Short Consensus Repeat-3 Domain of Recombinant Decay-accelerating Factor Is Recognized by *Escherichia coli* Recombinant Dr Adhesin in a Model of a Cell–Cell Interaction

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Summary

A bacterial pathogen that is important in both urinary tract and intestinal infections is *Escherichia coli* which expresses Dr or related adhesins. In this report, we present a model for testing cell–cell interaction, using both molecularly characterized laboratory cells that express recombinant molecules of human decay-accelerating factor (DAF), and recombinant bacterial Dr colonization factors. Dr adhesin ligand was identified as DAF (CD55), a membrane protein that protects autologous tissues from damage due to the complement system. Structure–function studies mapped the adhesin-binding site on the DAF molecule. A single-point substitution in the third short consensus repeat domain, Ser^{165} to Leu, corresponding to the Dr^a to Dr^b allelic polymorphism, caused complete abolition of adhesin binding to DAF.

The host–parasite interaction may occur via specific bacterial lectin-like molecules, called adhesins, fimbriae, or pili (1). Bacterial adhesin-mediated attachment may result in the colonization of host tissues and may lead to an infectious process (2). A bacterial pathogen that is important in both urinary tract (UT)[1] and intestinal infections is *Escherichia coli*, which expresses Dr or related adhesins, afimbrial adhesin I (AFA-I), afimbrial adhesin III (AFA-III), and diarrhea-associated F1845 (3–5). These adhesins have similar gene organizations, and their close relationship is confirmed by the nucleotide sequence of Dr adhesin with other Dr-like adhesins (6).

E. coli–bearing adhesins of the Dr family are found most frequently in the UT, and account for 27% of clinical *E. coli* isolates that cause symptomatic UT infection (7). Others frequently seen are P and type 1 fimbriae-positive *E. coli* (8, 9). Moreover, Dr adhesin is a possible virulence factor of symptomatic UT infection, including cystitis and protracted diarrhea-associated strains (3, 7, 10).

The Dr ligand for *E. coli*, Dr adhesins, has been found on erythrocytes and in several other tissues, including the uroepithelium of the lower and upper UT, renal tissue (basement membranes and Bowman's capsule), and colonic glands. Therefore, we propose that the Dr ligands in the aforementioned organs may support ascending colonization/infection of UT by endogenous flora (11).

We have previously identified a Dr adhesin ligand in human tissues based on the observation that the Dr adhesin does not bind to erythrocytes of the rare Inab phenotype (12), a null phenotype of the Cromer blood group (13). The latter was localized on a 70-kD glycoprotein (14) that subsequently was identified by three groups of investigators (15–17) as decay-accelerating factor (DAF, CD55), a membrane protein that protects autologous tissues from damage due to the complement system (18, 19). The identity of the Dr adhesin ligand as DAF-Cromer antigen was supported by the observation (17) that the Dr adhesin did not bind to normal human erythrocytes in the presence of purified DAF nor to affected erythrocytes in the disease paroxysmal nocturnal hemoglobinuria, a condition whereby the cells lack all glycosyl phosphatidylinositol (GPI)-anchored membrane proteins, including DAF (20–22). Thus, our hypothesis is that the Dr adhesin might recognize DAF (Cromer antigen).

Direct evidence for DAF–Dr adhesin recognition may be found via molecular characterization of the interaction of DAF and Dr adhesin. In this report, we present a model for testing cell–cell interaction, using both molecularly characterized laboratory cells that express recombinant molecules of human

[1] *Abbreviations used in this paper:* AFA, afimbrial adhesin; CHO, Chinese hamster ovary; DAF, decay-accelerating factor; GPI, glycosyl phosphatidylinositol; MHT, minimal hemagglutination titer; SCR, short consensus repeat (domain); S/T, serine/threonine-rich; UT, urinary tract.

J. Exp. Med. © The Rockefeller University Press • 0022-1007/93/12/2115/07 $2.00
Volume 178 December 1993 2115–2121

DAF, and recombinant bacterial Dr colonization factors. Our results with the model have led to the identification of DAF as the ligand of the family of recombinant Dr and related *E. coli* adhesins. Further, we have developed structure–function studies that have mapped the adhesin binding site on the DAF molecule.

