Isolation and Serologic Characterization of AIDA-I, the Adhesin Mediating the Diffuse Adherence Phenotype of the Diarrhea-Associated \textit{Escherichia coli} Strain 2787 (O126:H27)

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The adherence of diarrhea-associated \textit{Escherichia coli} to the small-bowel mucosa is an important step in the pathogenesis of diarrheal diseases. In tissue culture systems, diarrhea-associated strains show three distinct patterns of adherence: localized adherence, diffuse adherence (DA), and the recently described aggregative adherence. To study the molecular basis of the DA phenotype, we investigated the diarrhea-associated DA strain 2787 (O126:H27), isolated from a case of infantile diarrhea. The DA phenotype is mediated by a 6.0-kb DNA fragment derived from a 100-kb plasmid harbored by the wild-type strain. This fragment codes for a 100-kDa protein which can be released from the bacterial cell into the supernatant by mild heat treatment. Recombinant DA+ strains as well as the isolated 100-kDa protein were used to engender specific antisera in rabbits. As demonstrated by Western blotting (immunoblotting), the antibodies engendered by the recombinant DA+ strain recognized a 100-kDa protein in the wild-type strain 2787 and in all recombinant strains showing DA. Immunogold electron microscopy localized the 100-kDa protein to the bacterial cell surface.

Serologically related proteins of similar size were detected by Western blotting in other DA+ diarrhea-associated strains belonging to enteropathogenic \textit{E. coli} serotypes. The 100-kDa protein denoted AIDA-I (adhesin involved in diffuse adherence) binds in a saturable fashion to HeLa cells. AIDA-I-specific immunoglobulin G antibodies—and, to an even greater extent, Fab fragments derived thereof—inhibited bacterial attachment to HeLa cells. This is direct evidence that the 100-kDa protein is the adhesin mediating the DA phenotype of these diarrhea-associated strains and is representative of a group of serologically related proteins in other DA+ strains.

Enteropathogenic \textit{Escherichia coli} (EPEC) strains were the first \textit{E. coli} strains recognized as important pathogens in diarrheal diseases (14). Even today, EPEC strains are the major bacterial cause of neonatal and infantile gastroenteritis throughout the world, especially in developing countries. The pathogenic mechanisms involved in the development of persistent diarrhea caused by EPEC, however, still remain unclear.

Originally, EPEC strains were classified by O:H serotype. Recently, strains that do not produce heat-stable or heat-labile enterotoxin and are not invasive according to the Serény test but cause diarrhea by as yet unknown pathogenic mechanisms are classified as EPEC (13).

As indicated by histopathological studies, adherence of EPEC to the intestinal mucosa is of prime importance for the pathogenesis of EPEC-caused diarrhea. To study the attachment of EPEC strains, HeLa and HEP-2 cells are often used as model systems. Three distinct patterns of adherence have been described: localized adherence (LA), in which bacteria attach to and form microcolonies in distinct regions of the cell surface; diffuse adherence (DA), in which bacteria adhere evenly to the whole cell surface; and aggregative adherence, in which aggregated bacteria attach to the cell (17, 18, 21). These different attachment patterns imply distinct adherence mechanisms and recognition of different structures serving as receptors on the epithelial cell surface.

A large plasmid (ca. 100 kb, denoted pMAR2) was shown to mediate the LA phenotype (17). A 1-kb fragment of pMAR2 was proposed as a specific probe for the detection of the EPEC adherence factor (EAF) of LA EPEC strains (1). The EAF was suggested to correspond to a 94-kDa protein (15). However, not all EAF+ strains express a 94-kDa protein, and the expressed proteins of this size were further shown to be serologically different (5). All EAF+ strains induce attaching and effacing lesions in gut epithelial tissues. This phenomenon is characterized by the attachment of bacteria to enterocytes and local effacement of the microvilli of brush border membranes, followed by disruption of the cellular cytoskeleton (9, 10). Recently, the genetic locus on the chromosome responsible for attaching and effacing lesions (\textit{eae} locus) in tissue culture was identified (8). Some \textit{E. coli} strains show LA and attaching and effacing lesions but do not react with the EAF+ probe (11, 19). This strongly suggests that probably more than one mechanism, and thus probably also more than one type of adhesin, are involved in the attachment of EPEC bacteria to epithelial cells (11).

