Bacterial fibronectin-binding proteins and endothelial cell surface fibronectin mediate adherence of *Staphylococcus aureus* to resting human endothelial cells

Sharon J. Peacock,1,4 Timothy J. Foster,2 Brian J. Cameron1 and Anthony R. Berendt3,4

Adhesion of *Staphylococcus aureus* to human endothelial cells is implicated in the pathogenesis of invasive staphylococcal disease. The adhesion to endothelial cells of isogenic mutants defective in defined surface structures was studied. Three strains of *S. aureus* defective in fibronectin-binding proteins FnBPA and FnBPB showed reduced adhesion. This was fully restored by complementation of a FnBPA−FnBPB− mutant derived from strain 8325-4 with a multicopy plasmid encoding FnBPA or FnBPB. Adhesion of mutants defective in other surface structures was unaffected. Anti-fibronectin antibodies blocked adhesion of 8325-4 to endothelial cells, while adhesion of strains 8325-4, P1 and five clinical isolates was inhibited by the recombinant form of the binding domain of FnBPB (rFNBD) from *Streptococcus dysgalactiae*. Adherence of bacterial aggregates resulting from the presence of purified fibrinogen was also inhibited by rFNBD protein. Three strains of *S. aureus* defective in FnBPA and FnBPB were not internalized by endothelial cells. *S. aureus* FnBPs mediate adhesion to human endothelial cells and are required for subsequent internalization, interactions of potential relevance to pathogenesis and treatment.

**Keywords:** adherence, endothelium, fibronectin, *Staphylococcus aureus*

**INTRODUCTION**

*Staphylococcus aureus* is a major cause of community-acquired and nosocomial sepsis (Emori & Gaynes, 1993; Steinberg *et al*., 1996; Communicable Disease Surveillance Centre, 1998). The clinical manifestations are widespread and include bacteraemia, endocarditis, pneumonia, osteomyelitis, septic arthritis and abscess formation. In the hospital setting, *S. aureus* is the leading cause of post-operative wound infection, and is prominent in medical-device-related sepsis including intravenous devices, prosthetic joints and peritoneal dialysis catheters (Waldvogel, 1995). The effective treatment of *S. aureus* sepsis is threatened by the continued rise in the incidence of methicillin-resistant *S. aureus* (MRSA) infection (Panlilio *et al*., 1992; Speller *et al*., 1997), combined with the recent emergence of MRSA strains also demonstrating intermediate sensitivity to vancomycin (Centers for Disease Control and Prevention, 1997; Hiramatsu *et al*., 1997a, b). There is thus a pressing need to increase our understanding of the pathogenesis of staphylococcal disease, as a basis for developing novel therapeutic and preventive approaches.

An important feature of *S. aureus* sepsis is the frequency with which it seeds from the bloodstream to other body sites. Bacterial metastasis from blood to tissues such as bones, joints and solid organs is clinically apparent in 1–53% of individuals with staphylococcal bacteraemia (reviewed by Ing *et al*., 1997) and must involve interactions between circulating bacteria and vascular endothelial cells. *S. aureus* adheres to human endothelial cells in vitro (Vercellotti *et al*., 1984; Ogawa *et al*., 1985) but, surprisingly, both the endothelial surface receptor(s) and the bacterial-cell-wall-associated adhesin(s) responsible remain undefined. Once adhesion has occurred *S. aureus* cells undergo a process akin to phagocytosis (Lowy *et al*., 1988; Yao *et al*., 1995). This

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**Abbreviation:** FnBP, fibronectin-binding protein.
internalization initiates changes in cytokine expression (Yao et al., 1995, 1996), and induces hyper-adhesiveness for monocytes and granulocytes (Beekhuizen et al., 1997). Following endothelial cell uptake, S. aureus has been demonstrated both within vacuoles and free in the cytoplasm (Menzies & Kourteva, 1998). The subsequent fate of the endothelial cell varies between studies but appears to depend on the secretion of x-toxin, which is cidal to endothelial cells (Vann & Proctor, 1988). Lack of x-toxin production by small colony variants may explain why these can persist in the intracellular niche without causing endothelial cell death (Balwit et al., 1994; von Eiff et al., 1997).