Materials and Methods

Bacterial Strains. *E. coli* HB101 strains containing plasmids pBJN406, pILL22, pILL115, and pSSS1 that expressed Dr, AFA-I, AFA-III, and F1845 adhesins, respectively, were grown on L-agar plates supplied with appropriate antibiotics. Bacterial cells were collected from the plates and a washed suspension of each strain was made, with an OD of 0.6 at 600 nm. Twofold serial dilutions of suspension were prepared to measure the minimal hemagglutination titer (MHT) for each *E. coli* strain, using 3% (vol/vol) human group O erythrocytes in 2% α-methylmannose. Experiments were performed with suspensions of identical MHT (1/64) and/or having an identical OD of 0.6 at 600 nm.

Chinese Hamster Ovary (CHO) Cell Transfectants Expressing DAF and DAF-Deletion Mutants. CHO cell transfectant clones that stably express DAF cDNA or various DAF constructs have been previously reported (23). Briefly, the CHO cells were transfected with 10 μg of DNA in the expression vector SFFV.neo (24) and 100 μg of lipofectin (25), and selected in 0.25 mg/ml G418 (active drug). Clones were produced by a number of rounds of sterile sorting with flow cytometry, followed by cloning via limiting dilution (23). Surface expression of DAF in the clones was measured by flow cytometry of CHO cells treated with rabbit polyclonal or murine monoclonal anti–human DAF (23), followed by appropriate FITC-labeled second antibody.

Purification of Fimbriae. Dr fimbrial protein was isolated by heat-shock treatment, deoxycholate, urea, and sucrose purification steps, as described previously (26).

CHO Binding Assay. CHO cells stably transfected with cDNA for human DAF or DAF deletion mutants were cultured in Ham's F12 medium supplemented with 10% FCS and grown to ~70% confluence in eight chamber slides (23). Suspensions of live *E. coli* cells (300 μl in PBS and 2% α-methylmannose) were added to the chambers containing CHO cells in quadruplicate, and incubated for 30 min at room temperature; experiments were performed on fixed and/or live CHO cells. Both methods gave similar results, and therefore, fixed cells were used preferably. Chambers were washed five times with PBS. One set of chambers was fixed for 10 minutes with cold acetone and stained for 45 s with safranin. The stained chambers were examined under a phase-contrast microscope (Leica Inc., Deerfield, IL), and 100 CHO cells per well were counted. The results were expressed as the mean number of bacterial cells per CHO cell.

To count live bacterial cells, the other set of unfixed chambers was swabbed, placed in 1 ml of sterile, cold distilled H_2O, and vortexed for 20 s. Then 10-fold dilutions were made and 50 μl of each dilution was spread onto L-agar plates and incubated at 37°C overnight. CFU were determined and the mean number of bacterial cells binding a single CHO cell was calculated. Experiments were multiply performed, and although results with the two methods were similar, the microscopic method showed less variability than the CFU test, was highly reproducible, and therefore used preferably.

Binding of Purified Dr Fimbriae to the CHO Cells. Briefly, 300 μl of purified fimbriae (20 μg of protein per chamber) was added to the slide containing various CHO transfectants, incubated for 30 min at +4°C, washed five times with PBS, and evaluated by indirect fluorescence with rabbit IgG, anti–Dr adhesin, and fluorochrome-conjugated anti–rabbit IgG (27).

Results

Our previous data suggested that the *E. coli* Dr adhesin bound to human DAF (12, 17). To confirm this finding and to permit mapping of structure–function relationships in the adhesin–ligand interaction, we established a system using recombinant proteins for studying this interaction. *E. coli* HB101 was transformed with a plasmid encoding the Dr adhesin, and CHO cells were transfected with an expression plasmid encoding DAF. A photomicrograph of the results of a binding study showed that the Dr adhesin specifically bound to human DAF (Fig. 1, *upper panels*). Further negative controls, using *E. coli* transformed with the vector without the adhesin insert, demonstrated that binding was specific for the adhesin (data not shown). Several experiments of this type clearly confirmed that DAF was a ligand for the Dr adhesin (Fig. 1, *lower panel*). In addition, binding could also be seen using purified fimbriae and CHO transfectants expressing DAF (data not shown). Experiments were performed also with live and fixed CHO cells and/or bacterial cells at 4°C, 25°C and 37°C. Changes in the conditions of the experiment did not affect the binding results.