However, the term EPEC has recently been used in a more restricted sense to denote only those diarrhea-associated \textit{E. coli} strains which exhibit attaching and effacing lesions and are positive in the fluorescent-actin staining assay (11). Diarrheagenic \textit{E. coli} strains exhibiting the DA phenotype, at least two different adhesins have so far been identified. Bilge et al. (3) have characterized a region of chromosomal DNA coding for a fimbrial adhesin. Recently, we reported the identification of a plasmid-encoded afimbrial adhesin involved in diffuse adherence (AIDA-I) by molecular cloning (2). Here, we describe the isolation, localization, and serologic characterization of a 100-kDa protein encoded on the DNA fragment conferring the DA phenotype. This
protein is identified as the AIDA-I adhesin mediating diffuse adherence to tissue culture cells.

**MATERIALS AND METHODS**

**Bacterial strains and plasmids.** Strain 2787 (O126:H27), originally isolated from a case of infantile diarrhea, was kindly provided by H. Einecke (Hygiene Institut, Universität Heidelberg, Heidelberg, Germany). This strain exhibits a classical EPEC serotype (O126:H27). To our regret, its serotype was erroneously reported as O127 previously (2). All other clinical isolates in this study were obtained from H. Karch (Universität Würzburg, Würzburg, Germany). These EPEC strains all belong to serogroup O128 but show different plasmid profiles, indicating that they are distinct strains. *E. coli* K-12 C600 (F− thi-1 thr-1 leuB6 lacY1 tonA2I supE44 λ−) was used as the host strain for all recombinant plasmids. The plasmids constructed in the molecular cloning of the adhesin have been described previously (2). Plasmid pIB6 harbors an 11.0-kb fragment obtained by partial EcoRI restriction of plasmid DNA of the wild-type strain 2787. Plasmid pIB4 contains a 9.2-kb fragment derived from CiaI restriction of pIB6. Both plasmids transfer the DA phenotype to *E. coli* K-12 strains and are used interchangeably in this study. Bacteria were grown overnight at 37°C in liquid culture in Standard I medium (E. Merck AG, Darmstadt, Germany) with the appropriate antibiotic (100 µg of ampicillin per ml). Bacterial strains used to quantify adhesion assays were cultivated with the addition of 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce β-galactosidase activity.

**Preparation of total cell extract.** Bacteria were suspended in 62.5 mM Tris-HCl (pH 6.8)−20% glycerol−3% sodium dodecyl sulfate (SDS)−8% β-mercaptoethanol and incubated for 10 min at 100°C.

**Selective detachment and solubilization of the adhesin.** To detach and solubilize surface-associated adhesin proteins, the bacteria were incubated at 60°C for 20 min in 10 mM sodium phosphate (pH 7.0) (6). After removal of bacterial cells by centrifugation, the adhesin was isolated from the supernatant.

**Adhesin-specific rabbit antiserum.** Specific antibodies were prepared in a female chinchilla bastard rabbit which prior to immunization was devoid of antibodies reactive with the 100-kDa protein. For immunization, whole cells of the recombinant DA+ strain C600/pIB4, as well as isolated 100-kDa protein, were employed. Plasmid pIB4 contains a 9.2-kb DNA fragment conferring the DA phenotype on K-12 strains (2). Approximately 3 × 109 *E. coli* C600/pIB4 cells suspended in complete Freund’s adjuvant (0.5 ml) were injected intradermally at multiple sites. Two booster injections with the same dose in incomplete Freund’s adjuvant were administered 3 and 7 weeks later. One week after the last immunization, the rabbit was bled from the ear vein. Since the serum engendered by whole bacteria recognized various other *E. coli* proteins, it was exhaustively absorbed with *E. coli* C600/pBR322 (approximately 1012 cells per ml). Fab fragments were prepared from purified immunoglobulin G antibodies as described by Harlow and Lane (7).

**Detection of the adhesin by Western blotting (immunoblotting).** Proteins were separated by SDS-polyacrylamide gel electrophoresis in a discontinuous buffer system by the method of Laemml (12). The electrophoretic transfer was performed with 25 mM Tris-HCl (pH 7.2) essentially as described by Towbin et al. (20) and Burnette (4). After transfer, the nitrocellulose was blocked with 3% bovine serum albumin-phosphate buffered saline (BSA-PBS) for 30 min at room temperature. Antiserum was applied in a 1:5,000 dilution in 0.1% BSA-PBS. After 90 min, the nitrocellulose was washed three times (10 min each) with 0.06% Brij 35-PBS and incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated second antibody (Bio-Yeda, Israel) in 0.1% BSA-PBS. Bound antibody was visualized after repeated washings by incubating it with Nitro Blue Tetrazolium−5-bromo-4-chloro-3-indolyl phosphate (used as a substrate) in AP buffer (100 mM Tris-HCl [pH 9.5], 1 mM MgCl2). After color development, the reaction was stopped by the addition of 20 mM Tris-HCl (pH 8.0) containing 5 mM EDTA.

