accumulate cytotoxic material, thus promoting DNA mutation and carcinogenesis (547, 548). Consequently, the observed blockade of autophagosome maturation triggered by the Afa/Dr DAEC Sat toxin may play a role in carcinogenesis. It is noteworthy that this deleterious effect of autophagy has been observed for *H. pylori* in relation to its role in the development of gastric cancer (549), since the vacuolating cytotoxin VacA induces autophagy and disrupts autophagosome maturation after prolonged exposure (550). UPEC-induced cell exfoliation in turn induces the upregulation of urothelial turnover, and it has been postulated that it may play a role in patients' predisposition to bladder cancer (504, 551, 552). It is tempting to hypothesize that Afa/Dr DAEC is involved in the development of bladder cancer, given the observed cell exfoliation effect triggered by the Afa/Dr DAEC Sat toxin. Collectively, these results provide a group of facts but do not amount to evidence of a possible link between Afa/Dr DAEC infection and intestinal or bladder carcinogenesis.

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