Attempts to define the endothelial receptor for S. aureus adherence have yielded a 50 kDa membrane glycoprotein on human cells (Tompkins et al., 1990), and a 130 kDa membrane glycoprotein on bovine cardiac endothelial cells (Johnson, 1993). Neither has been characterized further. Acidic fibroblast growth factor has been reported to reduce bacterial adherence (Blumberg et al., 1988), which is also modulated by extracellular matrix heparan sulfate (Alston et al., 1997), while human tumour necrosis factor has been shown to enhance adhesion to glutaraldehyde-fixed endothelial cells in the presence of plasma (Cheung et al., 1991a). In the latter model, fibrinogen has been reported to act as a bridging molecule in adherence (Cheung et al., 1991b) without definition of the cognate ligands for it on either cell type.

The bacterial determinants that promote adhesion of S. aureus to endothelium have not been elucidated. The majority of clinical isolates produce capsular polysaccharide serotype 5 or 8 (Karakawa & Vann, 1982), which when purified has been shown to bind to endothelial cells and result in release of interleukin-6 and interleukin-8 (Soell et al., 1995). However, this is inhibited by the presence of pooled human serum from healthy blood donors (Soell et al., 1995). S. aureus also expresses a range of cell-wall-associated proteins that promote adherence to extracellular matrix proteins and/or soluble plasma components (Foster & McDevitt, 1994). The fibrinogen-binding protein Cla and collagen-binding protein have been shown to be important in the pathogenesis of experimental endocarditis (Moreillon et al., 1995; Hienz et al., 1996). The fibrinogen- and fibrinogen-binding proteins of S. aureus promote bacterial attachment to plasma clots (Raja et al., 1990; Moreillon et al., 1995), and to plastic coated with these host proteins in vitro and ex vivo (Vaudaux et al., 1989, 1993, 1995; Greene et al., 1995). It is possible that these observations have direct relevance to the mechanisms by which S. aureus adheres to host cells, given that endothelial cells synthesize and incorporate fibronectin into the extracellular matrix, and secrete it into the culture medium when grown in vitro (Jaffe & Mosher, 1978).

The aim of this study was to evaluate the role of a number of bacterial surface structures in adherence of S. aureus to endothelial cells in vitro. The approach taken was to compare the adherence of a range of defective mutants with that of the isogenic parent as a means of identifying candidate adhesins and cognate receptors for further study. Our results indicate that the interaction between the fibronectin-binding proteins (FnBPs) and endothelial-cell-associated fibronectin represents the dominant pathway for the adherence of S. aureus to human endothelial cells in vitro, and that FnBPs are required for subsequent bacterial internalization.

METHODS

Chemicals and reagents. All chemicals and reagents were obtained from Sigma-Aldrich, unless otherwise indicated. Culture media (Medium M199 and PBS) were from Gibco Life Technologies. Falcon tissue culture plasticware was obtained from Becton Dickinson.

Bacterial strains. The laboratory strains of S. aureus used, and their sources, are listed in Table 1. The clinical strains JR75, JR76, JR77, JR78 and JR80 were isolated in the Oxford microbiology department in 1995 from blood cultures of patients with native valve S. aureus endocarditis. Phillips and PH100 were gifts from Drs Magnus Höök and Jo Patti, Texas. The fnbA::Tc fnbB::Em mutations were co-transduced from DU5883 to strain P1 and JR80 by phase-85-mediated transduction (Asheshov, 1966). Transductants resistant to 2 µg tetracycline ml⁻¹ and 10 µg erythromycin ml⁻¹ were selected. The lack of adherence to purified human fibronectin at 10 µg ml⁻¹ (Sigma) was confirmed by microtitre adherence assay using an established method (data not shown) (Hartford et al., 1997).

Bacterial storage, growth and harvest. Bacterial isolates were stored in tryptone soy broth with glycerol (15%, v/v) at −80 °C. Isolates were inoculated from frozen stocks into 10 ml Todd–Hewitt broth (Difco Laboratories) contained in a 50 ml glass universal container and incubated for 15–18 h under constant rotation at 37 °C in air. S. aureus DU5883(pFNBA4) and DU5883(pFNBB4) were cultured in Todd–Hewitt broth supplemented with 10 µg chloramphenicol ml⁻¹ to maintain plasmid selection. Purity of stock cultures was checked by simultaneous subculture onto blood agar with visual inspection of colony morphology after overnight incubation at 37 °C in air. Bacteria were collected from broth culture by centrifugation at 3000 r.p.m. for 10 min, washed four times with PBS, resuspended in Medium M199 with Earle’s salt and 25 mM HEPES (M199) and filtered through a sterile 3 µm filter (Gelman Sciences) to remove bacterial aggregates. A subsequent Gram stain showed the cell suspension to comprise bacteria singly or in pairs. Final bacterial concentrations were adjusted to an OD₆₀₀ of 0·90±0·94, corresponding to a c.f.u. count of approximately 1×10⁸ c.f.u. ml⁻¹ on viability counts as performed by serial dilution, inoculation onto blood agar and colony count after overnight incubation at 37 °C in air.