A series of CHO cell transfectant clones expressing increasing amounts of DAF were used in binding assays with *E. coli* recombinant strains expressing the Dr adhesin or one of three other related adhesins, AFA-I, AFA-III, and F1845. All four strains bound in a dose-dependent fashion (Fig. 2); accordingly, DAF is a ligand for this entire family of adhesins. The AFA-I adhesin showed the strongest attachment capacity of these four recombinant strains, in that it was the only adhesin to bind to the CHO cell clone expressing the lowest amount of DAF.

Conversely, we also attempted to quantitate adhesins through the use of serial dilutions of recombinant strains (MHT 1/2 through 1/64). All four strains bound in a dose-dependent fashion to DAF-positive cells (data not shown). To eliminate potential differences in attachment to CHO cells due to the variation in expression of adhesins, suspensions of recombinant strains were used at the same MHT (as measured by the agglutination titer). When necessary, before the experiment, the MHT level of each strain was adjusted to the same hemagglutinating activity (1/64), by diluting or concentrating the suspension. For example, on the average, a suspension with an OD of 0.6 at 600 nm had an MHT titer of 1/64, and occasionally of 1/32. Therefore, clones were approximately equal and only occasionally was a minor adjustment in OD necessary.

We next attempted to localize the binding sites of the adhesins on the DAF molecule by using a series of DAF deletion mutants. Most of the extracellular domains of DAF are composed of four short consensus repeat (SCR) domains, each of ~60 amino acids and containing four conserved cysteines, as well as other residues (28). A set of deletion mutants that individually lack one of the four SCR units was expressed

