Chimeric Dr Fimbriae with a Herpes Simplex Virus Type 1 Epitope as a Model for a Recombinant Vaccine

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The potential of the major structural protein DraE of Escherichia coli Dr fimbriae has been used to display an 11-amino-acid peptide of glycoprotein D derived from herpes simplex virus (HSV) type 1. The heterologous sequence mimicking an epitope from glycoprotein D was inserted in one copy into the draE gene in place of a predicted 11-amino-acid sequence in the N-terminal region of surface-exposed domain 2 within the conserved disulfide loop (from Cys21 to Cys53). The inserted epitope was displayed on the surface of the chimeric DraE protein as evidenced by immunofluorescence and was recognized by monoclonal antibodies to the target HSV glycoprotein D antigen. Conversely, immunization of rabbits with purified chimeric Dr-HSV fimbriae resulted in a serum that specifically recognized the 11-amino-acid epitope of HSV glycoprotein D, indicating the utility of the strategy employed.

The display of peptide segments on the surfaces of bacteria offers new applications in medical research and the biotechnology of recombinant vaccines. Fimbriae are particularly attractive candidates for epitope display. They are adhesive bacterial surface structures that enable bacteria to target and to colonize specific host cells and tissues. Fimbriae are long, thread-like surface organelles, found in as many as ~500 copies per cell; each fimbrial fiber is a polymer composed of hundreds of structural subunits. Thus, expression of a foreign epitope in a fimbrial structural subunit may result in the production of an enormous number of copies of the antigen of interest. A number of different fimbrial types have been used to display heterologous proteins on the surfaces of bacteria (9). Vaccines based on wild-type fimbriae have been highly successful, for example, in protecting against various diarrheal-causing enteropathogenic Escherichia coli strains (10). Such results have indicated that fimbriae are very good immunogens in the context of live vaccine and purified proteins.

E. coli strains bearing Dr fimbriae or the related adhesin afimbrial adhesin I (AFA-I), afimbrial adhesin III (AFA-III), or F1845 adhesin are associated with urinary tract infections (UTI) and diarrhea, especially gestational pyelonephritis, cystitis, chronic diarrhea, and recurrent UTI (6, 14). Despite similar genetic organization, phenotypic expression in members of the Dr family of adhesins is associated with either fimbrial or afimbrial morphology. The dra gene clusters share a highly conserved region including the draA, draB, draC, draD, and draF genes. Unlike these genes, the structural adhesin-encoding gene, designated draE, is highly heterogeneous within the Dr family of adhesins. Adhesin DraE is associated with fimbrial morphology (13). The fimbrial and afimbrial adhesins of the Dr family recognize different epitopes on their common cellular receptor, decay-accelerating factor (DAF), which is present on erythrocytes and other cell types (7, 15, 16). The Dr hemagglutinin binds type IV collagen (23). The collagen-binding phenotype is unique to the DraE adhesin (4).

The Dr family of adhesins mediates mannose-resistant hemagglutination (MRHA), diffuse adherence to epithelial cells (1, 18), and binding to human polymorphonuclear leukocytes (8). MRHA mediated by the Dr hemagglutinin is inhibited by chloramphenicol, whereas MRHA mediated by F1845 fimbriae, AFA-I, and AFA-III is not (14). Carnoy and Moseley (4) have shown that the Dr hemagglutinin properties of type IV collagen binding and chloramphenicol sensitivity of binding are independent and separable phenotypes. The identification of specific amino acids associated with these phenotypes suggests the existence of a conformational rather than a linear receptor-binding domain.

Dr fimbriae have a conserved mechanism of translocation to the bacterial surface. The proteins encoded by the dra operon are produced as precursors with an N-terminal signal sequence. This element is removed during export across the inner membrane. Fimbrial proteins are secreted into the periplasm by means of the general secretory system (19). Two accessory proteins assist in construction of the fimbrial shaft. DraB is a periplasmically located chaperone that binds to nascently translocated DraE fimbrial subunits and prevents them from making incorrect interactions causing aggregation and proteolytic degradation. DraC, an usher protein localized in the outer membrane, is involved in organized polymerization of the structural components into fimbrial organelles.

Herpes simplex virus (HSV) infections are among the most frequent human infections, affecting both children and sexually active adults, with peak incidence from age 14 to 39. Neonatal HSV may be associated with a serious outcome to the fetus. Accordingly, an HSV vaccine is a target of very intense studies by many investigators. Glycoprotein D (gD) is the most exten-
sively studied HSV type 1 (HSV-1) antigen. gD interacts with at least three different receptors to enable productive entry of the virus into cells (3). gD is required for the spread of the virus from cell to cell by functions that may be related to those required for the initial entry of the virus into cells. gD also blocks apoptosis induced by HSV-1 early infection. Thus, gD has become the main research target for recombinant vaccines against HSV (3).