Endothelial cell culture. Endothelial cells were obtained from human newborn umbilical vein using an adaptation of a previously described method (Jaffe et al., 1973a). Both ends of the cord vein were cannulated using a Portex luerlock adaptor (Southern Syringe), after which the vessel was flushed with PBS. Cells were released by instilling M199 supplemented with 50 U penicillin ml⁻¹ (Gibco), 50 µg streptomycin ml⁻¹ (Gibco) and 0·5 mg collagenase type IA ml⁻¹ into the vessel lumen. After incubation for 20 min at 37 °C the cell suspension was collected by centrifugation, resuspended in M199 supple-
Table 1. Strains of *S. aureus* used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Properties</th>
<th>Reference</th>
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<tbody>
<tr>
<td>8325-4</td>
<td>fnbA::Tc&lt;sup&gt;a&lt;/sup&gt; fnbB::Em&lt;sup&gt;h&lt;/sup&gt;</td>
<td>NCTC 8325 cured of prophages</td>
<td>Novick (1967)</td>
</tr>
<tr>
<td>DU5883</td>
<td>fnbA::Tc&lt;sup&gt;a&lt;/sup&gt; fnbB::Em&lt;sup&gt;h&lt;/sup&gt; (pFNBA4:fnbA&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>Mutant strain of 8325-4 defective in FnBPs A and B</td>
<td>McDevitt et al. (1994)</td>
</tr>
<tr>
<td>DU5883(pFNBA4)</td>
<td>fnbB::Em&lt;sup&gt;h&lt;/sup&gt; (pFNBA4:fnbA&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>Mutant strain of 8325-4 defective in FnBPs, complemented with multicopy plasmid expressing fnbA&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Greene et al. (1995)</td>
</tr>
<tr>
<td>DU5883(pFNBB4)</td>
<td>fnbA::Tc&lt;sup&gt;a&lt;/sup&gt; fnbB::Em&lt;sup&gt;h&lt;/sup&gt; (pFNBB4:fnbB&lt;sup&gt;c&lt;/sup&gt; Cm&lt;sup&gt;b&lt;/sup&gt;)</td>
<td>Mutant strain of 8325-4 defective in FnBPs, complemented with multicopy plasmid expressing fnbB&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Greene et al. (1995)</td>
</tr>
<tr>
<td>DU5880</td>
<td>clfA1::Tn917 (Em&lt;sup&gt;h&lt;/sup&gt;)</td>
<td>Mutant strain of 8325-4 defective in fibrinogen-binding protein ClfA</td>
<td>Pati et al. (1994a)</td>
</tr>
<tr>
<td>DU5877</td>
<td>Δcoa::Em&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Mutant strain of 8325-4 defective in coagulase</td>
<td>Phonomdaeng et al. (1990)</td>
</tr>
<tr>
<td>DU5875</td>
<td>Δspa::Tc&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Mutant strain of 8325-4 defective in protein A</td>
<td>Patel et al. (1987)</td>
</tr>
<tr>
<td>Phillips</td>
<td></td>
<td>Clinical osteomyelitis isolate expressing collagen-binding protein</td>
<td>Pati et al. (1999a)</td>
</tr>
<tr>
<td>PH100</td>
<td>cna::Gm&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Mutant strain of Phillips defective in collagen-binding protein</td>
<td>Patti et al. (1994a)</td>
</tr>
<tr>
<td>Newman</td>
<td></td>
<td>High level of fibrinogen-binding protein ClfA</td>
<td>Duthie &amp; Lorenz (1952)</td>
</tr>
<tr>
<td>DU5917</td>
<td>cps1::Tn917 (Em&lt;sup&gt;h&lt;/sup&gt;)</td>
<td>Mutant strain of Newman defective in capsular polysaccharide</td>
<td>Sau et al. (1997)</td>
</tr>
<tr>
<td>P1</td>
<td></td>
<td>Isolated from a rabbit inoculated with ATCC 25923</td>
<td>Sherertz et al. (1993)</td>
</tr>
<tr>
<td>DU5947</td>
<td>fnbA::Tc&lt;sup&gt;a&lt;/sup&gt; fnbB::Em&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Mutant strain of P1 defective in FnBPs A and B</td>
<td>This study</td>
</tr>
<tr>
<td>DU5908</td>
<td>clfA1::Tn917 (Em&lt;sup&gt;h&lt;/sup&gt;)</td>
<td>Mutant strain of P1 defective in fibrinogen-binding protein ClfA</td>
<td>T. J. Foster, personal</td>
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<tr>
<td>JR80</td>
<td></td>
<td>Isolate from a patient with endocarditis</td>
<td>communication</td>
</tr>
<tr>
<td>DU5953</td>
<td>fnbA::Tc&lt;sup&gt;a&lt;/sup&gt; fnbB::Em&lt;sup&gt;h&lt;/sup&gt;</td>
<td>Mutant strain of JR80 defective in FnBPs A and B</td>
<td>This study</td>
</tr>
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Mentioned with fetal bovine serum (20%, v/v; Gibco), 90 µg heparin ml<sup>−1</sup>, 5 ng recombinant fibroblast growth factor ml<sup>−1</sup>, 50 U penicillin ml<sup>−1</sup> and 50 µg streptomycin ml<sup>−1</sup> and seeded into a 25 cm<sup>2</sup> tissue culture flask. Cells were maintained at 37 °C in 5% CO<sub>2</sub> and passaged twice on reaching confluence using a 1:3 split, before subculture onto 13 mm Theranova coverslips (Gibco) in 24-well tissue culture plates. All flasks and coverslips used in cell culture were pre-coated with gelatin (0.2%, v/v) overnight at 37 °C. The identity of the cells was confirmed as endothelial by their cobblestone appearance at confluence, and positive immunofluorescent staining for von Willebrand factor (Jaffe et al., 1973b).