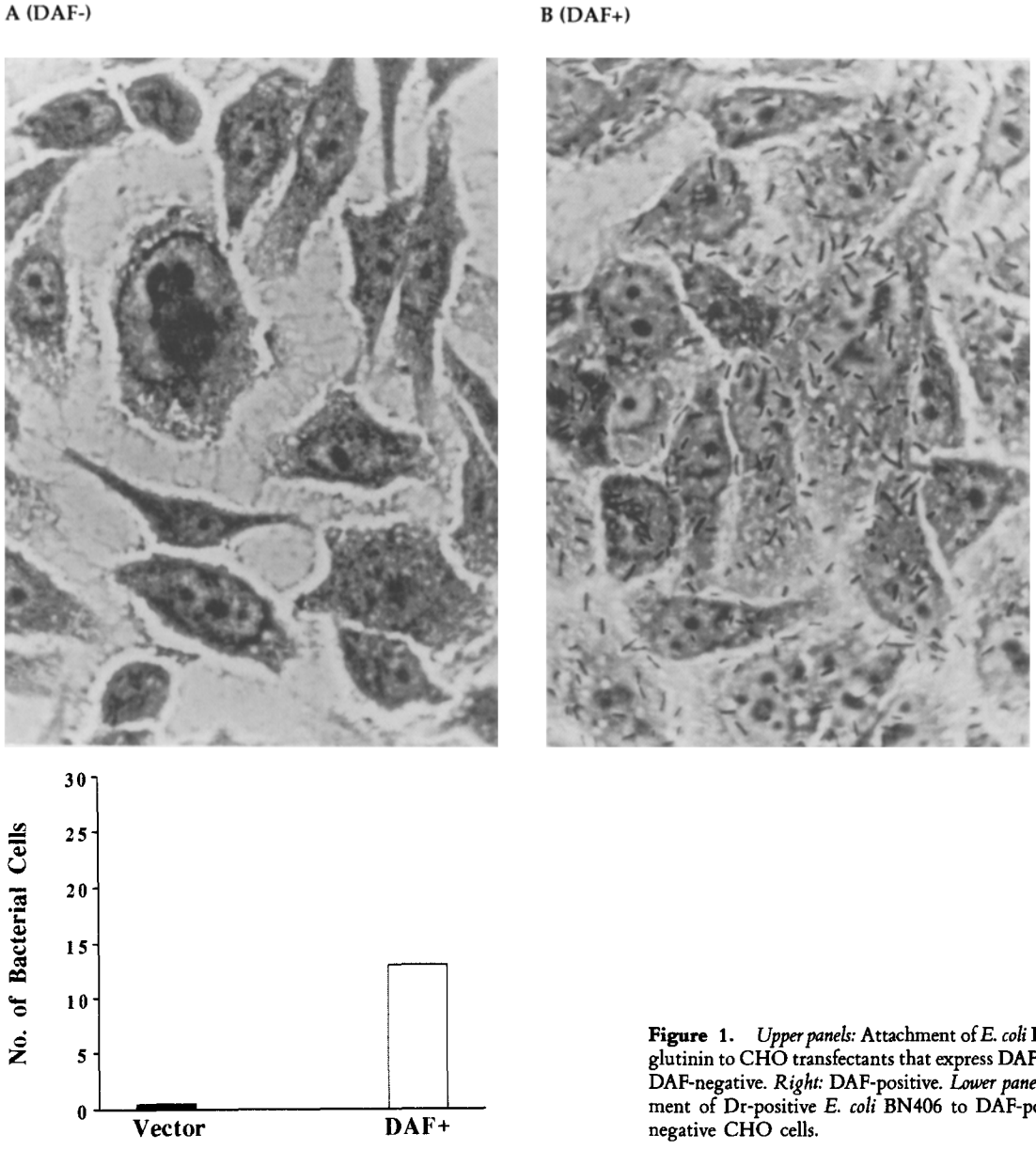

Figure 1. *Upper panels:* Attachment of *E. coli* BN406 bearing Dr hemagglutinin to CHO transfectants that express DAF or vector in control. *Left:* DAF-negative. *Right:* DAF-positive. *Lower panel:* Quantitation of attachment of Dr-positive *E. coli* BN406 to DAF-positive and control DAF-negative CHO cells.

in CHO cells (29). The surface expression of DAF by the individual clones was approximately equal; each deletion clone expressed an amount of DAF at least equal to the clone expressing wild-type DAF (varying less than twofold), as assessed by flow cytometry (29). The attachment of four recombinant *E. coli* strains that express Dr and related adhesins to these CHO transfectants expressing DAF and the DAF deletion mutants were investigated (Fig. 3). The binding patterns for all four recombinant *E. coli* strains were similar, with the exception of the binding to DAFΔSCR2. SCR1 and SCR4 were not required for binding of any of the adhesins, although AFA-I and AFA-III did show a 50% reduction in binding, with the removal of SCR4 (Fig. 3). Removal of SCR3 abolished binding of any of the adhesins, whereas removal of SCR2 abolished binding of all the adhesins, except for AFA-I, which retained full binding to the DAFΔSCR2 mutant (Fig. 3). Thus, the adhesin binding site on DAF is similar, but distinct, for the four adhesins of the Dr adhesin family, and involves SCR3 as well as SCR2 in some cases.

We had investigated previously a rare polymorphism of human DAF, designated Dr(a-), and we found that it arose from a point mutation that changed a single amino acid in SCR3: Ser^{165} in the high-frequency Dr^a allele to Leu in the rare Dr^b [Dr(a-)] allele (30). We also found that erythrocytes of the Dr^b phenotype did not bind Dr adhesin (12). However, because Dr^b erythrocytes expressed only one-third the levels of surface DAF found in Dr^a erythrocytes, it could not be ascertained if this lack of adhesin binding was a quantita-

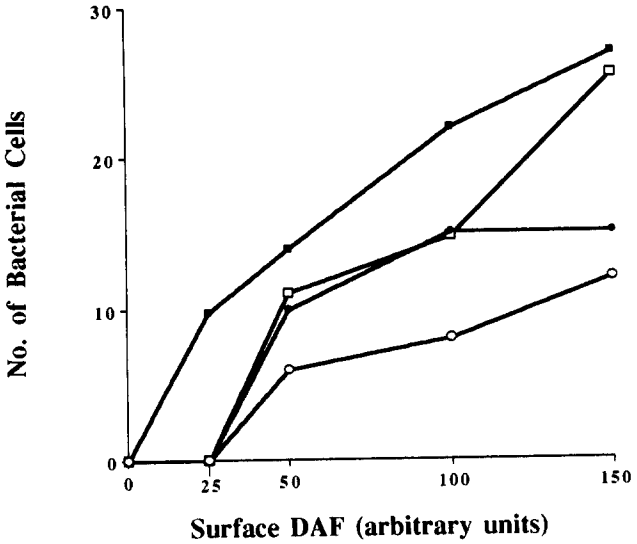

Figure 2. Dose-dependent binding of *E. coli* strains that express recombinant adhesins to CHO transfectants that express increasing amounts of surface DAF. ■, AFA-I; □, Dr; ●, F1845; O, AFA-III.

tive or a qualitative effect. This becomes particularly relevant because of the present results implicating SCR3 of DAF in the adhesin-binding site. Thus, we studied binding of all four of the Dr adhesin family members to CHO transfectants expressing equal amounts of the Dr^a or Dr^b allele of DAF. The results clearly showed that the single amino acid change between Dr^a and Dr^b completely abolished binding of all four of the adhesins (Fig. 4), suggesting that this amino acid position is part of the binding site.

