Fibrinogen Acts as a Bridging Molecule in the Adherence of *Staphylococcus aureus* to Cultured Human Endothelial Cells

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Abstract

The propensity of *Staphylococcus aureus* to cause acute endovascular infections during transient bacteremia is poorly understood. To examine the events leading to the attachment of staphylococci to endothelium, adherence assays were developed to study the role of blood factors in the mediation of staphylococcal adherence to cultured human umbilical vein endothelium in vitro. Results indicate that the preferential attachment of *S. aureus* to endothelial cells is mediated by fibrinogen adsorbed from plasma onto the endothelial surface. The binding is apparently specific because it could be blocked by goat anti-human fibrinogen antibody in a dose-dependent fashion while nonimmune goat IgG, mouse MAb against AG-1 (a platelet antigen found on the endothelial cell surface), nonspecific mouse MAb and rabbit antibodies to human vitronectin and fibronectin were not inhibitory. Our data also indicate that fibrinogen is a necessary but not the only blood constituent in the mediation of binding of *S. aureus* to endothelium. This was supported by the finding that fibrinogen alone, at a concentration equivalent to that in plasma, did not potentiate staphylococcal adherence as much as plasma while afibrinogenemic plasma reconstituted with fibrinogen did. Because fibrinogen is known to bind to endothelial cells, our data is consistent with the hypothesis that fibrinogen and additional plasma factor(s), possibly activated during inflammation, promote staphylococcal adherence to endothelium. In addition, the role of the fibrinogen binding receptor of *S. aureus* as an adherence factor to native endothelium is also suggested. (*J. Clin. Invest.* 1991. 87:2236–2245.) Key words: *S. aureus* • adherence • endothelial cells • fibrinogen • staphylococcal adherence

Introduction

Staphylococcus aureus continues to predominate as one of the most frequently encountered pathogens in nosocomial bacteremia (1, 2). In particular, *S. aureus* accounts for one-quarter to one-third of the episodes of bacteremia associated with intravascular devices (3). One intriguing aspect of the pathogenesis of *S. aureus* bacteremic infections in a human host is the unique ability of the organism to adhere to and colonize the endothelium (4) during transient bacteremia (5, 6). In the case of catheter-related bacteremia, the prevailing view is that bacterial colonization starts at the skin-catheter junction, advances down the fibrin-coated external surface of the device (7) and ultimately seeds the bloodstream (2). Once the bacteria are in the bloodstream, patients are at an increased risk of developing endocarditis (8, 9), especially those with underlying valvular heart diseases (congenital or rheumatic causes).

Acute infective endocarditis is a fulminant, life threatening disease of the endocardium frequently caused by *S. aureus*. In distinction to subacute endocarditis that commonly runs an indolent course, acute endocarditis can result in the rapid destruction of the infected valves and a high mortality (10, 11). Furthermore, patients who are at risk of acquiring this infection usually do not have underlying valvular abnormalities.

The propensity of *S. aureus* to cause acute bacteremic endocarditis is poorly understood. Nonetheless, one can speculate that the bacterium must attach to the surface of the endothelium bafore invasion can occur. However, the molecular events that characterize the nonspecific and specific interactions between bacterial surface constituents and the endothelium are poorly defined. These interactions are further complicated by the fact that the endothelium can adsorb blood macromolecules onto its surface through both nonspecific (12) and specific mechanisms (13–15). The adsorbed molecules, in turn, may serve as specific receptors for the adherence of *S. aureus*.

Several factors are known to potentiate interactions between endothelial cells (EC)[1] and the blood constituents that may serve as adhesion molecules for the underlying matrix. In particular, recent work has defined several receptors on the endothelium that are capable of recognizing an RGD (Arg-Gly-Asp) sequence in adhesion macromolecules (13, 14) such as fibrinogen, fibronectin, and vitronectin. Because *S. aureus* is known to have receptors for fibrinogen (16, 17), fibronectin (18–20), and vitronectin (21), it is conceivable that these bound adhesion molecules may serve as receptors for the adherence of microorganisms to endothelium. To investigate this possibility, we examined the role of blood factors in mediating staphylococcal adherence to cultured human umbilical vein EC in vitro. Our results indicate that fibrinogen may act as a bridging molecule in the mediation of *S. aureus* to cultured endothelium. Further, this study also implicates the fibrinogen binding receptor of *S. aureus* as an important adherence factor in the pathogenesis of endovascular invasion during staphylococcal bacteremia.

Methods

Bacterial strains. Two recent *S. aureus* human blood isolates, strain DB and LM, were from the Rockefeller University culture collection.

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1. *Abbreviation used in this paper:* EC, endothelial cell.

Our previous studies (22) have demonstrated the relative deficiency of protein A in strain LM as confirmed by a lack of reactivity of the cell wall antigens of this strain against chicken anti-protein A antibody on immunoblots. Because of this property, strain LM is useful in the examination of the role of blood proteins in mediating protein A–independent adhesion (22). A *S. epidermidis* control, strain 6937, which has been implicated in catheter-related infections was a gift from Dennis Maki at the University of Wisconsin Medical School. All strains were stored at −70°C in chemically defined medium (CDM) (23) with 20% glycerol (vol/vol).

Bacterial growth and harvest. S. aureus cells were grown in liquid CDM while *S. epidermidis* was cultured in CDM supplemented with 10% Todd Hewitt broth. For radiolabeling cells in liquid culture, [methyl-^{3}H] thymidine (sp act ≈ 50 Ci/mmol, New England Nuclear, Boston, MA) was added to the medium to yield 15 μCi/ml. After incubating the bacteria for 22 h at 37°C with slow agitation (80 rpm), cells were harvested by centrifugation (2,000 *g* for 10 min). Bacteria were then washed four times with 10 ml of cold sterile PBS, pH 7.4, resuspended in 5 ml of Medium 199 (M199) (Whittaker Bioproducts, Walkerville, MD), dispersed with a Vortex mixer for 30 s, and filtered through a 5-μm filter (Schleicher & Schuell, Keene, NH) to remove bacterial aggregates. A subsequent gram stain revealed the cell suspension to contain mostly single cells without cluster formation. Cells were then adjusted to an OD of 1.0 at 650 nm with M199 using an 18-mm borosilicate culture tube. CFU corresponding to the OD (≈ 1.5×10^9 CFU/ml for an OD of 1.0 at 650 nm) were obtained by serial tube dilution with subsequent plating on blood agar plates. The usual specific activities were ~ 1,000 cpm/2 × 10^6 for *S. aureus* strains and 1,000 cpm/1.5 × 10^6 for *S. epidermidis*. Preliminary experiments also revealed > 95% of the radioactivity of the cell suspension to be cell-associated.