**Endothelial cell adhesion assay.** Sterile 24-well flat-bottomed tissue culture plates were blocked with BSA (1%, w/v) for 1 h and rinsed twice with PBS. A bacterial inoculum of 10<sup>8</sup> c.f.u. suspended in 500 µl M199 was added to each well. This inoculum was selected on the basis of dose response curves (data not shown). Confluent endothelial cells coating 13 mm Theranova coverslips were added after dip-washing three times in M199 to remove traces of culture media. The 24-well plates were incubated at 37 °C in CO<sub>2</sub> for 1 h. This incubation period was selected on the basis of time-course studies (data not shown). The coverslips were then dip-washed three times in M199 and once in PBS to remove non-associated bacteria. The endothelial cells were fixed with Cytofix (Cellpath), air-dried, and stained with crystal violet (0.5%, w/v) for 5 min. Following dip-rinsing in water, the coverslips were air-dried and mounted on glass slides. The number of endothelial-cell-associated bacteria was quantified using a visual method. The bacterial count included both adherent and internalized bacteria, but for simplicity bacteria are referred to as adherent in the remainder of the text. Bacteria prepared for the adhesion assay as above were shown not to divide over the course of an hour in binding medium (data not shown), making the number of visualized bacteria an accurate representation of those that had adhered. Each coverslip was scanned under low power to ensure confluence and integrity of the monolayer. Using a calibrated graticule under oil immersion and a ×100 magnification lens, the number of bacteria associated with 1 mm<sup>2</sup> of confluent endothelial cells was enumerated by a standardized counting procedure. Without prior visual inspection at high power, five high-power fields were selected for counting, as follows: (1) the centre of the coverslip; (2) two points between the centre and left edge; and (3) two points between the centre and right edge.

**Endothelial cell internalization assay.** Internalization of *S. aureus* by endothelial cells was evaluated by incubating the monolayer with lysostaphin at the end of the adherence assay. This has been shown to remove *S. aureus* that is adherent to the monolayer but does not affect the viability of internalized bacteria (Yann & Proctor, 1987). The endothelial cell adhesion assay was performed using the method described above, at the end of which coverslips were rinsed three times in M199 and placed into 24-well plates containing lysostaphin at 10 µg ml<sup>−1</sup> in M199 or M199 alone. These were incubated for 20 min at 37 °C in CO<sub>2</sub> then fixed and stained as before. Crystal violet
was shown to penetrate the endothelial cell and stain internalized bacteria. The numbers of internalized bacteria per mm² in the lysostaphin-treated monolayers were compared with the total bacterial count (adherent plus intracellular) for the controls. Internalization was also examined in a second assay in which 10⁶ c.f.u. were centrifuged onto the surface of the endothelium during a 5 min spin at 1000 r.p.m. The adherence assay was then allowed to continue over 1 h at 37 °C in CO₂, followed by lysostaphin treatment as described above. The 60 min time point used to examine internalization in this assay was selected on the basis of time-course studies (data not shown). Coverslips were rinsed, fixed and stained, and the numbers of bacteria per mm² were counted using the standardized counting procedure.