The SCR domains that have been analyzed are the NH_2-terminal part of the DAF molecule, the remainder of which consist of a 70-amino acid serine/threonine-rich (S/T) domain that is the site of extensive O-linked glycosylation (31) and a GPI anchor (28). Binding studies showed no attachment of any of the adhesins to a CHO cell transfectant expressing a DAFΔS/T deletion mutant (Fig. 5). This was somewhat surprising as this domain is distant from the implicated SCR3 domain. However, previous results had suggested that this heavily O-glycosylated S/T domain serves as a spacer projecting the complement regulatory SCR domains away from the cell membrane (29). Reasoning that the same effect might be operating with the *E. coli* attachment, adhesin binding was also measured for a CHO transfectant expressing a construct designated DAFΔS/T + HLA, a chimeric molecule that consisted of the four SCR domains of DAF (but lacking the S/T domain) and most (COOH terminus) of the HLA-B44 molecule including the transmembrane and cytoplasmic domains (29). This chimeric molecule that repositions the four SCR domains of DAF on HLA-B44 completely restored and even improved adhesin binding (Fig. 5).

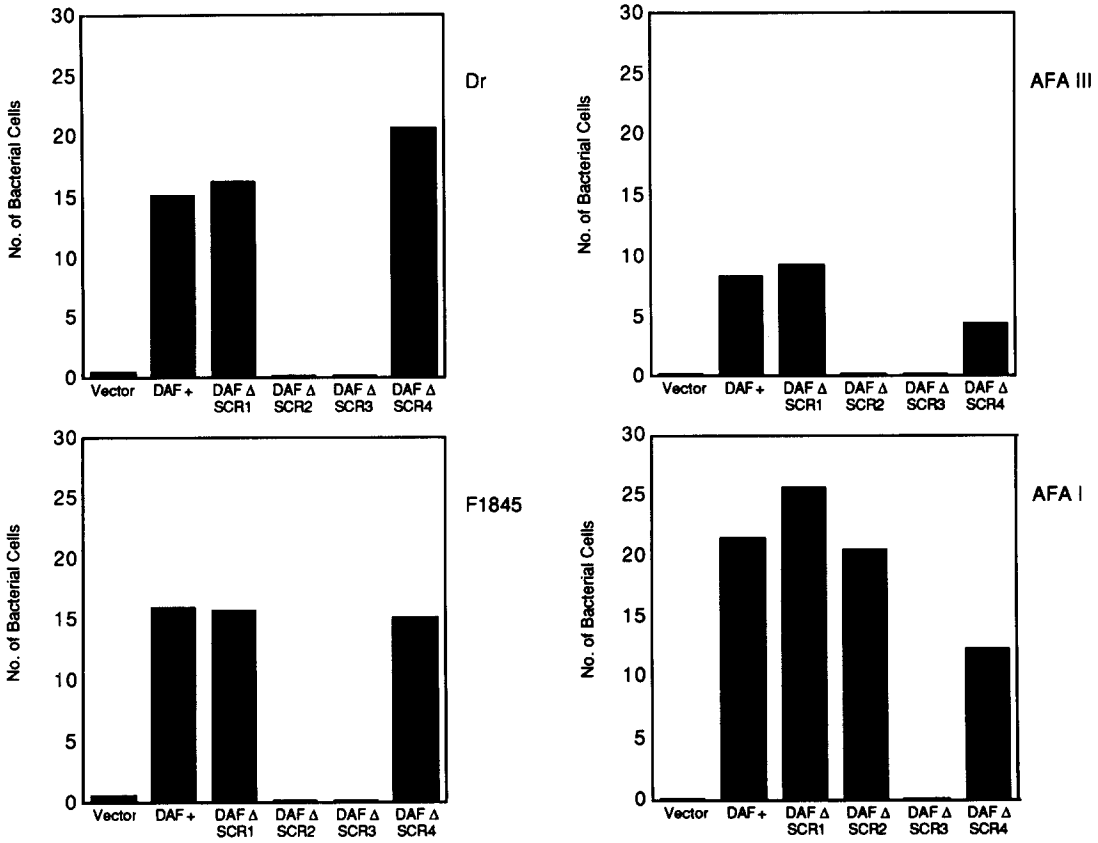

Figure 3. Attachment of *E. coli* strains that express recombinant Dr and related adhesins AFAI, AFAIII, F1845 to CHO transfectants DAFΔ SCR1, 2, 3, and 4 deletion mutants.