Endothelial cell tissue culture. EC from human umbilical cords were obtained and cultured as previously described (24, 25). In most studies, EC were grown to confluence on gelatin-coated 96-well microtiter plates (Corning Glass Works, Corning, NY) in the absence of antibiotics in M199 supplemented with 16% FCS, 4% human serum, endothelial cell growth factor, and heparin as described (24, 25). In some studies, monolayers were grown on 20 × 20 mm coverslip or six well tissue culture dishes (Corning Glass Works) in the absence of antibiotics. All experiments reported here were carried out with human EC in the second or third passage.

Assay for adherence to endothelial cells. Unless otherwise stated, all reagents and incubations were at 37°C. To enumerate the number of endothelial cells in each well, two wells from each microtiter plate were treated with 200 μl of 0.05% collagenase, 0.01% EDTA for 5 min followed by counting in a hemacytometer after testing for trypan blue exclusion for cell viability. In preparing cells for the adherence assays, confluent human EC monolayers in microtiter wells were washed once with 200 μl of M199 and then lightly fixed with 0.05% glutaraldehyde in M199 for 5 min followed by two washes with M199 (150 μl each). In addition to removing glutaraldehyde from EC, washes with M199 which contains glycine and other amino acids served to quench the binding capacity of residual glutaraldehyde that may be present on EC. The concentration of glutaraldehyde that was used for fixation has been determined in pilot studies to be the minimal amount necessary. Preliminary studies also revealed that glutaraldehyde-treated EC were able to bind to bacterial cells approaching ≈ 50–75% of nontreated controls while retaining adherent characteristics similar to that of nonfixed endothelium (data not shown). Glutaraldehyde fixed cells have the advantage of minimizing endothelial detachment in the adherence assays during the ensuing lengthy washing procedure. Because of these properties, most of the adherent assays were performed with fixed cells.

For the adherence assays, wells containing glutaraldehyde-fixed EC were preincubated with either 100 μl of M199 or heparinized plasma diluted with an equal volume of M199 (100 U of heparin in 10 ml of whole blood) at 37°C for 30 min with agitation (200 rpm) followed by two washes with 150 μl of M199 each to remove excess proteins in plasma-treated wells. Bacterial cells (≈ 1–2 × 10^8 CFU in 100 μl) were mixed with the monolayers and further incubated for 1 h with agitation. After incubation, the monolayers were washed three times with 200 μl each of M199. With this washing method, residual radioactivities associated with nonadherent cells in each well (e.g., *S. epidermidis* in M199) approached that of background. The EC and adherent bacteria were subsequently lysed with 200 μl of 2.5% SDS and 0.2 M NaOH (lysis buffer) and 100 μl of this lysate was neutralized with 400 μl of 0.05 M acetic acid and counted for radioactivity in the presence of scintillation fluid (Readysafe, Beckman Instruments, Inc., Fullerton, CA). The radioactivities of a known quantity of a standard suspension of bacteria and the samples were counted in a Beckman liquid scintillation spectrometer (model LS 5000TD). The number of adherent bacteria in each well was derived from a standard curve of CFU vs. radioactivity. With most studies, ≈ 1% of the labeled cell inoculum showed nonspecific adherence to the tissue culture wells. Pilot studies also revealed that the presence of lysis buffer did not result in any appreciable degree of quenching.

In preliminary studies, various concentrations (1×10^6—1×10^9 CFU/ml) of *S. aureus* cells, LM and DB, were incubated with endothelial cells in the presence of M199 for 1 h with agitation. Given the available specific activities of the labeled bacteria, 10^8 CFU in 100 μl of M199 generated optimal binding of bacteria to the microtiter wells. On the basis of these studies, all adherence assays were performed at 37°C with agitation with 100 μl of bacterial cells (≈ 10^8 CFU).

Because of the potential problem with staphylococcal adherence to the side wall of the microtiter wells (with or without plasma treatment) as a source of error, several pilot experiments were undertaken to validate the accuracy of our adherence assays. First, radiolabeled bacteria were allowed to interact with clean microtiter wells for 1 h with agitation followed by three washes with M199. Residual radioactivity associated with bacteria adherent to the plastic alone was minimal. Second, experiments were performed in which microtiter wells were preincubated with different volumes of plasma (25–100 μl in 25-μl increments). Our findings indicated that there was at most ≈ 20% reduction in bacterial adherence when the plasma volume was reduced from 100 to 25 μl. In comparison to a daily variation of ≈ 10% in adherence results with similar samples that were performed under identical conditions, the contribution from nonspecific adherence to plasma coated wells appeared small. Third, confluent six-well tissue culture dishes were used to minimize the side-wall/bottom surface area ratio in the adherence assays. By preincubating cells with a plasma volume (≈ 0.8 ml) that minimally covers the monolayer, we were able to demonstrate a significant increase in the adherence of *S. aureus* with plasma-treated wells in comparison to untreated controls. More importantly, the degree of enhancement with plasma is comparable with that seen in microtiter wells with EC. Fourth, there were occasional endothelial cell lines with which preincubation with plasma did not result in an appreciable enhancement in bacterial binding when compared with nontreated controls. Taken all together, these findings would suggest that nonspecific adsorption of *S. aureus* to microtiter wells (with or without plasma factors) was not a major source of error in our adherence assays.

To determine the effect of blood proteins in the mediation of staphylococcal adherence to EC, endothelial monolayers grown to confluence in microtiter plates were washed and fixed as described above. To ensure reproducibility in adherence results, all plasma and serum samples in subsequent experiments came from a single donor. Microtiter wells in quadruplicate were incubated for 30 min with agitation with 100 μl of the following solutions: plasma diluted 1:1; serum diluted 1:1; HSA at 1 mg/ml (Sigma Chemical Co., St. Louis, MO); fibrinogen (Sigma catalog No. F4883) and HSA at 1 mg/ml each; fibronectin (at 250 μg/ml from Boehringer Mannheim Biochemicals, Indianapolis, IN) and HSA 1 mg/ml; or vitronectin (at 30 μg/ml from Calbiochem-Behring Corp., San Diego, CA) and HSA (1 mg/ml). The fibrinogen preparation used in these experiments showed intact α, β, and γ chains on polyacrylamide gel in the presence of SDS and reducing agent (26) without any contamination with fibronectin and vitronectin as determined by both Coomassie blue and silver stain criteria. To evaluate the effect of fibrin degradation products in serum on bacterial adherence,

EC were also preincubated with treated serum devoid of fibrin degradation products. The treated serum was prepared by incubating serum sample with latex particles coated with affinity purified anti-human fibrinogen fragments D and E (Wellcome Diagnostics, Research Triangle, NC) in a 10 × 15-mm column overnight at 4°C with rotation. The absence of fibrin degradation products in the fall through was verified by a dot blot assay in which 25 μl of the treated serum samples together with controls (untreated serum and plasma) were allowed to react with affinity purified goat anti-human fibrinogen antibody (see below for method of preparation) followed by development with substrate (27). In other experiments, the microtiter wells were incubated with 100 μl of various concentrations of fibrinogen (2.5–0.1 mg/ml) in the presence of HSA (1 mg/ml) after fixation with glutaraldehyde. Several blood factors in combination were also tested to evaluate for the promotion of bacterial adherence to glutaraldehyde-fixed EC. After preincubation with the respective blood protein or proteins, the monolayers were washed with M199, incubated with radiolabeled bacteria for 1 h, washed three times with M199, lysed, and counted for radioactivity as previously described.