The aim of this study was to construct a model expression system for Dr chimeric fibriniae with a gD epitope derived from HSV-1. Carnoy and Moseley (4) analyzed the sequence of DraE adhesin for predicted surface domains by using the method of Emini et al. (5) and located surface-exposed domain 2. They chose the N-terminal region of surface-exposed domain 2 for site-directed mutagenesis because this region of the DraE fimbrial subunit was implicated in the binding process (4). Based on these site-directed mutagenesis studies, our strategy anticipated insertion of an 11-amino-acid epitope (QPELAPEDPED) into DraE adhesin in place of the N-terminal region of the last family members that form a DAF binding epitope (4). Therefore, it was unlikely that the insertion position could interfere with bioassembly of the fibriniae, because every subunit would carry a foreign peptide segment. The other predicted advantages of such an approach were the excellent immunogenic properties of Dr fibriniae, which could boost the immune response against a passenger epitope, and production of large amounts of major subunit protein with the foreign antigen, which can be easily purified.

We report here that the recombinant strain obtained, E. coli BL21(DE3)/pDraE-HSV3/pCC90D54stop, showed expression of the fusion fimbrial protein DraE-HSV displaying an immunoreactive HSV-1 epitope. The expression system also allows the insertion of foreign sequences (other epitopes) into DraE adhesin in the position corresponding to the BamHI restriction site in the cloned DNA sequence of an HSV-1 epitope.

MATERIALS AND METHODS
Bacterial strains, plasmids, enzymes, and reagents. Overexpression was carried out in E. coli strain BL21(DE3), which does not express Lon and OmpT proteases (Novagen, Nottingham, United Kingdom). Restriction enzymes were purchased from BioLabs. The reagents for PCR were obtained from DNA-Gdańsk II s.c. (Gdańsk, Poland). Isopropyl-β-D-thiogalactopyranoside (IPTG), agarose, and other reagents were purchased from Sigma.

Bacterial cells were grown on solid medium (Luria agar [LA], containing, per liter, 5 g of yeast extract, 10 g of tryptone-pancreatic digest of casein, 10 g of sodium chloride, and 15 g of agar) or liquid broth (Luria broth [LB] medium, containing, per liter, 5 g of yeast extract, 10 g of tryptone-pancreatic digest of casein, and 10 g of sodium chloride) supplemented with the appropriate antibiotics (Sigma).

Plasmid pBJN406, containing an 11.4-kb HindIII-HindIII DNA fragment (previously constructed by B. Nowicki) (11, 12) carrying the whole dra operon, which is responsible for the uropathogenic properties of bacteria, was used for PCR amplification.

Plasmid pCC90, corresponding to the Dr hemagglutinin operon, which had its promoter region and its regulatory genes upstream of draB gene deleted, was from S. Moseley, University of Washington (Seattle). This plasmid was constructed in order to eliminate differences in protein expression among mutants as a result of methylation-dependent phase variation, which occurs in the promoter regions of adhesins (2, 21). In the first step, an EcoRI restriction site was inserted by site-directed mutagenesis 45 bp upstream of the draB gene. The resulting 2.8-kb EcoRI fragment was deleted, and the EcoR-HindIII fragment containing the truncated draB operon was cloned into the pACYC177 vector, allowing transduction of the dra gene cluster from the kanamycin resistance gene promoter. The plasmid carries the origin of replication from plasmid p15A, enabling it to coexist in cells with vectors, such as pBR322 and pUC19, that carry a ColE1 compatibility group origin.

Plasmid pCC90D54stop, which contains the dra gene cluster with a deletion of the region upstream of draB and a mutated draE gene (Dr-D54stop mutant; by site-directed mutagenesis, the GAC triplet of the draE gene encoding Asp-54 was replaced with a stop codon), was from S. Moseley.

Plasmid pET30 EκLIC, an expression vector with a strong T7 promoter, a kanamycin resistance gene, and a pBR322 origin of replication, was from NovaGen.

Plasmid DNAs were isolated from E. coli cultures by using the Mini-prep Plus kit (A&A Biotechnology, Gdynia, Poland).

PCR amplification. The draE gene with its signal sequence, encoding bacterial DraE adhesin, was prepared by PCR amplification using the DNA of plasmid pBJN406 as a template. The primers enabled the introduction of a DNA sequence encoding an HSV-1 epitope into the draE gene. The draE gene with the HSV-1 epitope was amplified in two DNA fragments: DNA fragment I, encoding the N-terminal region of the DraE adhesin with the HSV-1 epitope, and DNA fragment II, encoding the C-terminal region of the DraE adhesin with the HSV-1 epitope. The sequences of the primers designed on the basis of the draE gene with its signal sequence (GenBank accession no. AF239316) are shown in Fig. 1B.