**Localization of the adhesin by immunogold electron microscopy.** Copper grids were coated with bacteria for 90 s. After the removal of excess bacterial suspension, the grid was placed face down on a drop of 1:1,000-diluted antiserum for 30 min, washed with PBS, and placed on goat anti-rabbit antibodies adsorbed to 5-nm colloidal gold particles (Janssen Pharmaceutica, Beerse, Belgium) for an additional 30 min. After washing, the grids were negatively stained with 1% uranyl acetate. Samples were examined with a Philips EM 400 transmission electron microscope.

**Direct binding of the 100-kDa protein to HeLa cells.** HeLa cells were cultured in a 96-well tissue culture plate. After the formation of monolayers, they were washed with PBS and fixed with 0.25% glutaraldehyde for 10 min. After a 20-min incubation with 0.2% glycine to remove excess residual glutaraldehyde, the plates were blocked with 3% BSA-PBS. Purified 100-kDa protein in PBS was added in serial dilutions (100 µl per well). Plates without added 100-kDa protein were used as controls. The plates were washed with 0.06% Brij 35-PBS after 1 h at 37°C. After the addition of antiserum (1:10,000 in 0.1% BSA-PBS), the plates were incubated for 1 h at 37°C, and the wells were then washed several times with 0.06% Brij 35-PBS. Bound antibody was detected enzymatically with alkaline phosphatase-conjugated second antibody, with p-nitrophenyl phosphate used as a substrate. The enzyme reaction was evaluated by determining the optical density at 405 nm. All assays were run in triplicate.

**Adhesion assay.** To assay the adherence of bacteria to HeLa cells, the method described by Minami et al. (16) was slightly modified. Confluent HeLa cell monolayers in 96-well tissue culture plates were washed with PBS prior to the addition of bacteria. For the induction of β-galactosidase activity, the bacteria were first cultivated in the presence of 0.2 mM IPTG. About 107 bacteria in 100 µl of PBS containing 0.5% mannose were added per microtiter well. After an incubation at 37°C for 30 min, the cells were washed three times with PBS and subjected to the β-galactosidase assay. To disrupt the bacterial cell wall, 80 µl of assay buffer (100 mM sodium phosphate [pH 7.0], 1 mM MgSO4, 100 mM β-mercaptoethanol) saturated with toluene was added to each well and incubated for 10 min at room temperature. As a substrate, o-nitrophenyl galactoside (130 µl of a 2.5-mg/ml solution in assay buffer) was added to each well. After incubations at 37°C for various times (2 to 4 h), the absorbance at 405 nm was determined in an automated enzyme-linked immunosorbent assay (ELISA) reader (TiterTek). For inhibition assays, the bacteria were incubated with antiserum or Fab fragments directed at the AIDA-I protein for 30 min prior to the addition to HeLa cells. The data shown are averages of assays run in triplicate.
RESULTS

Serologic identification of the cloned adhesin AIDA-I as a 100-kDa protein in DA+ recombinant strains. The molecular cloning of an adhesin mediating DA to tissue culture cells has been described previously (2). In these studies, a protein of about 100 kDa was identified that corresponded with the DA phenotype, since this protein was detected in the original wild-type strain and the DA+ recombinant strains derived thereof. Recombinant DA+ strains harboring partially deleted versions of the putative AIDA-I operon did not express the 100-kDa protein. Thus, to verify AIDA-I as the 100-kDa protein and for serologic characterization, specific antisera were engendered in rabbits. For this purpose, whole cells of the DA+ recombinant strain C600/pIB4 as well as the purified 100-kDa protein were used as immunogens. The antiserum raised against C600/pIB4 bacteria was exhaustively absorbed with C600/pBR322 bacteria to remove antibodies directed at proteins not encoded on the DNA insert mediating the DA phenotype. The two antisera obtained showed no apparent differences in specificity, as shown by Western blotting. As demonstrated in Fig. 1, absorbed antibodies raised against whole cells detected five proteins of about 140, 100, 90, 50, and 38 kDa in whole-cell extracts of the wild-type isolate 2787 and the recombinant strain C600/pIB4. However, only the protein of 100 kDa was recognized in the protein fraction obtained from DA+ diarrhea-associated E. coli strains by mild heat extractions.