**Competitive endothelial binding inhibition assay with the recombinant form of the ligand-binding domain of FnBP.** Endothelial cell adhesion assays were performed using the method described above, with the exception that the recombinant form of the ligand-binding domain of FnBP of *Streptococcus dysgalactiae* (Joh et al., 1994) (rFNBD-B; a gift from Dr Magnus Höök, Texas, referred to below as rFNBD protein) was added to the wells immediately prior to bacterial inoculation at a final concentration of 1 µg ml⁻¹, 10 µg ml⁻¹ or 50 µg ml⁻¹. To control for the possibility that recombinant protein non-specifically interfered with bacterial adherence, parallel assays were performed in the presence of a recombinant truncated ClfA protein (Clf41, residues 221–559) (O’Connell et al., 1998) at a final concentration of 25 µg ml⁻¹. A second control was bacteria in the absence of recombinant protein. The total volume was maintained at 500 µl for all wells.

**Effect of anti-human fibronectin antibodies on adhesion to endothelial cells.** The effect on adherence of anti-human fibronectin antibodies was evaluated using 8325-4 defective in protein A (DU5875) so as to control for the confounding interaction between protein A and the Fc region of IgG. The following antibodies were used at a concentration of 10 µg ml⁻¹: rabbit polyclonal antibody to purified human fibronectin (F3648; Sigma); sheep polyclonal antibody to purified human fibronectin (Serotec UK; Oxford); mAb recognizing an epitope located within the 5th type III repeat of human plasma fibronectin (mouse IgG1 clone IST-4; F 0916, Sigma); and mAb recognizing the N-terminus of fibronectin (mouse IgG3 clone 10B7; Biogenesis). The following control antibodies were used at a concentration of 10 µg ml⁻¹: normal sheep IgG (I 5131, Sigma); normal rabbit IgG (I 5006, Sigma); mouse IgG1, Kappa (MOPC-31C; M 9035, Sigma); and mouse IgG3, Kappa (FLOPC 21; M 3645, Sigma). All antibodies were added to the wells immediately prior to bacterial inoculation.

**Bacterial adhesion to solid-phase purified fibronectin.** Purified human fibronectin at 10 µg ml⁻¹ (Sigma) in TBS was spotted onto tissue culture grade plastic Petri dishes and incubated at 37 °C in air for 1 h, then flooded with 1 % BSA in TBS and maintained overnight at 4 °C. Dishes were rinsed twice in M199 immediately prior to use. After bacterial preparation as described above, strains were suspended in M199 with 1 % BSA to a final concentration of 5 x 10⁸ c.f.u. ml⁻¹ and 1.5 ml was added to each dish. Following incubation at 37 °C in air for 1 h, the dishes were rinsed four times with M199, fixed with glutaraldehyde (2 %, v/v) in M199 for 2 h and stained with 0.5 % crystal violet for 5 min. Adhesion was quantified using a visual method. The purified fibronectin spots were examined using a calibrated graticule under oil immersion with a x 100 magnification lens. The number of bacteria adherent to an area of 1 mm² purified protein were enumerated by a standardized counting procedure. Without prior visual inspection at high power, five high-power fields were selected for counting, as follows: (1) the centre of the protein spot; (2) two points between the centre and left edge; and (3) two points between the centre and right edge.

**Endothelial cell adhesion assays after pre-coating with, or in the presence of, fibrogenin and fibronectin.** Adherence assays were performed using one of two modifications. (1) Confluent endothelial cells coating Thermomax coverslips were pre-coated for 30 min at 37 °C in air with either purified human fibronectin at 300 µg ml⁻¹ (Sigma), or purified human fibrinogen at 1 mg ml⁻¹ (Sigma). The coverslips were then dip-rinsed in M199 four times prior to use in the adherence assay. (2) Adherence assays were performed in the presence of either purified human fibrinectin at 300 µg ml⁻¹ or purified fibrinogen at 1 mg ml⁻¹, with or without rFNBD protein. Contamination of commercially available human fibrinogen by fibronectin is a potential confounder in endothelial cell adherence assays. The human fibrinogen used in this study was evaluated and purified prior to use, as follows. The presence of contaminating fibronectin was confirmed by Western blotting following fractionation by SDS-PAGE of a 15 µl aliquot of fibrinogen solution, 2 mg ml⁻¹, dissolved in M199, as previously described by McDevitt et al. (1992) (data not shown). Fibrinogen was removed by passing the fibrinogen solution through a gelatin-Sepharose column (Pharmacia Biotech) using the procedure described by the manufacturer. The absence of contaminating fibronectin in the eluate was confirmed by Western blot. Western blotting did not demonstrate the presence of contaminating immunoglobulin.