All data were derived from analysis of samples performed in quadruplicate. Unless otherwise stated, the data presented represent a single experiment with an analysis performed on quadruplicate wells. The results of each study were repeated at least two times to confirm the validity of the data. The number of adherent bacteria was derived from a standard curve of cpm plotted against CFU for each bacterial strains tested. Within each set of data that compared variables, the experiment was performed concurrently from the same batch of bacterial cells grown on the same day. Because of the variability of results from individual EC cell lines, critical experiments with relevance to each other were accomplished with the same cell line from the same passage.

Preparation of affinity purified goat anti-human fibrinogen antibody. Goat anti-human fibrinogen antibody was prepared as previously described (28). Briefly, 10 mg of human fibrinogen (Sigma Chemical Co.) dissolved in PBS was added to 1 g of glutardialdehyde activated beads (Boehringer Mannheim Biochemicals). After incubation overnight at 4°C, the beads were washed sequentially with 1.5% NaCl, 0.3 M ethanolamine, 0.9% NaCl, 0.5 M propionic acid, 0.9% NaCl, and PBS, as described (29). Goat anti-human fibrinogen IgG (50 mg in 3 ml of PBS [Cappel Laboratories, Cochranville, PA]) was adsorbed to the fibrinogen-linked affinity column by rotating the mixture at 4°C overnight (8). After washing the column extensively with PBS, adsorbed antibodies were eluted under acid conditions (pH 2.5), neutralized, and concentrated. The monospecifity of the affinity purified goat anti-human fibrinogen IgG was verified by an immunoblot using purified fibrinogen and plasma as antigens as previously described (28). The protein concentration of the affinity-purified antibody was determined by the BCA protein assay reagent (Pierce Chemical Co., Rockford, IL).

Conjugation of affinity-purified goat anti-human fibrinogen antibody with alkaline phosphatase. Affinity-purified goat anti-human fibrinogen antibody prepared above was conjugated to alkaline phosphatase (Sigma Chemical Co.) as described by Voller et al. (30).

Preparation of afibrinogenemic plasma using affinity chromatography. Affinity-purified goat anti-human fibrinogen antibody (5 mg) was covalently attached to 1 mg of glutardialdehyde-activated beads as described above. 5 ml of heparinized plasma was allowed to rotate with the beads in a column (1.5 × 20 cm) at 4°C overnight followed by the collection of fibrinogen-deficient plasma in the fall through. After acid elution to remove adsorbed fibrinogen on the column (28), the fall through was reapplied to the same column. The procedure was repeated twice to assure complete removal of fibrinogen from the plasma sample. The absence of fibrinogen in the sample was verified by a dot blot assay in which 25 μl of the fall through was applied to a nitrocellulose membrane in a dot blot apparatus (Bio-Rad Laboratories, Inc., Richmond, CA). After blocking the membrane with blocking buffer (Tris 0.01 M with 0.5 M NaCl and 0.5% Tween 20, pH 8.2) for 1 h at RT, alkaline phosphatase conjugate of the affinity-purified goat anti-human fibrinogen antibody diluted 1:1,000 in blocking buffer was incubated with the blot for 1 h followed by development with substrate as described by Blake et al. (27). Dilutions of plasma and purified fibrinogen serve as positive controls, whereas BSA was included as a negative control.

Determination of the fibrinogen concentration in plasma. Fibrinogen concentration in plasma was determined by the modified thrombin time test (diagnostic kit 880, Sigma Chemical Co.) as described in the manufacturer insert.

Studies on the inhibition of staphylococcal adherence to EC with competitive assays. To evaluate the specificity of fibrinogen in mediating staphylococcal adherence to EC, adherence assays were performed with monolayers in microtiter wells that have been preincubated with 100 μl of plasma diluted with an equal volume of M199 followed by two brief rinses with M199. In preliminary studies, the effect of staphylococcal adherence to plasma-treated EC in the presence of fibrinogen-containing media was assessed. Within a range of fibrinogen concentration tested (10 mg–1 μg/ml), bacterial clumping was found to occur and thus the direct influence of fibrinogen in the medium on the inhibition of bacterial adherence to EC cannot be tested. To investigate the effect of antibody in blocking bacterial adherence, the wells containing EC were allowed to incubate with bacterial cells to which 50 μg (in a 25-μl volume) of the following had been added: (*a*) affinity-purified goat anti-human fibrinogen IgG; (*b*) affinity-purified goat anti-mouse IgG (Jackson Immunoresearch, West Grove, PA); (*c*) rabbit polyclonal anti-human vitronectin IgG (Calbiochem-Behring Corp.); (*d*) rabbit polyclonal anti-human fibronectin IgG (Calbiochem-Behring Corp.); or (*e*) mouse MAb IgG_1 directed to M protein of group of A streptococcus. In some assays, a MAb to AG-1 (a gift from J. L. Miller at the State University of New York Health Science Center at Syracuse [31]), a platelet antigen which has been found to be on the surface of EC in high concentration (Jaffe, E. A., unpublished observation), was used in place of goat anti-fibrinogen antibody to further define the specificity of fibrinogen in the mediation of staphylococcal adherence to EC. In other experiments, EC precoated with plasma were incubated with radiolabeled LM or DB cells to which various concentrations of affinity-purified goat anti-human fibrinogen antibody or nonimmune goat IgG had been added in a competitive assay. After a 1-h incubation period, EC were washed, lysed, and counted as described.

Assays of staphylococcal adherence to EC in the presence of afibrinogenemic plasma. To further confirm fibrinogen as an important mediator of staphylococcal adherence, glutaraldehyde-fixed EC were preincubated with 100 μl of either plasma or afibrinogenemic plasma diluted with an equal volume of M199 for 30 min followed by two rinses with M199. In other assays, various concentrations of fibrinogen were added to the afibrinogenemic plasma before preincubation with EC. After these incubations, labeled *S. aureus* cells were added to the monolayers as described. The monolayers were then washed, lysed, and counted.