PCR reactions were performed on a Perkin-Elmer 2400 thermocycler. PCRs were carried out in 50-μl volumes containing 1 μl of DNA template (plasmid pBJN406; 0.25 μg/μl), 5 μl of 10× reaction buffer (100 mM Tris-HCl [pH 8.8], 20 mM MgCl₂, 500 mM KCl, 1% Triton X-100), 5 μl of deoxynucleoside triphosphates (10 mM), 2 μl of each primer (10 μM), and 1 U of thermostable Pwo DNA polymerase (DNA Gdansk II s.c.). Reactions were started with 300 s of denaturation at 94°C, followed by 10 cycles, each consisting of 30 s at 94°C, 40 s at 52°C, and 40 s at 72°C, and then by 25 cycles, each consisting of 30 s at 94°C, 40 s at 68°C, and 40 s at 72°C, with an additional extension step at 72°C (300 s). After amplification, 10-μl samples were subjected to electrophoresis in a standard 2% agarose gel to confirm the presence of the amplified products. The specific 192-bp DNA fragment I and 357-bp DNA fragment II were obtained.

Cloning. The amplified DNA fragment I (encoding the N-terminal part of the DraE adhesin with the HSV-1 epitope) and the amplified DNA fragment II (encoding the C-terminal part of the DraE adhesin with the HSV-1 epitope) were purified from an agarose gel band by using the DNA Gel-Out kit (A&A Biotechnology). DNA fragment I was digested with the KpnI and BamHI endonucleases (BioLabs), purified using the DNA Clean Up kit (A&A Biotechnology), and cloned directionally into KpnI and BamHI sites of the pET30LIC/Ek vector, producing recombinant plasmid pETDraE-HSV3 (the gene is out of frame in this construct). This plasmid was then digested with the NdeI endonuclease and ligated in the presence of the KpnI endonuclease to provide the gene in frame. The resulting recombinant plasmid encoding the N-terminal part of the DraE adhesin with the HSV-1 epitope was designated pDraE-HSV2. After that, the resulting plasmid was digested with the BamHI and HindIII endonucleases, permitting cloning of DNA fragment II, which was digested with the same restriction enzymes. The resulting recombinant plasmid encoding the whole draE gene with the HSV-1 epitope was designated pDraE-HSV3. The recombinant plasmids were selected by electrophoretic mobility analysis and confirmed by restriction analysis. The draE-HSV1 gene of the pDraE-HSV3 plasmid was sequenced using the ABI Prism 377 automated system, and the nucleotide sequence was analyzed using the GeneDoc and ClustalX computer programs.

Expression of the DraE adhesin with the HSV-1 epitope. The pDraE-HSV3 recombinant plasmid was transformed into E. coli BL21(DE3). One liter of LB medium supplemented with 20 μg of kanamycin/ml was inoculated with 20 ml of an overnight culture of E. coli strain BL21(DE3) containing the recombinant pDraE-HSV2 plasmid. The inoculated culture was grown with agitation at 30°C to an optical density of 0.2, induced by addition of IPTG (final concentration, 0.1 mM), and grown as described above overnight. The culture was harvested by centrifugation at 6,000 × g and 4°C and was stored at −20°C for further use.

For sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins during cultivation, 1-ml samples were collected and the cells were harvested by centrifugation. Next, the pellets were resuspended in 100 μl of phosphate-buffered saline (PBS [pH 7.5], containing 80 mM sodium hydrogen orthophosphate, 20 mM sodium dihydrogen orthophosphate, and 100 mM sodium chloride). A 10-μl portion of each cell solution was then used directly for SDS-PAGE in 15% polyacrylamide gels stained with Coomassie brilliant blue R-250 (Sigma).
Purification of chimeric Dr-HSV fimbriae and Dr fimbriae. Recombinant plasmid pDraE-HSV3 encoding the DraE-syg adhesin (with the signal sequence) with the HSV-1 gD epitope was introduced into the DraE adhesin present in chimeric DraE-HSV protein is shown (boxes labeled draE-N and draE-C, respectively). Between them, the location of the DNA fragment (33 bp) of the draE gene deleted in chimeric draE-HSV (the site of HSV fragment sequence insertion) is indicated. The 5′ overhanging sequences of primers 1-DraE-HSV and 2-DraE-HSV are also indicated. The BamHI site is used to connect two amplification products (DNA fragments I and II) and to facilitate later manipulations and insertion of other heterologous peptides into the DraE adhesin. (B) Sequences of the PCR primers used to amplify the recombinant DraE-HSV gene. Underlining indicates the recognition sequences for appropriate restriction endonucleases permitting cloning into a pET30LIC/Ek plasmid. Boldfaced portions of primer sequences are complementary to the nucleotide sequence of the 5′ gene. DNA fragments encoding the N- and C-terminal regions of the DraE adhesin present in chimeric DraE-HSV protein are shown.