Comparison of proteins in strains C600/pIB4 and C600/pBR322 detected by these antisera demonstrated that expression of the two proteins of about 140 and 50 kDa, besides that of the 100-kDa adhesin, was dependent on the presence of the 9.2-kb DNA fragment containing the AIDA-I operon. The signals corresponding to proteins of approximately 90 and 38 kDa resulted from residual unspecific binding of antibodies, since they were also detected in whole-cell extracts of C600/pBR322 bacteria.

Reaction of DA+ EPEC strains with AIDA-I-specific antisera by Western blotting. To investigate whether other diarrhea-associated DA+ strains express proteins serologically related to AIDA-I, various wild-type isolates with classical EPEC serotypes were subjected to Western blotting. Membrane-associated proteins from several strains were isolated by mild heat treatment and examined for their reactions with AIDA-I-specific antibodies. Specific recognition of a protein(s) of about 100 kDa by absorbed antiserum engendered by C600/pIB4 was demonstrated for some but not all diarrhea-associated DA+ strains (Fig. 2). About 100 additional nonadhering E. coli wild-type isolates were tested with the antiserum, but they did not exhibit cross-reactive proteins. The corresponding preimmune serum did not recognize proteins of 100 kDa (data not shown). This serologic cross-reactivity might be indicative of a group of related proteins associated with the DA phenotype. However, whether these proteins are also structurally related to the AIDA-I protein has to await further investigation since, interestingly, these strains did not hybridize to an AIDA-I-encompassing DNA fragment used as a probe (2).

Localization of the 100-kDa adhesin (AIDA-I) on the bacterial cell surface. To localize and identify AIDA-I on the bacterial cell surface, immunogold electron microscopy was employed. As demonstrated (Fig. 3) for the DA+ recombinant strain C600/pIB6 (2), the gold label indicating the presence of the 100-kDa protein is spread evenly on the bacterial cell surface. In the wild-type strain 2787, the number of gold particles per bacterial cell was about 20-fold less than in the recombinant C600/pIB6 (data not shown). The higher number of adhesin molecules per cell in the recombinant strains is probably due to the increased gene dosage caused by the high copy number of the pBR322-cloning vector, compared with the few copies of the pBR322 plasmid carrying the AIDA-I operon (2).

Direct binding of the 100-kDa adhesin (AIDA-I) to HeLa cells. Thus far, the results obtained represent only indirect
FIG. 3. Localization of the 100-kDa protein to the surface of bacteria by immunogold electron microscopy. Grids were coated with a suspension of C600/pIB6 in PBS, and, after the removal of excess liquid, they were incubated with immune serum (A) or preimmune serum (B). Bound antibodies were detected by gold-labeled protein A. To visualize the bacteria by negative staining, the grids were incubated with 1% uranyl acetate. Bar, 0.5 μm.

evidence that the surface-associated 100-kDa protein corresponds to AIDA-I, the adhesin of wild-type strain 2787. All deletion mutants of the original recombinant plasmid mediating the DA phenotype also produced the 100-kDa protein (2). Direct evidence that the 100-kDa protein is the adhesin mediating DA (AIDA-I) was obtained by incubating HeLa cell monolayers with isolated 100-kDa protein. Bound protein was detected by pIB4-specific antiserum by using alkaline phosphatase-conjugated second antibody (Fig. 4). The 100-kDa protein bound to HeLa cells in a saturable fashion, which points to a finite number of putative receptor moieties. In assays run as negative controls, the protein extract obtained by mild heat treatment from C600/pBR322 bacterial cells was used. No binding of proteins to HeLa cells was detected with nonabsorbed antiserum, indicating that the binding is specific for the pIB4-encoded 100-kDa protein.