Detection of fibrinogen on EC by immunofluorescence and ELISA. To verify the presence of fibrinogen on EC, endothelial monolayers were fixed with 0.05% glutaraldehyde for 5 min in six-well tissue culture dishes. The monolayers were then incubated with plasma (1:1 dilution with M199) or M199 for 30 min followed by three washes with M199 with agitation for 5 min each. Fluorescein-conjugated $F(ab)_2$ fragments of goat anti-human fibrinogen IgG (1:5 dilution in M199) (Cappel Laboratories) were then added and allowed to incubate with EC for another 30 min. After four additional washes with M199, the EC were examined by immunofluorescent microscopy as described (32). As a control, EC were incubated with the fluorescein conjugate without prior treatment with plasma.

ELISA was also used to ascertain the presence of fibrinogen on EC after preincubation with plasma. EC in microtiter wells were fixed with 0.05% glutaraldehyde as described. After blocking the EC with ELISA blocking buffer (0.01 M Tris with 0.15 M NaCl and 0.1% BSA, pH 7.4) for 1 h, the monolayers were incubated with 100 μl of plasma in different dilutions at 37°C for 45 min (1:200 to 1:12,800 in fourfold dilutions). Serum at 1:200 dilution or HSA (1 mg/ml) was used as controls. After two washes with M199, the wells were incubated with affinity-purified goat anti-human fibrinogen or goat anti-mouse IgG diluted

1:1,000 in blocking buffer for 3 h at 37°C with shaking. After six washes with blocking buffer, rabbit anti-goat alkaline phosphatase diluted 1:2,000 was added and incubated at room temperature (RT) overnight. The EC cells were again rinsed six times with blocking buffer. Developing substrate (ρ-nitrophenol) was added to the wells and the plate was read at 405_{nm} at timed intervals.

Visual confirmation of bacterial adherence to EC. To visually confirm staphylococcal adherence to EC, endothelial monolayers were grown to confluence in 20 × 20-mm coverslips placed in six-well tissue culture dishes. After fixing the EC with 0.05% glutaraldehyde, the wells were incubated with 1 ml of either M199 or plasma (diluted with an equal volume of M199) for 30 min at RT with agitation followed by two washes with 1.5 ml of M199 each. *S. aureus* cells and the *S. epidermidis* control (1 ml volume) were added and further incubated for 1 h at RT with agitation. In some assays, 500 μg of either affinity-purified goat anti-fibrinogen IgG, goat anti-mouse IgG, or M199 control (in a 250-μl volume) was added to the bacterial cells before incubation with EC. After incubation, the coverslips were washed four times with M199 and gram-stained. In other studies, various concentrations of affinity-purified goat anti-human fibrinogen antibody were added to EC in the presence of bacteria. In these experiments, the number of bacteria per field was enumerated under oil immersion (×1,000) by averaging the number of bacteria counted in 10 visual fields. Correlation of this result with cpm obtained from similar experiments performed in microtiter wells was determined by linear regression analysis (33).

Data and statistics. Adherence data were expressed as the number of CFU bound per endothelial cell±SEM. Student's *t* test was used for comparison of data with appropriate controls when necessary.

Results

Staphylococcal adherence to EC. Two *S. aureus* strains, LM and DB, and a *S. epidermidis* control were used to examine the effect of the time of incubation on bacterial adherence to EC. In the presence of M199 alone, prolonging the time of incubation had no effect on bacterial adherence among the bacterial species for up to 2 h (Fig. 1). In contrast, preincubation of the monolayers in microtiter wells with plasma revealed a significant increase in adherence for both LM and DB, whereas the *S. epidermidis* control was not affected. The adherence of *S. aureus* with plasma-coated EC is time-dependent. The binding data seems to suggest a bimodal or amplified pattern of adherence after 30 min for both staphylococcal strains. However, gram strain studies of bacteria on EC indicated that this amplified pattern of bacterial adherence was not due to bacterial clumping. Continued incubation beyond 1 h revealed that strain DB reached a plateau, whereas strain LM continued to display augmentation in bacterial adherence to EC. Based on these findings, all subsequent assays with microtiter wells were performed for 1 h at 37°C with agitation.

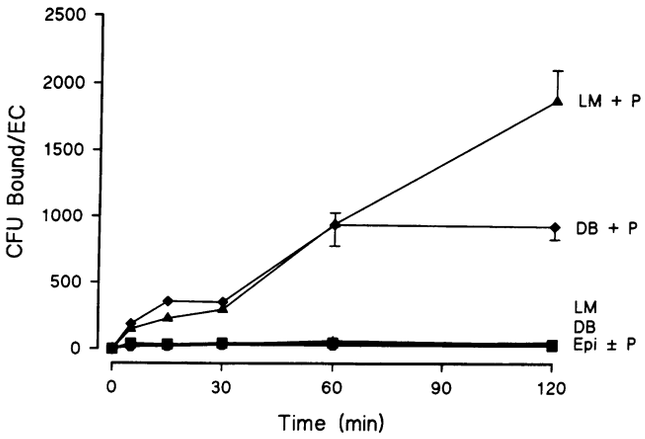

Figure 1. The effect of time of incubation on bacterial adherence to EC. Microtiter wells containing confluent human EC monolayers were precoated with either M199 or diluted plasma (*P*) for 30 min followed by two washes with M199. *S. aureus* strains, LM and DB, or a *S. epidermidis* control (*Epi*) were then incubated with the endothelium for varying periods of time. The number of bacterial cells bound is shown as CFU per endothelial cell (mean±SEM, $n = 4$). In points where SEM bars are not visible, the bars are smaller than the symbols.

The influence of endothelial cell density on staphylococcal adherence to plasma-treated endothelium in microtiter wells was evaluated. The number of endothelial cells used to seed each microtiter well can affect the result of the adherence assays. In particular, an inoculum of 7,500 EC per microtiter well generated optimal bacterial binding. This inoculating cell density would translate to an average cell count of ≈ 30,000–50,000 per well at confluence. It was also determined that preconfluent monolayers generally bound fewer bacterial cells than monolayers at confluence (data not shown).

To further validate the adherence results with radiolabeled bacteria in microtiter wells, experiments were conducted to visually confirm bacterial adherence in a semiquantitative manner. Confluent endothelial monolayers on 20 × 20-mm coverslips fixed with glutaraldehyde were pretreated with plasma followed by incubation with LM cells as described. In some assays, various concentrations of goat anti-fibrinogen antibody were added to EC to block bacterial adherence (see result below). After gram stain, the average number of bacteria per visual field on these coverslips was obtained by enumerating the total number of microorganisms in 10 randomly selected visual fields. Results demonstrated good correlation between the number of bacteria per visual field and cpm of adherent bacteria obtained from the adherence assays in microtiter wells. The correlation coefficient between two variables in a number of experiments ranged from 0.87 to 0.99.