**FIG. 1.** (A) Construction strategy for a recombinant draE gene with inserted DNA encoding an antigenic sequence (for example, an HSV-1 gD epitope). DNA fragments encoding the N- and C-terminal regions of the DraE adhesin present in chimeric DraE-HSV (the site of HSV fragment sequence insertion) is indicated. The 5′ overhanging sequences of primers 1-DraE-HSV and 2-DraE-HSV are also indicated. The BamHI site is used to connect two amplification products (DNA fragments I and II) and to facilitate later manipulations and insertion of other heterologous peptides into the DraE adhesin. (B) Sequences of the PCR primers used to amplify the recombinant draE-HSV gene. Underlining indicates the recognition sequences for appropriate restriction endonucleases permitting cloning into a PET30LIC/Ek plasmid. Boldfaced portions of primer sequences are complementary to the nucleotide sequence of the 5′ or 3′ end of the draE gene. The 5′ overhanging sequences of the 2-DraE-HSV and 3-DraE-HSV primers encoding the 11-amino-acid HSV-1 gD epitope are italicized.

**Purification of chimeric Dr-HSV fimbriae and Dr fimbriae.** Recombinant plasmid pDraE-HSV3 encoding the DraE-syg adhesin (with the signal sequence) with the HSV-1 gD epitope was introduced into the DraE mutant E. coli BL21 (DE3)pCC90D54stop, which contains the dra gene cluster with deletion of a region upstream of draB and with a mutated draE gene (Dr-D54stop mutant). The recombinant E. coli BL21(DE3)pCC90D54stopE-HSV cells expressing chimeric Dr-HSV fimbriae were grown on LA plates with appropriate antibiotics (20 μg of kanamycin/ml and 100 μg of ampicillin/ml) and IPTG (final concentration, 6 mM) at 37°C for 24 h, harvested in PBS (15 ml for 10 150-mm-diameter plates), and heat shocked at 65°C for 1 h in a water bath. At the end of the incubation, the bacterial suspension was centrifuged at 6,000 × g for 20 min, and the supernatant was collected in fresh tubes. After that, a 40% solution of ammonium sulfate in PBS was added to a final concentration of 20% and mixed overnight at 4°C to precipitate the fimbriae. The next day, the suspension was centrifuged at 3,000 × g for 30 min at 4°C. The supernatant was poured out, and the pellet was redissolved in a 20% solution of ammonium sulfate and rotated overnight at 4°C. The precipitated protein (obtained after centrifugation) was resuspended in 2 ml of PBS. The suspension was poured into a dialysis bag (molecular weight cutoff, >100,000) and dialyzed overnight against PBS. The protein was then passed through a Sepharose 4B column (Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, United Kingdom), where the Dr-HSV fimbriae were eluted in the void volume. The purity of the preparation was evaluated by SDS-PAGE in a 15% polyacrylamide gel stained with Coomassie brilliant blue R-250 (Sigma). The Coomassie brilliant blue R-250-stained polyacrylamide gel was photographed and scanned. The concentration of the protein was determined by densitometric analysis using the ScanPack (version 2.0) and BioDoc (version 1.0) programs (Biometa) with a low-molecular-weight marker (Amersham Pharmacia Biotech) as a standard.

Recombinant plasmid pCC90, which contains the dra gene cluster with a deletion of the region upstream of draB, was transformed into E. coli BL21 (DE3). The recombinant E. coli BL21(DE3)pCC90 cells were used for expression and purification of Dr fimbriae by the method described above for chimeric Dr-HSV fimbriae. The recombinant strain was used as a positive control. The recombinant strain E. coli BL21(DE3)pCC90D54stop, which does not express Dr fimbriae, was used as a negative control.

**Hemagglutination assay.** In order to determine whether purified Dr protein and DraE-HSV protein bind to DAF, 15 μl of each preparation (at a concentration of 1 μg/ml) was mixed with 15 μl of a 3% (vol/vol) solution of human erythrocytes in PBS with 2% a-methylmannose on glass slides. The mixture was rotated for 5 min, and agglutination was observed using a light microscope. Erythrocytes mixed with the Dr-D54 stop mutant were used as a negative control.