Inhibition of bacterial attachment by antibodies and Fab fragments directed at the 100-kDa adhesin. Further direct evidence that the 100-kDa protein isolated from DA+ recombinant strains represents AIDA-I was provided by inhibition experiments. Whole DA+ bacteria (C600/pIB6) were incubated with absorbed antiserum before being added to the HeLa cell monolayer. The number of adhering bacteria was determined by measuring the β-galactosidase activity, as described in Materials and Methods. As demonstrated in Fig. 5, absorbed anti-C600/pIB4 antiserum inhibited the adherence of C600/pIB6 recombinant bacteria to HeLa cells. To exclude possible interference with binding due to steric hindrance or cross-linking of bacterial cells, the inhibition experiment was also performed with purified Fab fragments generated from the immunoglobulin G fraction of pIB4-specific antiserum (Fig. 5). The inhibition of bacterial attachment by Fab fragments was even more efficient than with whole serum. These results prove directly that AIDA-I is the 100-kDa protein associated with the bacterial cell surface.

DISCUSSION

As with other pathogenic bacteria, the ability of diarrhea-associated E. coli to adhere to epithelial tissues represents an important virulence trait. Although genetic elements responsible for the expression of LA or DA have been identified for some isolates by molecular cloning (1–3), the actual adhesins mediating the particular phenotypes have in most cases remained controversial (5, 15). A fimbrial adhesin has been reported to be responsible for the DA phenotype of strain F1845 (3), and earlier reports from this laboratory (2)
pointed to a 100-kDa plasmid-encoded nonfimbrial protein as the AIDA-I.

The aim of this study was the identification and the serologic characterization of the protein factor(s) mediating the DA phenotype exhibited in tissue culture by the wild-type isolate 2787. Previously, we showed that this factor is encoded on a 6.0-kb DNA fragment derived from the largest of the 2-MDa plasmids harbored by strain 2787. Expression of a 100-kDa protein in the wild-type strain and in the recombinant DA+ strains, but not in mutant DA− strains, indicated a correlation between DA and the presence of the 100-kDa protein. To obtain antiserum against surface-exposed plasmid-encoded proteins, recombinant DA+ strain C600/pIB4 was used to immunize rabbits. The antiserum was exhaustively absorbed with C600/pBR322 to minimize unspecific binding. The absorbed antiserum recognized five distinct proteins by Western blotting. Three of these proteins, including a protein of 100 kDa, were present only in strains carrying the DA-conferring DNA fragment. These proteins were also detected in the DA+ recombinant strain C600/pIB264, which contains the minimal insert of 6.0 kb (data not shown). A fragment of 6.0 kb, however, does not have the capacity to encode three proteins of this size. Thus, it seems more likely that the proteins detected represent the 100-kDa protein, an approximately 140-kDa precursor, and a main degradation product of about 50 kDa. Recognition of the 100-kDa protein and both other bands (~140 and ~50 kDa) by Western blotting with antibodies raised against the heat-extracted AIDA-I protein supports this assumption.

As a prerequisite for its function as an adhesin, the 100-kDa protein was localized to the surface of the bacteria by electron microscopy using colloidal gold-labeled protein A. The distribution of the gold particles indicated an even distribution on the bacterial cell surface and further showed that the 100-kDa adhesin is not part of a filamentous piluslike structure. Direct evidence for the adhesive properties of the 100-kDa protein stemmed from saturable specific binding of the isolated protein to HeLa cells. Thus, we conclude that the adhesin involved in diffuse adherence (AIDA-I) is the 100-kDa protein. Further direct evidence originated from inhibition experiments. Specific antiserum directed at the 100-kDa protein was able to inhibit bacterial attachment of DA+ recombinant strains in tissue culture. Reduction of binding was even more pronounced when Fab fragments were used for inhibition. Since the adherence studies were performed solely with recombinant bacteria, the involvement of other factors in the attachment mechanism can be excluded.

Serologically related proteins of similar size can be found in other diarrhea-associated wild-type strains exhibiting the DA phenotype. Also, in these strains, ~100-kDa proteins cross-reacting with the absorbed anti-C600/pIB4 antibodies could be isolated by mild heat extraction. These results point to a group of serologically related proteins which might also be associated with the DA phenotype of these diarrhea-associated E. coli strains. The ease of isolation is further indicative of a similar mode of association of these proteins with the outer membrane. One of the strains tested expressed a serologically related 100-kDa protein which, however, did not adhere to HeLa cells and might thus represent an interesting functional mutant of an AIDA-I-related protein. As yet, nothing is known about possible receptor moieties on target cells which are recognized by DA-mediating adhesins. The determination of the DNA sequence of the putative AIDA-I operon, as well as the investigation of functional domains of AIDA-I, is currently under way in our laboratory.

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REFERENCES


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DIFFUSE ADHERENCE OF DIARRHEA-ASSOCIATED E. COLI

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