The role of blood proteins in the mediation of adherence of S. aureus to EC. In an effort to identify the important plasma mediator or mediators of staphylococcal adherence to EC, different isolated plasma proteins were used to coat EC before the addition of radiolabeled bacteria. As displayed in Table I *A*, fibrinogen (1 mg/ml) in the presence of HSA, although less effective than that of plasma, resulted in a threefold increase in bacterial adherence in comparison to the M199 control. In contrast, there was only a slight increase in adherence to EC with either fibronectin or vitronectin. Similarly, preincubating EC with HSA alone did not increase bacterial attachment. Results with serum (which contains IgG) differed between the two *S. aureus* strains because adherence with serum was higher with the protein A–containing strain DB than with LM, a protein A–deficient strain (22). The small increase in adherence with serum on DB appeared to be mediated by protein A reacting with adsorbed IgG on EC because the enhancement effect with DB could be inhibited by adding soluble Cowan I protein A (Calbiochem-Behring Corp.) in the assay to compete with the bacteria for available binding sites on adsorbed IgG (data not shown). By comparison, none of these blood protein preparations had any effect on the binding of *S. epidermidis* to EC.

The effect of other blood proteins in conjunction with fibrinogen was also studied. Combinations of fibrinogen with

Table I. Effect of Blood Factors on Staphylococcal Adherence to Endothelial Cells

Test solutions	LM cells	DB cells	*S. epidermidis* cells
	No. CFU bound per endothelial cells		
A			
Medium 199	20±2*	22±1	60±5
Serum (diluted 1:1 with M199)	34±4	72±8	68±4
Plasma (diluted 1:1 with M199)	341±44‡	754±68‡	53±7
HSA (1 mg/ml) in M199	20±2	24±1	48±3
Fibrinogen and HSA (1 mg each) in M199	77±3§	85±9§	40±5
Fibronectin (250 μg/ml) and HSA (1 mg/ml) in M199	25±3	43±3	66±9
Vitronectin (30 μg/ml) and HSA (1 mg/ml) in M199	30±4	27±3	60±3
B			
Medium 199	14±3	28±2	31±2
Plasma (1:1)	195±54	1270±132	44±4
Fibrinogen (1 mg/ml) with serum (1:1) and 2 U heparin in M199	113±8‖	646±50‖	37±2
Fibrinogen (1 mg/ml) with fibronectin (250 μg/ml) and HSA (1 mg/ml) in M199	48±4	264±54	34±6
Fibrinogen (1 mg/ml) with vitronectin (30 μg/ml) and HSA (1 mg/ml) in M199	64±8	132±22	29±3

Confluent human endothelial monolayers in microtiter wells were fixed lightly with 0.05% glutaraldehyde, washed, and then incubated with either blood proteins or M199 (100 μl) at 37°C for 0.5 h with agitation as described. After washing the well twice with M199, tritiated *S. aureus* (strains LM and DB) and *S. epidermidis* cells (≈ 1–2 × 10^8 CFU in 100 μl) were mixed with the monolayer and further incubated for 1 h at 37°C with agitation. After washing the monolayers three times with M199, cells were lysed with 200 μl of 2.5% SDS and 0.2 M NaOH, neutralized with acetic acid, and portions counted for radioactivity. The number of adherent bacteria in each well was derived from a standard curve of CFU vs. radioactivity. *A* and *B* represents separate experiments. * Values are given in number of CFU bound per endothelial cell (mean±SEM, $n = 4$). ‡ The increase in CFU bound per endothelial cell was statistically significant for both staphylococcal strains ($P < 0.005$ by two-tail t test) when compared with medium control. § This value is statistically significant ($P < 0.05$) for both staphylococcal strains in comparison to HSA controls. ‖ The increase in bacterial adherence with fibrinogen and serum factors was significant for both LM and DB strains in comparison to the fibronectin- and vitronectin-treated wells (in the presence of fibrinogen and HSA) in *B*.

fibronectin, vitronectin, or serum were preincubated with EC to determine if they could enhance the binding of *S. aureus* to the endothelium. As shown in Table I *B*, serum together with fibrinogen produced significant increase in the adherence of *S. aureus* to EC while the effect of fibrinogen in conjunction with either fibronectin or vitronectin was less. The augmentation effect of serum with fibrinogen on EC was not due to fibrin degradation products or human IgG found in serum. This was verified by adherence assays in which fibrinogen together with serum that has been treated (see Methods) to remove fibrin degradation products were found to potentiate bacterial adherence to the same degree as that of a control containing untreated serum and fibrinogen (data not shown). Similarly, the addition of polyclonal human IgG at serum concentration to fibrinogen did not enhance staphylococcal adherence when compared to fibrinogen alone (data not shown). When EC were incubated with a combination of fibrinogen, fibronectin, and vitronectin, adherence results did not differ significantly from those obtained when fibrinogen was used in conjunction with either fibronectin or vitronectin.

To evaluate the effect of fibrinogen concentrations on staphylococcal adherence to EC, the level of fibrinogen in the plasma sample (diluted 1:1 with M199) was first determined to be 1.55 mg/ml. Using a range of fibrinogen concentrations (0.1–2.0 mg/ml) diluted in M199, it was found that the number of *S. aureus* cells adhering to EC, although contingent upon the fibrinogen concentration, did not approach that of plasma (diluted 1:1) even at a high concentration of fibrinogen (2 mg/ml) (Fig. 2). At 0.1 mg/ml, the number of adherent bacteria per EC approached that of the M199 control. On the other hand, serum (diluted 1:1) in combination with fibrinogen at 1.5 mg/ml could promote staphylococcal adherence to a level comparable with that of the plasma sample for strain DB, whereas the effect on LM was less. Additional studies with fibrinogen (1.5 mg/ml) and serum heated at 56°C for 0.5 h to inactivate complements yielded similar results (data not shown).