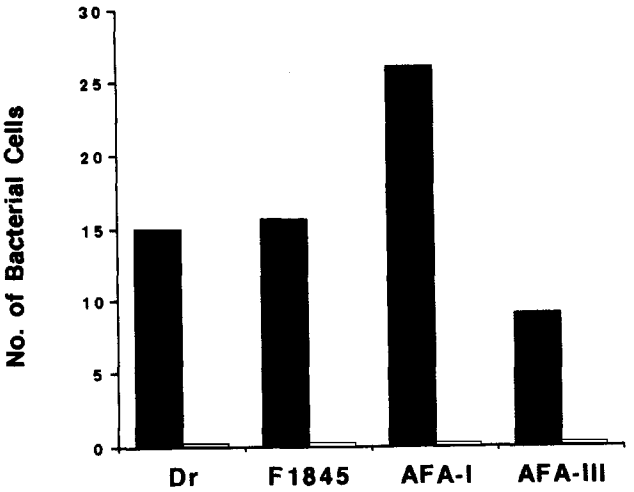

Figure 4. Attachment of *E. coli* that express recombinant Dr and related adhesins to CHO transfectants expressing equal amounts of (■) Dr^a and (□) Dr^b allele of DAF.

Discussion

The present report describes a molecular model to study host–pathogen interactions. Our approach permits an analysis of the cell–cell, adhesin–ligand, interaction in a molecularly characterized system, in which both *E. coli* adhesin and human tissue ligand have been isolated by molecular cloning and expressed in well-characterized cell types. Studies on the interaction between *E. coli* fimbriae and tissue ligands have been performed predominantly on human erythrocytes, purified glycoconjugates, or synthetic carbohydrates (1, 2, 32, 33). In such approaches, however, the antigenic complexity of human red blood cells or the use of selected glycoconjugates that are not necessarily a natural receptor present in human tissue, may affect the identification of natural receptor molecules. The present experiments indicated that DAF served as a ligand for the family of *E. coli* Dr adhesins.

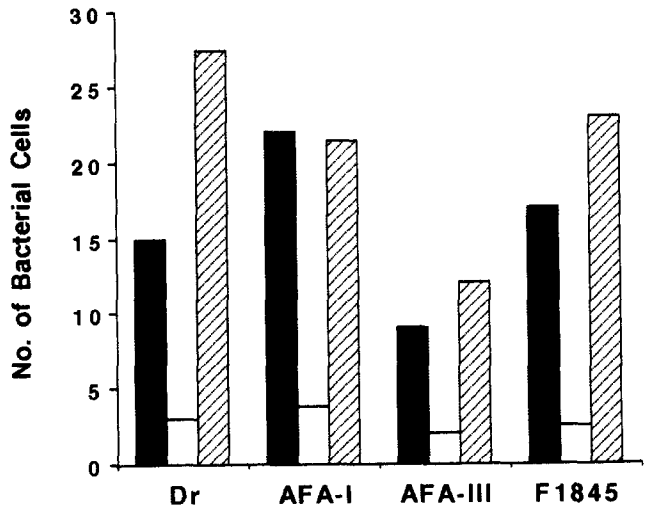

Figure 5. Attachment of *E. coli* strains that express recombinant Dr and other related adhesins to CHO transfectants expressing (*open bar*) DAFΔ S/T deletion mutant and (*hatched bar*) DAFΔ S/T +HLA chimeric molecules. (*Solid bars*) DAF.

DAF is a GPI-anchored membrane protein that protects host tissues from complement-mediated damage (28). DAF regulates complement activation at the critical C3 convertase step by preventing the association or by disassociating the two components on the enzyme (34). The DAF molecule contains four SCR domains, a S/T region, as well as a GPI anchor. We have demonstrated previously that SCR2, SCR3, and SCR4, but not SCR1 and Ser/Thr domains are each necessary to protect transfected CHO cells from human complement-mediated cytotoxicity, and that the level of protection increases with the expression of surface DAF (29).