**Statistical analysis.** Results for the numbers of bacteria adherent to or internalized by endothelium, or adherent to purified fibronectin, were expressed as the mean count per 1 mm² surface area. All points were performed in triplicate and each experiment was performed three times. Statistical analysis was carried out using the Statview 4.5 statistical software package (Abacus). Comparison of the mean count between bacterial strains was performed using an unpaired t-test with correction for multiple comparisons.

**RESULTS**

**Isogenic mutants deficient in FnBP demonstrate reduced binding to endothelial cells.** Adhesion to live endothelium was compared for the isogenic mutants and parental strains shown in Table 1. Evaluation of 8325-4 and related mutants demonstrated a significant reduction in the number of adherent bacteria for the 8325-4 FnBP-deficient mutant (Fig. 1). This contrasted with 8325-4 mutants defective in coagulase, protein A or fibronogen-binding protein ClfA, for which adhesion was unaffected (Fig. 1). There was no difference between strain Newman and its isogenic mutant defective in capsular polysaccharide (96 % of that for the parent, P > 0.05), or strain Phillips and its isogenic mutant defective in collagen-binding protein (104 % of that for the parent, P > 0.05) (data not shown). The reduction in adherence of the 8325-4 FnBP-deficient mutant was reproducible using FnBP-deficient mutants of strains P1 and JR80. The bacterial count for *S. aureus* P1 and JR80 defective in FnBPA and FnBPB was 9.2 % and 14.7 %, respectively, compared with that...
for the parent strains ($P < 0.0001$ in both cases) (data not shown).

**Complementation of the 8325-4 FnBP-deficient mutant with a multicopy plasmid restores endothelial binding**

Further evidence for the involvement of *S. aureus* FnBP in bacterial adherence to endothelial cells was provided by adhesion assays using the 8325-4 FnBP-deficient mutant complemented with a multicopy plasmid encoding either fnbA+ or fnbB+ on adhesion to endothelial cells was assessed. FnBPA+B is the double FnBP mutant complemented with pFNBA4 encoding fnbA, and FnBPA+B is the double FnBP mutant complemented with pFNBB4 encoding fnbB. Both complemented strains demonstrated significantly greater levels of adhesion than that of the FnBP-deficient mutant ($P < 0.0001$ and $P = 0.0003$, respectively), and the wild-type parental strain ($P = 0.0012$ and 0.0024, respectively).

**The recombinant form of the ligand-binding domain of FnBP-B from Streptococcus dysgalactiae inhibits endothelial binding of *S. aureus***

The effect of 10 µg rFNBD protein ml⁻¹ on adherence of *S. aureus* 8325-4, P1 and five recent clinical isolates is shown in Fig. 2. There was a significant reduction in count in the presence of rFNBD protein for all isolates, while the recombinant truncated ClfA protein (Clf41, residues 221–559) had no effect on adherence (range 89.6–95.5% of the control, $P > 0.05$) (data not shown). These results imply that the binding domain (D repeat region) of *S. aureus* FnBP participates in the interaction between *S. aureus* and the endothelial monolayer. Although markedly reduced, bacterial adhesion to the monolayer was not abolished at any of the concentrations of rFNBD protein used (data not shown). This concurs with the observation that FnBP-defective mutants can adhere to the monolayer, albeit at a reduced level compared to the parent strain.
Inhibition of endothelial binding with anti-fibronectin antibodies

The above findings suggest that fibronectin is the host cell surface receptor for adherence of *S. aureus* to endothelial cells in vitro. This was evaluated indirectly by examining the effect of a panel of anti-human fibronectin antibodies on adherence of the protein-A-defective 8325-4 mutant of *S. aureus* (Fig. 3). A significant reduction in adhesion was seen in the presence of rabbit or sheep polyclonal anti-human fibronectin antibodies, or a mAb to the N-terminus of human fibronectin. The number of adherent bacteria was not affected by a mAb recognizing the 5th type III repeat of human fibronectin.