**Antisera.** Rabbit anti-Dr adhesin antibodies raised against purified native Dr fimbriae have been described previously (17). HSV-Tag mouse monoclonal immunoglobulin G1 (IgG1) antibodies with specificity and affinity for the 11-amino-acid peptide derived from HSV-1 gD were from Novagen. All other anti-IgG antibodies were against the whole molecule. Anti-rabbit IgG, anti-mouse IgG, and anti-human IgG antibodies conjugated to horseradish peroxidase were purchased from Sigma. Anti-rabbit IgG conjugated to tetramethylrhodamine iso-
Samples were mixed with sample buffer, run in a 15% bisacrylamide gel containing SDS, and transferred to a nitrocellulose membrane in a transfer buffer (pH 8.3; 12.5 mM Tris, 96 mM glycine, 10% methanol) for 2 h by using a semidry blotting apparatus (0.5 V/cm2 of gel). All subsequent steps were carried out in PBS containing 0.05% Tween 20 (PBS-T) at room temperature with gentle rocking. After transfer, the membrane was blocked with 3% nonfat milk in PBS-T buffer for at least 30 min and then incubated with rabbit antibodies or anti-mouse IgG antibodies conjugated to horseradish peroxidase at a 1:1,000 dilution for 1 h. After three washes with PBS-T, protein bands were visualized by using a diaminobenzidine (DAB) substrate for horse-radish peroxidase at a 1:250 dilution (anti-HSV-Tag) in PBS of the secondary antibody at room temperature for 1 h. The reaction mixtures were then washed again three times with PBS-T and then incubated with anti-rabbit IgG antibodies conjugated to horseradish peroxidase at a 1:250 dilution (rabbit anti-DraE-HSV) in PBS of the primary antibody at room temperature for 1 h. The reaction products were slate gray to black.

**Immunofluorescence microscopy.** Cells from cultures grown on L-agar plates at 37°C for 24 h were harvested and washed gently in PBS. Bacterial suspensions (100 μl; 107 to 108 cells/ml) were incubated with 50 μl of a 1:500 dilution (anti-Dr fimbriae) or a 1:250 dilution (anti-HSV-Tag) in PBS of the primary antibody at room temperature for 1 h. The reaction mixtures were then washed three times with PBS containing 10% glycerol and incubated with 50 μl of a 1:25 dilution (anti-rabbit IgG conjugated to TRITC) or a 1:50 dilution (anti-mouse IgG conjugated to FITC) in PBS of the secondary antibody at room temperature for 1 h. The reaction mixtures were then washed again three times with PBS containing 10% glycerol. Bacterial suspensions (10 μl) were loaded onto glass slides and observed with an immunofluorescence microscope (Olympus BX-60). A bacterial suspension of *E. coli* BL21(DE3)/pCC90D54stop was used as a negative control. A bacterial suspension of *E. coli* BL21(DE3)/pCC90 (expressing wild-type Dr fimbriae) was used as a positive control.

**Electron microscopy.** Cells of the *E. coli* strain BL21(DE3) carrying the recombinant plasmids under study were grown on LA plates. With a platinum loop, a small portion of cells was scraped off the plate and very gently suspended in a 10-μl drop of PBS buffer. Carbon-coated copper grids (300 mesh) were floated for 1 to 2 min on the cell suspensions and then washed twice by being floated on 50-μl drops of PBS buffer; after that, they were negatively stained with 1.5% potassium phosphotungstate for 90 s. The preparations were analyzed with a Philips CM 100 transmission electron microscope at 60 kV at a magnification of ×3,805.

**Immunization with fimbriae.** The purified fimbriae were used to immunize rabbits. One rabbit was immunized with purified chimeric (DraE-HSV) fimbriae, and one rabbit was immunized with wild-type Dr fimbriae (control). Two doses of a suspension of 0.1 ml of fimbriae (0.1 mg of fimbriae/ml) mixed with equal parts of Freund’s incomplete adjuvant were administered subcutaneously at 2-week intervals. Blood was collected from animals before and 3, 6, and 9 weeks after immunization.
RESULTS

Construction of the recombinant plasmid pDraE-HSV3 encoding the DraE-syg adhesin with the HSV-1 epitope. The first step of our strategy was production of recombinant plasmid pDraE-HSV3 encoding the DraE adhesin (with its signal sequence) with an HSV-1 gD epitope derived from HSV-1. The sequence encoding the HSV-1 epitope consisting of 11 amino acids (QPELAPEDPED) was introduced into the draE gene in place of the N-terminal region of surface-exposed domain 2 (VAKTRGQLTDA), which is implicated in the DAF binding process. Sequence analysis and site-directed mutagenesis (4) located the surface-exposed domain 2. In the expression system described here, the draE gene (with its signal sequence) with the nucleotide sequence encoding the HSV-1 epitope was prepared by PCR amplification, with primers designed to clone the target gene into pET30LIC/Ek. The draE gene with the HSV-1 epitope was amplified by use of plasmid pBJN406 (harboring the dra gene cluster) in two DNA fragments: DNA fragment I, encoding the N-terminal region of the DraE adhesin with the HSV-1 epitope, and DNA fragment II, encoding the C-terminal region of the DraE adhesin with the HSV-1 epitope. The internal primers 2-DraE-HSV and 3-DraE-HSV enabled the introduction of the nucleotide sequence encoding the HSV-1 epitope into the draE gene with the signal sequence by PCR amplification (Fig. 1). The amplified draE-HSV gene was cloned in two DNA fragments into plasmid pET30LIC/Ek under the control of a strong T7 promoter (Novagen). The genetic construct obtained, harboring a single copy of the HSV-1 insert, retained the open reading frame, as confirmed by restriction and DNA sequence analyses. The chimeric fimbrial protein DraE-syg-HSV encoded by the recombinant plasmid has a molecular mass of 17,258 kDa and a calculated pI of 4.5.