Immunofluorescent microscopy was used to verify the presence of fibrinogen on plasma-treated EC. Bright immunofluorescence of plasma-treated EC was observed, whereas the control without prior plasma treatment exhibited no immunofluorescence (Fig. 3). To further confirm the presence of fibrinogen

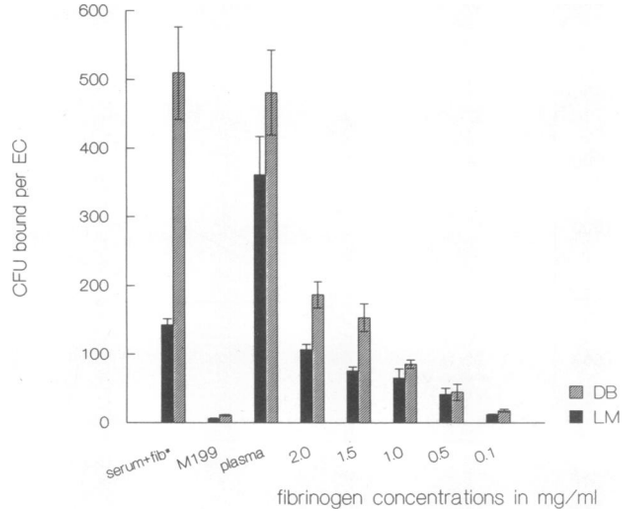

Figure 2. The effect of fibrinogen concentrations on staphylococcal adherence to EC. EC in microtiter wells were preincubated with various concentrations of fibrinogen in the presence of HSA (1 mg/ml) before the addition of radiolabeled LM (*solid bars*) or DB (*hatched bars*) cells. *In some assays, serum diluted with an equal volume of fibrinogen in M199 (final concentration at 1.5 mg/ml) was used in place of fibrinogen to coat the endothelium. The number of adherent bacteria was expressed as CFU bound per EC (mean±SEM, $n = 4$).

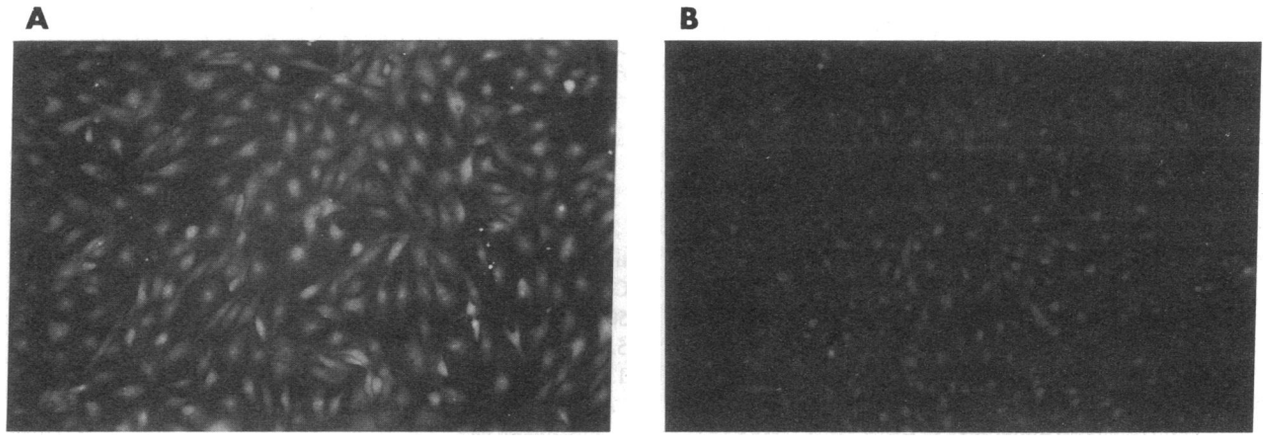

Figure 3. Immunofluorescence of plasma-treated (*A*) or M199-treated (*B*) EC stained with the fluorescein conjugate of the $F(ab)_2$ fragments of goat anti-human fibrinogen antibody. Magnification, 100.

on EC, a modified ELISA was performed using fixed EC as the solid phase support. Results indicated that the amount of fibrinogen adsorbed from plasma to EC was dependent on the dilution of plasma (data not shown). Control wells using serum or HSA to coat the EC and nonimmune goat IgG as the control antibody did not reveal OD readings significantly above background.

Blocking adherence to EC. Inhibition experiments with antibodies were used to demonstrate the specificity of binding of *S. aureus* to adsorbed fibrinogen on EC. EC preincubated with plasma were allowed to incubate with tritiated *S. aureus* or *S. epidermidis* cells in microtiter wells in the presence of various antibodies for 1 h in a competitive assay. Results showed that neither antibodies to human fibronectin and vitronectin nor nonspecific polyclonal and monoclonal antibodies (IgG_1) were able to block the attachment of *S. aureus* to EC (Table II *A*). Likewise, a MAb directed against AG-1, a platelet antigen found on the surface of EC in high concentration, did not block the adherence of *S. aureus* to EC (Table II *B*). In contrast, affinity-purified goat anti-human fibrinogen blocked adher-

Table II. Effect of Various Antibodies on Staphylococcal Adherence to Plasma-treated Endothelial Cells

Presence of blocking agents	LM cells	DB cells	*S. epidermidis* cells
	No. CFU bound per endothelial cell		
A			
None	396±98	1434±106	55±8
Affinity-purified goat anti-fibrinogen IgG (50 μg)	47±3*	320±43*	90±30
Affinity-purified goat anti-mouse IgG (50 μg) (control)	352±57‡	886±154‡	50±7
Polyclonal rabbit anti-human vitronectin antibody (50 μg)	659±77	1090±70	62±2
Polyclonal rabbit anti-human fibronectin antibody (50 μg)	428±37	1716±216	38±4
Nonspecific mouse MAb IgG_1 (40 μg)	304±71	1252±76	51±10
B			
None	306±35		
Affinity-purified goat anti-fibrinogen IgG (20 μg)	73±10*		
Affinity-purified goat anti-mouse IgG (20 μg) (control)	385±32		
MAb anti-AG-1 IgG_1 (10 μg)	428±117§		
Nonspecific mouse Mab IgG_1 (10 μg)	356±54		

Glutaraldehyde-treated EC in microtiter wells were preincubated with 100 μl of plasma diluted with an equal volume of M199 as described. After washing the wells twice with M199, tritiated bacteria were mixed with the monolayer and futher incubated for 1 h at 37°C with agitation. In some assays in *A*, various antibodies (≈ 25 μl in volume) were mixed with bacteria (100 μl) and added to the monolayer whereas M199 was added to the control well before bacterial incubation. In separate experiments (*B*), various antibodies (≈ 10 μl) were mixed with bacteria (40 μl) before they were added to the monolayer for further incubation. A smaller incubation volume was chosen for the latter experiments to conserve the amount of antibody employed while maintaining a reasonable concentration in the assay. After incubation, the monolayers were washed, lysed, and portions counted for radioactivity as described in the text. The amount of adherent bacteria was deduced from a standard curve of CFU vs. radioactive counts. Values are given in number of CFU bound per endothelial cell (mean±SEM, $n = 4$). * The reduction in CFU bound per endothelial cell was statistically significant for the tested strains ($P < 0.04$ in *A* and $P < 0.03$ in *B* by two-tail *t* test) when compared with non-treated controls. ‡ Not statistically significant (*t* test) for both LM and DB strains when compared with nontreated controls. § Not statistically significant for the tested strain when compared with nontreated controls.

ence for both strains of *S. aureus* (Table II, *A* and *B*). The reduction in adherence was 88 and 78% of controls for strains LM and DB, respectively, when 50 μg of anti-fibrinogen antibody was used in the assay (Table II *A*). Similarly, studies with EC grown on coverslips which were gram-stained after bacterial incubation (with or without antibody) also supported these results (Table III). None of the antibodies had an effect on the adherence of *S. epidermidis* to EC (Table II). This was also confirmed by gram-stained studies (Table III). The blocking activity of the anti-fibrinogen antibody was dose dependent (Table IV), whereas control goat IgG had no effect (Tables II and IV).

