

Foreign Body Infection: Role of Fibronectin as a Ligand for the Adherence of *Staphylococcus aureus*

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Foreign bodies made of polymethylmethacrylate coverslips were implanted subcutaneously into guinea pigs, were explanted four weeks later, and were tested for in vitro adherence of *Staphylococcus aureus* strain Wood 46. In the presence of serum, the level of staphylococcal adherence to explanted coverslips was 20 times higher than that of adherence to unimplanted coverslips. Adherence to explanted coverslips was caused by fibronectin deposits on the foreign body surface and was inhibited in a dose-related fashion by specific antibodies to fibronectin.

Implantation of prostheses and insertion of catheters into humans are associated with an enhanced risk of bacterial infection in the immediate vicinity of the synthetic foreign material; when such infection develops it compromises the function of the prosthesis or catheter [1-4]. In animal models foreign body infections have been provoked by 10^2 cfu of *Staphylococcus aureus*, whereas even 10^6 bacteria have failed to cause infection in the absence of foreign material [5-7].

Foreign body infections are not cured by antimicrobial treatments that are considered to be effective in the absence of foreign material [1-4]. This observation has led to the assumption that the synthetic material creates a microenvironment that favors bacterial multiplication and reduces accessibility to antibiotics [8]. Ultrastructural studies of the surface of pacemakers and catheters removed from humans have indeed revealed extensive growth of bacterial microcolonies embedded in a thick matrix of polysaccharides [9, 10]; this

matrix is probably of bacterial origin. A number of other studies have documented the immediate formation of a conditioning layer of host proteins on the surface of various synthetic materials exposed to blood proteins [11, 12]. Such protein layers are known to affect the subsequent adsorption of microorganisms on a variety of natural or artificial substrates [13, 14]. Therefore, these protein layers may control bacterial attachment during foreign body infection.

We previously developed an experimental model of foreign body infection [15] by implanting polymethylmethacrylate (MTA, Plexiglass) "tissue cages" sc into guinea pigs and then infecting the animals with 10^3 cfu of *S. aureus* strain Wood 46, which is devoid of protein A [16]. In the present study of bacterial adherence to MTA, we placed MTA coverslips inside tissue cages. Using an in vitro assay for the measurement of adherence of *S. aureus* strain Wood 46 to MTA coverslips, we compared the adhesive properties of explanted coverslips with those of unimplanted coverslips. We found evidence that fibronectin, which is deposited on the coverslips after their sc implantation, is a major determinant of adherence of *S. aureus* strain Wood 46.

Materials and Methods

Implantation of MTA coverslips into guinea pigs. MTA coverslips (1 × 1 cm, ethanol-cleaned) were inserted into rigid MTA tissue cages, which were then sterilized and implanted sc into guinea pigs [15]. After four weeks the tissue cages were explanted under anesthesia [15] and opened at both

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ends for removal of the coverslips, which were rinsed twice in PBS before being used in the adherence assay.

Assay for adherence of *S. aureus* strain Wood 46. A total of 2×10^7 cfu of *S. aureus* strain Wood 46 were incubated with 100 μ Ci of [*methyl*- 3 H]thymidine in 1 ml of Mueller-Hinton broth for 3 hr at 37 C and were grown to a concentration of $1-2 \times 10^8$ cfu/ml. After removal of the unbound radioactivity by centrifugation, the radiolabeled strain was suspended in 1 ml of 0.15 M NaCl (saline). Thereafter, aliquots of *S. aureus* in 1 ml of the selected attachment medium were incubated with either unimplanted or explanted MTA coverslips at 37 C with agitation. At the end of the attachment period, the fluids containing unbound bacteria were drained, and the coverslips were transferred into new tubes containing 1 ml of saline. This transfer procedure minimized the carry-over of fluid contaminated by unbound bacteria. After incubation for 5 min at 20 C, a second wash was performed with 1 ml of fresh saline for 30 min at 20 C. Finally, the coverslips were transferred into new tubes containing 1 ml of PBS and were incubated for another 30 min at 37°C with agitation. This last procedure released <20% of the bacteria from the coverslips. Thereafter, drained slides were immersed into scintillation fluid (Dimilume®; Packard Instruments, Houston), and their radioactivity was counted in a liquid scintillation counter (model no. LS-3145 T; Beckman Instruments, Fullerton, Calif). The cpm values were then multiplied by the cfu/cpm ratio of the radiolabeled culture of *S. aureus* strain Wood 46 (average ratio, 43 ± 14 cfu/cpm; 40 experiments). This procedure allowed the quantification of the bacteria adherent to the coverslips.