Adhesion of *S. aureus* to purified fibronectin correlates with endothelial binding

Adherence to endothelial cells was compared with adherence to solid-phase purified human fibronectin for 8325-4 and related fibronectin mutants P1, JR80 and Newman. Adhesion to endothelial cells correlated with adherence to purified fibronectin (data not shown). As predicted, the 8325-4 FnBP-deficient mutant showed no binding to purified fibronectin. The same mutant complemented with a multicopy plasmid expressing either *fnbA* (pFNBA4) or *fnbB* (pFNBB4) showed enhanced binding to fibronectin compared with the isogenic parent. This has been reported previously (Greene et al., 1995), and may be due to the expression of a higher number of FnBPs. *S. aureus* P1 adhered strongly to endothelial cells and fibronectin, while Newman adhered poorly to both substrates. The weak adherence of Newman to solid-phase fibronectin in this study has been observed by others (Vaudaux et al., 1995), and occurs despite the presence of two apparently functional *fnb* genes that express FnBP protein detectable by ligand-affinity blotting (Greene, 1995). The association between the ability to adhere to solid-phase purified human fibronectin and endothelial cells provides further indirect evidence for fibronectin as the endothelial cell receptor for *S. aureus*.

Effect of plasma proteins on adherence of *S. aureus* to endothelial cells

Previous studies have shown that plasma proteins are involved in bacterial adherence to endothelium. We evaluated the role of such proteins by pre-coating endothelial monolayers with purified human fibronectin or fibrinogen, or by adding these host proteins into the assay.

Pre-coating the monolayer with fibronectin followed by rinsing prior to use in the adherence assay had no effect (Fig. 4). This contrasted with the effect of actually adding fibronectin to the assay, which led to agglutination of bacteria as described previously (Proctor et al., 1984) and adherence of aggregates to the monolayer (data not shown).
Pre-coating the endothelial monolayer with fibrinogen also failed to influence adherence of 8325-4 or P1 (Fig. 5). The adherence assay was repeated for mutants of 8325-4 and P1 defective in ClfA. There was no significant difference in adherence to fibrinogen-pre-coated endothelium between these two pairs (Fig. 5).

The relative importance of FnBP and ClfA in the adherence of \textit{S. aureus} to endothelial cells \textit{in vitro} in the presence of fibrinogen was then assessed by assays in which fibrinogen (1 mg ml\(^{-1}\)) was added, with or without rFNBD protein (10 µg ml\(^{-1}\)). This was performed for 8325-4, P1 and the five clinical isolates (JR75–78 and JR80), which all behaved similarly. Fibrinogen alone resulted in the formation of a latticework of bacterial aggregates adherent to the monolayer. The entire inoculum appeared to become consumed into the aggregates, with virtually no bacteria adherent to the monolayer in singles, pairs or small clusters. In the presence of rFNBD protein and fibrinogen together, large bacterial aggregates formed but these were not adherent to the monolayer and were readily rinsed off at the end of the assay. Thus, \textit{S. aureus} FnBPs appear to predominate in importance over the fibrinogen-binding protein ClfA during bacterial adherence to endothelial cells in the presence of fibrinogen.

\textbf{Endothelial cells do not internalize \textit{S. aureus} mutants deficient in FnBP}

Using the adherence assay in which \(10^8\) c.f.u. were incubated with the monolayer for 1 h with no prior centrifugation of bacteria onto the endothelium, the number of bacteria internalized by endothelial cells was 9%, 25% and 21% of the total (intracellular + adherent) for wild-type 8325-4, P1 and JR80, respectively (data not shown). This contrasted with the three isogenic FnBP-deficient mutants, for which no intracellular bacteria were visualized either in the standard 1 mm\(^2\) surface area of endothelium examined, or during detailed scanning of the monolayer in multiple fields. It is possible that the apparent lack of internalization of the FnBP-defective mutants resulted from the low number of bacteria adherent to the monolayer rather than interruption of a specific uptake pathway. This was examined by centrifuging \(10^8\) c.f.u. onto the monolayer at the start of the adherence assay. The numbers of internalized bacteria per mm\(^2\) of endothelium after 60 min incubation at \(37^\circ\text{C}\) were 397, 826 and 790, respectively, for 8325-4, P1 and JR80. This compared with <1 bacterium per mm\(^2\) for FnBP-defective mutants of 8325-4, P1 and JR80. The lack of intracellular bacteria for all three mutants indicates that FnBPs are critical for internalization of \textit{S. aureus} by endothelial cells \textit{in vitro}.

\textbf{DISCUSSION}

We have shown for the first time that the \textit{S. aureus} FnBPs play an important role in adhesion to live human endothelial cells. Isogenic mutants defective in expression of FnBPs all showed marked reductions in adhesion to endothelial cells. This was true in two laboratory strains and a recent clinical isolate. Restoration, through plasmid complementation, of the fibronectin-binding ability of a FnBP-defective isogenic mutant also restored endothelial binding. Adhesion was inhibited by anti-fibronectin polyclonal and monoclonal antibodies, and by a recombinant fibronectin-binding domain from the FnBP of \textit{Streptococcus dysgalactiae}. In addition to providing evidence that staphylococcal FnBPs interact with endothelial fibronectin, our data also indicate that this is through the known interaction of the D region of FnBP with the N-terminal five type I domains of fibronectin (Sottile \textit{et al}., 1991). Bacterial uptake by the endothelial monolayer was also dependent on the presence of FnBPs.