Bacterial adherence with afibrinogenemic plasma. To further substantiate the role of fibrinogen as an important plasma mediator of staphylococcal adherence to EC, studies were done with fibrinogen-deficient plasma. Afibrinogenemic plasma was prepared from heparinized plasma with an affinity column containing anti-fibrinogen antibody-linked beads. Dot blot showed that the afibrinogenemic plasma displayed no reaction with anti-human fibrinogen antibody, whereas plasma and purified fibrinogen (1 mg/ml) controls were reactive at 1:1,000 dilution. When EC were preincubated with afibrinogenemic plasma (diluted 1:1), bacterial adherence did not differ from that of the M199 control (Fig. 4). However, addition of fibrinogen to afibrinogenemic plasma in the incubation medium was able to restore bacterial adherence to EC to a level approaching that of plasma in a concentration-dependent manner (Fig. 4).

Discussion

It has been estimated that acute endocarditis involves normal heart valves in at least 50% of the cases (10, 11), whereas the subacute form occurs most often in patients with underlying valvular abnormalities. Because of this difference in target tissue involvement, an investigation into the pathogenesis of acute endocarditis should focus on the interaction between the bacteria and undamaged endothelial surface. For this reason, we chose confluent monolayers of human endothelium in tissue culture as an analogue of the host surface encountered in vivo. In addition, the constant and immediate contact of blood with EC warrants examination of bacterial adherence in the presence of blood factors.

Table III. Adherent CFU to EC per Visual Field on Coverslip

Strain	Preincubation medium	Blocking agents	CFU
LM	M199	None	2±0.7
LM	Plasma	None	750±93
LM	Plasma	Goat anti-fibrinogen tibody (50 μg/100 μl)	56±19
LM	Plasma	Goat anti-mouse antibody (50 μg/100 μl)	608±90
S. epidermidis	M199	None	30±6
S. epidermidis	Plasma	None	7±4

Confluent endothelial monolayers on coverslips were pretreated with plasma followed by incubation with LM or *S. epidermidis* cells. In some assays, goat anti-fibrinogen antibody or nonimmune goat IgG was added to the incubation medium. After gram stain, the average number of bacteria per visual field was obtained by enumerating the number of microorganisms in 10 randomly selected visual fields under oil immersion. Values are given in CFU per visual field (mean±SEM, n = 4).

Table IV. Effect of Various Concentrations of Affinity-purified Goat Anti-Human Fibrinogen IgG on Staphylococcal Adherence to Plasma-treated Endothelial Cells

	LM cells	DB cells
	No. CFU bound per endothelial cell	
A. Amount of affinity-purified goat anti-human fibrinogen IgG		
Control	525±39	489±36
50 μg	67±15	136±10
5 μg	96±14	179±4
1 μg	200±18	237±28
B. Amount of affinity-purified goat anti-mouse IgG		
50 μg	399±50	616±36
5 μg	458±80	610±83
1 μg	410±72	638±94

Endothelial monolayers which have been fixed with glutaraldehyde were preincubated with diluted plasma as described. Tritiated staphylococcal cells were added to the microtiter wells to which various concentrations of affinity-purified goat anti-human fibrinogen IgG or nonimmune goat IgG (control) (in a 25-μl volume) have been added in a competitive assay. After a 1-h incubation period, the monolayers were washed with M199, lysed, and portions counted for radioactivity as before. Values are given in number of CFU bound per endothelial cell (mean±SEM, n = 4).

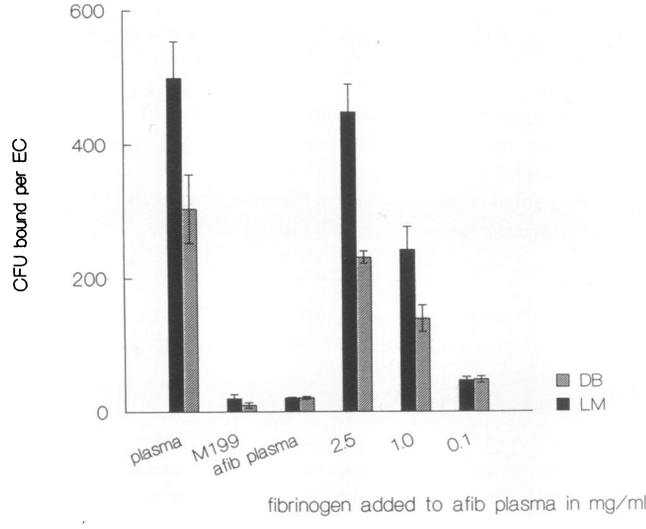

Figure 4. The effect of addition of fibrinogen to afibrinogenemic plasma on the adherence of *S. aureus* to EC. EC were preincubated with either afibrinogenemic plasma or afibrinogenemic plasma plus fibrinogen before the addition of labeled LM (*solid bars*) or DB (*hatched bars*) cells. EC pretreated with plasma were included as a positive control. Values were given as CFU bound per EC (mean±SE, n = 4).

Our study into staphylococcal attachment to cultured EC preincubated with various blood factors demonstrates that the preferential attachment of *S. aureus* to EC appears to be mediated by fibrinogen adsorbed from plasma onto the endothelial surface. In contrast, *S. epidermidis,* an organism generally not known to cause acute intravascular infection on native endothelium did not manifest binding to fibrinogen.

The attachment of *S. aureus* to fibrinogen bound on EC appeared to be specific because it could be blocked by goat anti–human fibrinogen antibody in a dose-dependent fashion, whereas nonimmune goat IgG, nonspecific mouse MAb, and antibodies to human vitronectin and fibronectin were not inhibitory (Tables II *A* and IV). Whether blocking is due to steric hindrance or competitive binding by the antibody to the portion of the fibrinogen molecule where staphylococcus adheres cannot be absolutely determined by these studies. Nevertheless, the fact that a MAb directed against AG-1, a platelet antigen found on EC in abundance, did not block staphylococcal adherence to EC would seem to suggest specific binding between the bacteria and fibrinogen adsorbed onto the endothelial cell receptor (14) while minimizing the role of steric hindrance due to anti–fibrinogen antibody. Although our results provide strong evidence that fibrinogen is an important plasma mediator of staphylococcal adherence to EC, our data also suggested that the presence of fibrinogen is necessary but not the only requirement for the significant promotion of staphylococcal adherence to EC. This was supported by the finding that fibrinogen alone at serum concentration did not potentiate staphylococcal adherence to a degree that was comparable with that of plasma (Fig. 2).