DraE proteins. The products were 90% pure. The concentration, with primers designed to clone the target gene into pET30LIC/Ek, was employed as the secondary serum. Anti-HSV serum was used as the primary serum, and a peroxidase-anti-rabbit serum was employed as the secondary serum. (B) A mouse strain assayed by Western blotting. (A) A rabbit anti-Dr (anti-fimbrial) serum was employed as the primary serum, and a peroxidase-coupled anti-mouse serum was employed as the secondary serum. Purified fimbrial proteins were prepared as described in the text. Lanes M, low-range color marker (Sigma) for 45.0, 29.0, 20.0, 14.2, and 6.5 kDa; lanes 1, elution fraction of chimeric Dr-HSV fimbriae (collected from the Sepharose 4B column); lanes 2, elution fraction of wild-type Dr fimbriae (collected from the Sepharose 4B column); lanes 3 to 5, dialysis fractions of purified chimeric Dr-HSV fimbriae after induction with 3, 4, and 6 mM IPTG, respectively; lanes 6, dialysis fraction of purified wild-type Dr fimbriae; lanes 7, whole-cell lysate of the E. coli BL21 (DE3) host harboring plasmid pCC90D54stop (carrying the dra operon with a deleted region upstream of draB and a mutated draE gene).

Expression of the DraE-HSV adhesin with and without the signal sequence. E. coli strain BL21(DE3) transformed with plasmid pDraE-HSV3 was grown at 30°C in 800 ml of LB medium containing 20 μg of kanamycin/ml to an OD600 of 0.2. IPTG was then added to a final concentration of 0.1 mM. The culture was then cultivated further at 30°C. The cells were harvested after 6 h of cultivation. Optimization of the expression conditions revealed that a 6-h induction and growth at 30°C in LB medium led to substantial overproduction of the DraE-HSV adhesin with (17,258 kDa) and without (15,622 kDa) the signal sequence. Bands corresponding to a ~17-kDa protein and a 15.5-kDa protein were observed in SDS-PAGE profiles of crude extracts of E. coli BL21(DE3)/pDraE-HSV3 culture after IPTG induction (Fig. 2, lanes 2 to 6). These bands were absent in the control crude extract of E. coli BL21(DE3)/pET30LIC/Ek culture (Fig. 2, lane 1). Western blot analysis of E. coli BL21(DE3)/pDraE-HSV3 whole-cell lysates with anti-Dr (Fig. 3A) and anti-HSV (Fig. 3B) antibodies revealed the presence of chimeric proteins with and without the signal sequence, with the expected molecular masses.

Construction of two-plasmid expression system of chimeric Dr-HSV fimbriae (pDraE-HSV3, encoding the DraE-syg-HSV protein, and pCC90D54stop, containing the dra operon with a mutated draE gene). To create a model expression system for chimeric Dr fimbriae with a gD epitope derived from HSV-1, recombinant plasmid pDraE-HSV3 was introduced into a DraE+ mutant [E. coli BL21(DE3)/pCC90D54stop] that contained the dra gene cluster with deletion of a region upstream of draB and with a mutated DraE gene (Dra-D54stop mutant). When both plasmids pDraE-HSV3 and pCC90D54stop were present in a given strain, complementation occurred: the recombinant E. coli BL21(DE3)/pDraE-HSV3/pCC90D54stop strain showed expression of chimeric Dr-HSV fimbriae that in quality and quantity were similar to those produced by E. coli BL21(DE3)/pCC90 containing the dra gene cluster with deletion of a region upstream of draB (as described below).

Expression of chimeric Dr-HSV fimbriae and immunological detection of the HSV-1 epitope inserted into the DraE adhesin. Expression of chimeric Dr-HSV fimbriae and wild type Dr fimbriae was performed as described in Materials and Methods. We obtained about 2 mg each of the DraE-HSV and DraE proteins. The products were >90% pure. The concen-
Concentrations of the proteins were determined by densitometric analysis using the ScanPack (version 2.0) and BioDoc (version 1.0) programs (Biometra) with a low-molecular-weight marker (Amersham Pharmacia Biotech) as a standard.