Our findings are important because they cast light on a key interaction in the pathogenesis of metastatic \textit{S. aureus} infection, the adherence of bacteria to endothelial cells. Since this organism is able to infect apparently normal bone and joint tissues, a direct interaction with endothelial cells is likely as a first step in the invasion of these deeper tissues. Fibronectin is well placed to act as a receptor in this regard. It is a normal component of the extracellular matrix on the luminal surface of an endothelial monolayer and our findings are consistent with a previous report of the ultrastructural localization of fibronectin between bovine endothelial cells and adherent \textit{S. aureus} (Vann \textit{et al}., 1989). The observation that \textit{S. aureus} 8325-4 has two FnBPs, FnBPA and FnBPB, encoded by the closely linked genes \textit{fnbA} and \textit{fnbB} (Signas \textit{et al}., 1989; Jonsson \textit{et al}., 1991) underscores the importance of interactions with fibronectin in the biology of the organism. Given that the defect in endothelial cell adhesion seen with the FnBP-deficient mutant was restored by the presence of a multicopy plasmid encoding one of \textit{fnbA} or \textit{fnbB}, either FnBPA or
Adhesion of *S. aureus* to live endothelial cells is rapidly followed by internalization, a process which requires the presence of bacterial FnBPs. Fibronectin, through its known interactions with integrin receptors present on endothelium (Albelda et al., 1989), is an ideal candidate molecule to orchestrate these events. Many other invasive pathogens use integrins as cellular receptors (for review see Berendt & McCormick, 1997) and *Streptococcus pyogenes* has already been shown to invade a number of epithelial cell lines through the interaction between fibronectin and epithelial-cell-surface integrins (Ozeri et al., 1998). Our observations thus prompt the speculation that *S. aureus* invades endothelial cells through similar mechanisms.

Under physiological conditions, the interaction of *S. aureus* with endothelial cells takes place at greatly lower bacterial density, in whole blood and under conditions of flow. The relevant activation status of the endothelial cells in vivo is unknown and their phenotype will vary according to site. We previously showed that there are important differences between microvascular and large vessel endothelium in the expression and function of host receptors for the adhesion of malaria-infected erythrocytes (McCormick et al., 1997). Furthermore, for adhesion of both leukocytes and malaria-infected erythrocytes, endothelial receptors show differential adhesion under shear flow conditions, with ‘rolling’ and ‘static’ receptors. It therefore remains possible that under conditions of flow and cytokine activation, additional adhesion pathways operate independently of, or alongside, the fibronectin pathway. Elucidating this under the full range of conditions that might prevail in vivo requires further detailed studies.

Plasma proteins are also an important component of these interactions. We were surprised to find no convincing role in adhesion for fibrinogen, which has been previously reported to act as a bridging molecule (Cheung et al., 1991b). There are a number of differences between the previous study and our own that may explain this, including the bacterial strains used and the method of quantifying bacterial adherence. An additional difference is that the earlier group used an assay where the endothelial cells were fixed with glutaraldehyde. This may have modified the affinity or accessibility of the binding sites on the fibronectin molecules, rendering them unable to interact with the FnBPs on the bacteria. In support of this, we find that glutaraldehyde fixation of purified fibronectin that has been immobilized on plastic reduces the adherence of *S. aureus* 8325-4 and the clinical isolate JR80 to 5% and 8%, respectively, of that for the non-treated control. We cannot exclude the possibilities that under different conditions of growth or activation, the fibronectin pathway plays a lesser role or that it is inoperative, secondary adhesion mechanisms become important. Indeed, in our system, low levels of residual bacterial binding (approx. 10–20% of control) are seen when the fibronectin-binding pathway is non-functional due to mutation, rFNBD protein or anti-fibronectin antibodies. The receptor for this secondary pathway remains unidentified.

Finally, it is important to note that FnBPs are not unique to *S. aureus*, being widely distributed among the streptococci (reviewed by Patti et al., 1994b). Whether other organisms capable of causing complicated bacteraemias also adhere to endothelial cells by this route remains a matter for speculation and further study.

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Staphylococcus aureus adherence to endothelial cells


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