Previous in vitro studies have shown that *S. aureus* is more adherent than enteric gram-negative bacilli to the native endocardial surface (4, 5, 34). However, these studies are largely accomplished in systems where the assays were performed either in the presence of serum or devoid of plasma factors altogether. More recently, several studies have implicated fibronectin and vitronectin in the mediation of adherence of gram-positive bacteria to EC. Immunoelectron microscopic studies by Vann et al. (35) suggests that fibronectin found in areas between *S. aureus* and cultured bovine endothelial cells might be involved in the adherence of *S. aureus,* thus implicating a possible role for fibronectin in endocarditis. Valentin-Weigand et al. have also shown that vitronectin may play a mediatory role in the adherence of streptococci to cultured human endothelial cells (36). In distinction to these studies, our results have minimized the role played by vitronectin and fibronectin, whereas the importance of fibrinogen adsorbed from plasma in the mediation of staphylococcal adherence to EC was emphasized. This discrepancy may have been due to experimental design in that we have used plasma and fibrinogen at a level approaching physiologic concentrations in our adherence assays. In addition, we have also allowed for the competition among various plasma components in their binding to EC before bacterial attachment. Further, our bacterial cells were filtered, rather than sonicated, to disperse bacterial aggregates before the adherence assay. This approach was undertaken to avoid any loss in bacterial fibrinogen binding capacity as a result of sonication (37). An alternative explanation for the discrepancy in results may be due to differences in receptor expression for fibrinogen, vitronectin, and fibronectin between strains used in different studies.

Using a technique based on the pH-dependent affinity of iminobiotin for streptavidin, Tompkins and colleagues (38) have identified and isolated a 50-kD endothelial cell membrane protein that appears to bind to *S. aureus* in vitro. Of note is the finding in their study that preincubation of bacteria with a physiological concentration of fibrinogen in M199 did not appear to interfere with the binding of staphylococci to confluent human endothelial cell monolayers. Because of significant differences in the methodology used for the adherence assay, a direct comparison of the data cannot be made between their studies and ours. For instance, in this study fibrinogen was used to preincubate EC, whereas fibrinogen was allowed to coat the bacteria before incubation with EC in the other study. Nevertheless, preincubation of the endothelium with fibrinogen alone in our study results only in a mild increase in staphylococcal adherence (Table I) when compared with that of plasma. Thus, despite assay differences, the similarity in trend is apparent between the results of these two studies.

Recently, the fibrinogen binding component on *S. aureus* has been isolated and is reported to be a protein complexed with carbohydrate (mol wt 420 kD) (16). However, the molecular size of the uncomplexed form of the protein was not determined. Purification studies by Usui et al. (17) reported the fibrinogen binding component to be proteins with molecular masses of 62, 61, and 59 kD. Similarly, the results of our previous study also confirm the fact that the fibrinogen receptor on the staphylococcal surface is likely to be a protein (28). Its relative resistance to trypsin and sensitivity to proteinase K also suggest that it may exist as a compact structure in which the arginine and lysine residues present in the molecule (16) are not exposed.

Although the fibrinogen binding receptor on *S. aureus,* also called clumping factor, has been suggested as a virulence factor on the basis of more severe infection in clumping factor positive strains (40), the mediatory role of this protein in endoscular invasion by *S. aureus* is not well defined. The current study has provided strong evidence that the mediation of staphylococcal adherence to EC during transient bacteremia (e.g., catheters) probably occurs through a bridging molecule such as fibrinogen that is adsorbed on EC through both specific (14, 39) and nonspecific mechanisms (12, 41). Because adherence is a prerequisite to endovascular invasion, the results of our study strongly implicate the fibrinogen binding receptor of *S. aureus* to be an important mediator of staphylococcal adherence to endothelium. With the knowledge that fibrinogen is an acute phase reactant that is frequently elevated in critically ill patients (42), increased plasma fibrinogen levels may potentially increase the adsorption of this molecule onto the endothelial surface in susceptible patients, thereby allowing more *S. aureus* cells to adhere through the fibrinogen receptor.

In previous studies examining the interaction between fibrinogen and cultured EC (39, 43), it was shown that the association of fibrinogen with EC was generally rapid and reached a plateau within 20 min. The binding affinity of fibrinogen to EC in monolayers was moderate, yielding a value of $\approx 1.5 \times 10^{-6}$ M (40). The minimal fibrinogen concentration required to promote EC binding to a fibrinogen-covered substrate was approximately one-tenth that of plasma (i.e., between 0.2 and 0.4 mg/ml) (44). This is in agreement with our result on the amount of fibrinogen necessary in the preincubation medium to promote staphylococcal attachment to EC (Fig. 2).

In addition to fibrinogen, our data also indicate that other plasma factor(s) might play mediatory roles in the adherence of *S. aureus* to EC. This hypothesis is supported by the observation that preincubation with serum and fibrinogen (Table I *B*) or afibrinogenemic plasma in the presence of fibrinogen (Fig. 4) enhance staphylococcal adherence to a level comparable with that of plasma and that this level of adherence is more than that obtained with fibrinogen alone. Our result would be consistent with a model in which the binding of fibrinogen to EC would increase in the presence of another yet unknown, but possibly activated, plasma factor(s). This model would explain the discrepancy in bacterial adherence between the mild promotional role of fibrinogen alone and the effective blocking of staphylococcal adherence to EC in the presence of anti-human fibrinogen antibody.

The findings in this study may be particularly relevant to acute endocarditis and other intravascular infections in patients who develop transient staphylococcal bacteremia (e.g., from catheters). One would expect in this situation that the endothelium would be "stimulated" before the attachment of the staphylococci. The requirement of a second blood factor, possibly activated during inflammation, for the promotion of staphylococcal adherence to EC via enhanced fibrinogen binding is a hypothesis that is concordant with the results of this investigation. Our findings also reveal that the contributory plasma factor(s) is unlikely to be vitronectin, fibronectin, immunoglobulin, or serum complement (Tables I and II). Whether other blood coagulation factors play a role in the promotion of fibrinogen binding to EC is not determined in these studies. Nevertheless, it is tempting to speculate that fibrinogen binding to endothelium during acute inflammation (e.g., during transient bacteremia) plays a role in the potentiation of endothelial invasion for staphylococci.

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