The ability of *E. coli* strain BL21(DE3) containing plasmids pDraE-HSV3 and pCC90D54stop to express chimeric Dr-HSV fimbriae was assayed by immunoblotting, hemagglutination, immunofluorescence microscopy, and electron microscopy. The *E. coli* strain expressing wild type Dr fimbriae was used as a positive control in the experiments. As a negative control we used the *E. coli* strain containing the *dra* gene cluster with a mutated *draE* gene. In Western blots the purified fimbria-specific rabbit anti-Dr antibody (Fig. 4A), the mouse anti-HSV antibody (Fig. 4B), and sera obtained from women infected with HSV-1 (see Fig. 8) recognized chimeric DraE-HSV proteins without signal sequences. Immunofluorescence staining (anti-Dr–anti-TRITC and anti-HSV–anti-FITC staining) of *E. coli* cells expressing chimeric Dr-HSV fimbriae
showed that the specific antibodies recognized chimeric DraE-HSV protein (Fig. 5C and E). This experiment proved that the foreign HSV-1 epitope was exposed on the surfaces of the host cells. The surface localization and organelle assembly of the chimeric DraE-HSV adhesin were investigated by electron microscopy (Fig. 6). The results obtained revealed that chimeric DraE-HSV proteins were assembled into fimbral organelles and that the shape and structure of these fimbrae were the same as those of native DraE fimbrae (Fig. 6; compare panels A and B). The microscopic data proved that the subunit proteins produced in the cytoplasm as precursors with the N-terminal signal sequence were secreted into the periplasma and then exported across the inner membrane on the surfaces of bacterial cells. Electron microscopy for E. coli BL21(DE3)/pCC90D54stop cells (negative control) showed bacterial cell surfaces without any fimbrae (data not shown).

**Hemagglutination assay.** We mixed equal volumes of purified chimeric Dr-HSV fimbrae or wild type Dr fimbrae (as a positive control) isolated from the recombinant bacterial strains and the erythrocyte suspension to determine hemagglutination ability in the presence of α-mannose. This experiment showed that the purified DraE adhesin gave a positive hemagglutination reaction. Introduction of the HSV-1 epitope into the DraE adhesin in place of the N-terminal region of surface-exposed domain 2 abolished the hemagglutination capacity of the DraE-HSV fimbral protein.

**Immunogenicity of chimeric Dr-HSV fimbrae.** Chimeric Dr-HSV fimbrae purified from the bacterial host containing plasmids pDraE-HSV3 and pCC90D54stop were used to immunize a rabbit. Another rabbit was immunized with wild type Dr fimbrae as a control. The serum from the rabbit immunized with the chimeric fimbrae was able to recognize the HSV-1 epitope in chimeric fimbrae (DraE-HSV protein) and in a 31.1-kDa target HSV-1 protein (Novagen) by use of an immunoblotting technique, but the serum obtained from the control rabbit immunized with the wild type fimbrae was not able to recognize the HSV-1 epitope (Fig. 7).

**DISCUSSION**

The work described here represents the first model expression system for chimeric Dr fimbrae with the immunoreactive HSV-1 epitope (OPELAPEDPED) derived from HSV-1 gD inserted into the DraE fimbral subunit in place of the N-terminal region of surface-exposed domain 2 (VAKTRGQLTDA), which is involved in DAF-mediated adhesion (15) and invasion (B. Nowicki, personal communication).

In the absence of an X-ray structure of the fimbral subunit, the insertion region was chosen based on computer localization of the surface-exposed regions (5) and site-directed mutagenesis analysis (4). The insertion position chosen was not critical in the subunit-subunit interaction or in bioassembly of the fimbrae, as suggested by electron microscopic results.

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**FIG. 6.** Electron micrographs of negatively stained preparations of E. coli BL21(DE3) cells harboring plasmid pCC90 (encoding the *dra* operon with a deleted region upstream of *draB*) (A) and E. coli BL21(DE3) cells harboring plasmid pCC90D54stop (encoding the *dra* operon with a deleted region upstream of *draB* and a mutated *draE* gene) and plasmid pDraE-HSV3 (encoding the DraE-HSV adhesin) (B). Magnification, ×3,805.
showing good expression of fimbriae with unchanged fiber morphology. The replacement of hydrophilic domain 2 of DraE with the HSV epitope abolished the ability of the chimeric DraE-HSV protein to bind to a DAF receptor present on the surfaces of erythrocytes. The ability of the DraE adhesin to bind to the DAF receptor is determined mainly by the conformational features of the protein subunit rather than by the defined consecutive sequence (4). Recently, Van Loy et al. (22) showed that amino acids important in the interaction with DAF are located mainly at the C terminus of the DraE adhesin (the region from amino acid 63 to 81 of the mature DraE adhesin). Probably the replacement of an 11-amino-acid sequence with the foreign HSV epitope caused some structural protein rearrangement.