The present study of DAF mutants has allowed us to define the adhesin binding site on DAF. First of all, this site is composed exclusively of a peptide sequence. DAF has three classes on nonpeptide modification: a single N-linked oligosaccharide (31) at the border of SCR1 and SCR2, multiple O-linked oligosaccharides attached to the S/T domain, and a GPI anchor at the carboxy terminus (29). The DAFΔSCR1 mutant lacks the N-linked oligosaccharide, and thus adhesin binding to this molecule (Fig. 3) demonstrates that N-linked oligosaccharides are not required. The DAFΔS/T + HLA construct lacks the O-linked oligosaccharides; therefore, adhesin binding to this molecule (Fig. 5) demonstrates that O-linked oligosaccharides are not required. Finally, this latter construct has a transmembrane domain instead of a GPI anchor, and thus the GPI anchor is not required for adhesin binding.

The peptide sequences in DAF that comprise the adhesin binding site were localized by study of the DAFΔSCR mutants. SCR3 is critical for the binding of all four adhesins (Fig. 3). Furthermore, a single-point substitution in SCR3, Ser^{165} to Leu, corresponding to the Dr^a to Dr^b allelic polymorphism (30), caused complete abolition of adhesin binding to DAF (Fig. 4). This shows that our previous finding (12) of a lack of reactivity of the Dr adhesin with Dr^b erythrocytes (that express only one-third the normal levels of DAF) was due to the absence of an adhesin binding site rather than to the lower expression of DAF. The Dr^a and Dr^b change does not appear to affect the complement regulatory function of DAF (30), so the adhesin and complement binding sites may not be the same.

The four adhesins exhibited some differences in their binding sites on DAF, as the DAFΔSCR4 mutant showed a moderately reduced binding of the AFA-I and AFA-III adhesins but normal binding of the Dr and F1845 adhesins (Fig. 3). Thus, this study supports our former proposal (5) that the binding sites on DAF are similar but distinct for some of the adhesins. This finding suggests that either the SCR2 or SCR4 peptide sequence is directly necessary for binding of certain of the adhesins or that the presence of these SCR domains affects the conformation or accessibility of the binding site in SCR3. Accessibility is also a factor for the S/T domain. The data (Fig. 5) demonstrate that this region is acting as a nonspecific spacer (in that it can be replaced by an unrelated sequence from HLA-B44), presumably projecting the DAF binding

site above the membrane for interaction with the adhesin. A similar effect was seen for DAF interaction with its natural ligands C3b and C4b (29).

Among the important features of our model is the expression of adhesins and ligands by the cells. This creates an opportunity to study cell–cell interaction, not only with respect to receptor–ligand recognition, but it may also allow us to design experiments on cell-to-cell interaction. The system has the advantage of assigning occurring phenomena to the particular gene and the gene product; e.g., responses of live DAF-bearing cells to colonization by live *E. coli* expressing Dr adhesins, toxins, or their mutants.

The finding that Dr fimbriae recognize a specific SCR-3 epitope(s) of DAF is very intriguing and may represent an example of bacterial colonization mechanisms that utilize human immune system molecules. A well-known example of this process whereby a human pathogen invades and alters the immune system is that of human immunodeficiency virus' binding to CD4 and streptococcal M protein, binding the factor H (35). The broad distribution of DAF at several tissue sites, including in leukocytes, erythrocytes, the urogenital tract, and other epithelial and endothelial tissues, may create several prime areas for bacterial colonization/infection, and perhaps may alter DAF function. Binding of monoclonal antibodies to the SCR3 region inactivates the DAF regulatory function (30), and the same antibody blocks the attachment of Dr fimbriae to DAF (unpublished observation). Further studies will determine whether the DAF–Dr adhesin interaction results in the alteration of the complement regulatory function of DAF and leads to immunopathology of tissues colonized by the Dr^+ bacteria. The recombinant approach used in this report can now be extended to an analysis of the binding site on the Dr adhesin molecule.

We are grateful to Ms. Cathy Gehret, Ms. Julia Frazier, and Ms. Mary Lou Mraz for excellent secretarial support.

This work was supported by National Institutes of Health grants DK-42029 to Dr. B. Nowicki and AI-15322 to Dr. D. M. Lublin.

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Received for publication 6 July 1992 and in revised form 26 August 1993.

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