The inserted HSV-1 epitope (in one copy) was recognized in the context of the chimeric protein exposed on the surfaces of bacterial host cells by immunofluorescence microscopy. Most importantly, the HSV-1 epitope of the purified DraE-HSV fimbrial protein was recognized by monoclonal antibodies against the 11-amino-acid peptide domain derived from HSV-1 gD, by the serum raised against the DraE adhesin, and by sera obtained from women infected with HSV-1 (Fig. 8). The purified chimeric Dr-HSV fimbriae also elicited a specific immune response in an immunized rabbit. The anti Dr-HSV antibodies decorated intact chimeric E. coli fimbriae, as evidenced by fluorescence microscopy. The serum obtained reacted with the HSV-Tag extract containing a 31.1-kDa target protein, used as a positive control. The recognition of the HSV-1 gD antigen is the most critical evidence of the utility of the strategy proposed here. Finally, the recombinant E. coli strain expressed surface exposed fimbriae composed of the recombinant DraE subunits with the foreign HSV-1 epitope. In this way each homopolymeric fimbria contained the HSV-1 epitope amplified in a thousand copies.

The expression system constructed allows insertion of foreign sequences into the DraE adhesin in the position corresponding to the BamHI restriction site in the cloned DNA sequence encoding an HSV-1 epitope. The usefulness of the strategy described here was successfully examined for other epitopes including the c-myc epitope (derived from the human proto-oncogene product p62 c-myc) and V5 (derived from a small epitope, Pk, present on the P and V proteins of the paramyxovirus of simian virus 5) (unpublished data).

Recombinant plasmid pDraE-HSV3, encoding the DraE adhesin with the HSV-1 epitope, is currently being modified in our laboratory for further studies to produce the adhesin-chaperone protein complex DraE-HSV-DraB, which should be very stable and easy to purify (unpublished data).

A strategy similar to the fimbrial DraE-HSV protein insertions within surface-exposed hydrophilic domains of various adhesins of the Dr family and/or other adhesins, involved in initial binding to a specific receptor or facilitating cell entry, may become useful in displaying functional epitopes for the context of the fimbrial protein expressed on the surfaces of bacterial host cells by immunofluorescence microscopy.

FIG. 7. Immunoblots showing the immune responses of rabbits immunized with wild-type purified Dr fimbriae (serum dilution, 1:5,000) (A) and purified chimeric Dr-HSV fimbriae (serum dilution, 1:500) (B). Lanes M, low-range color marker (Sigma) for 45.0, 29.0, 20.0, 14.2, and 6.5 kDa; lanes 1, dialysis fraction of purified wild-type Dr fimbriae; lanes 2, dialysis fraction of purified chimeric Dr-HSV fimbriae after induction with 6 mM IPTG; lane 3, HSV-Tag extract containing a 31.1-kDa target protein (Novagen), used as a positive control.

FIG. 8. Western blot analyses with sera obtained from four women infected with HSV-1. Sera were diluted 1:100. Each of the four panels shows results obtained with a primary serum sample from a different patient. The blots were developed with anti-human IgG (whole molecule) antibodies conjugated to horseradish peroxidase (serum dilution, 1:1,000) and were then visualized using DAB and nickel chloride. Lanes M, low-range color marker (Sigma) for 45.0, 29.0, 20.0, 14.2, and 6.5 kDa; lanes 1, 3, 5, and 7, elution fractions of wild-type Dr fimbriae (collected from the Sepharose 4B column); lanes 2, 4, 6, and 8, elution fractions of chimeric Dr-HSV fimbriae (collected from the Sepharose 4B column).
development of recombinant vaccines for *E. coli* upper and lower UTI.

The fimbria-assisted peptide display may have multiple applications, especially in the biotechnology of recombinant vaccines. During the past 10 years, different fimbrial types have been used to display immune-relevant sections of various foreign proteins (9). Chimeric fimbrial vaccines can be used in the context of purified proteins or for the development of live recombinant vaccines. Vaccines based on wild type fimbriae have been successful in protecting farm animals against various diarrhea-causing enterotoxigenic *E. coli* strains (10). On the other hand, corresponding studies for human applications are significantly less advanced.

Dr fimbriae may have several advantages as carriers of foreign epitopes over other fimbrial systems such as type 1 or P pili. Type 1 and P pili are heteropolymers encoded by four or six different genes, respectively. The major components of type 1 and P pili are repeating FimA or PapA subunits arranged in a right-handed helix to form a wide fiber with an axial hole. The heteropolymeric structures of these pili are determined mainly by the head-to-tail interactions between the consecutive subunits. The second-order interactions between subunits are also implicated in the maintenance of the complex helical structures of type 1 and P pili (20). Construction of chimeric type 1 and P pili must be preceded by careful analysis of the accessible crystal structures in order to avoid the destruction of interactions important in the bioassembly of the pili. The Dr fimbriae are relatively simple homopolymeric structures encoded by the *draE* gene. These fimbriae are thick, flexible, hair-like filaments without a helical structure that is stabilized by head-to-tail interactions. Therefore, construction of chimeric Dr fimbriae is simpler and can be based on mutational and computer analyses of the DraE sequence.

In summary, future research on construction of the most effective recombinant vaccines and evaluation of their efficiency in animal models should help determine whether Dr display systems are effective in preventing HSV and other recurrent infections, including infections of the upper urogenital tract in women and men of reproductive age.

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