Purification of human fibronectin and fibrinogen. Fibronectin was purified from pooled human plasma by affinity chromatography on gelatin-Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) [17]. Plasma proteins that bound nonspecifically to the matrix were first removed with a column packed with unmodified Sepharose [18]. Plasma proteins that were weakly bound to the gelatin-Sepharose were washed off with 1 M urea. Thereafter, fibronectin was eluted with 4 M urea, concentrated by ultrafiltration, and stored as described previously [19]. Purified fibronectin was shown to be free of contaminants by SDS-PAGE [20] (figure 1). The concentration of the purified

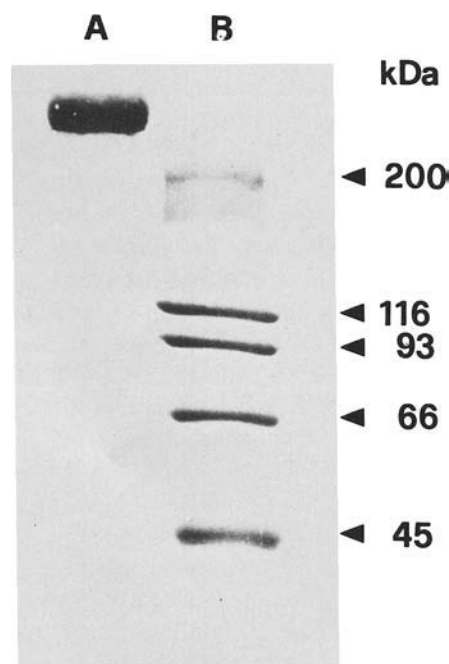


Figure 1. SDS-PAGE of purified fibronectin on a discontinuous slab gel. A gradient gel was made (5%-15% in acrylamide), and the samples were tested under reducing conditions. Lane A shows the results obtained with 4 μ g of purified human fibronectin, and lane B shows those obtained with molecular weight standard (myosin, β -galactosidase, phosphorylase B, bovine serum albumin, ovalbumin).

fibronectin was measured spectrophotometrically by use of an absorbance value of 1.28 at 280 nm [21]. Fibronectin was also measured with the fibronectin turbidimetric immunoassay from Boehringer (Mannheim, Federal Republic of Germany) and was found to be active in a macrophage phagocytic assay [22].

Fibrinogen was prepared from a cryoprecipitate of pooled human plasma, which was extracted twice with a buffer containing 10% ethanol. The unextracted material (containing fibrinogen) was then solubilized in citrate-glycine buffer and freed from particulate material by centrifugation at 2,000 g for 20 min. The supernatant was stored in lyophilized form and then dissolved again in buffer and freed from fibronectin by three passages over a gelatin-Sepharose column [17]. This preparation contained 5 mg of protein/ml, with <2 μ g of fibronectin/ml (as determined with the aforementioned turbidimetric immunoassay).

Preparation of monospecific antibodies to

fibronectin. Rabbit antibodies to human fibronectin were prepared essentially according to Zardi et al. [18]. Antiserum from immunized rabbits was adsorbed onto insolubilized fibronectin-free plasma proteins [18] and was shown to be monospecific by immunoelectrophoresis. The following immunoglobulin fractions were obtained from the monospecific antiserum by ammonium sulfate precipitation: (1) total immunoglobulins (IgG); (2) immunoglobulins depleted of antifibronectin activity, which were designated dIgG and were obtained after selective removal of antibodies to fibronectin by affinity chromatography on immobilized fibronectin; and (3) affinity-purified antibodies to fibronectin, which were designated anti-FN IgG and were obtained by elution of the immunoglobulins previously adsorbed on the fibronectin-Sepharose gel. After weakly binding antibodies had been removed with 3 M KSCN, affinity-purified anti-FN IgG was eluted from the column with 8 M urea.

Modification of S. aureus adherence to coverslips. All adherence assays were performed in Dulbecco's PBS solution supplemented with 1 mM CaCl₂ and 0.5 mM MgSO₄ (D-PBS; Gibco, Biocutt, Glasgow, Scotland). When serum, plasma, or albumin from humans or guinea pigs was included in the attachment-phase medium, the protein concentration was adjusted to 5 mg/ml of D-PBS.

Unimplanted coverslips were treated with protein solutions according to the experimental protocol and were rinsed in PBS before the adherence assay. Unimplanted coverslips were coated with purified fibronectin by incubation for 1 hr at 37 C in a solution containing 125 µg of fibronectin/ml of PBS and subsequent rinsing in PBS.

Some explanted coverslips were trypsinized before the adherence assay by incubation for 20 min at 37 C in 1 ml of a solution containing 10 µg of purified trypsin/ml (Sigma Chemical, St. Louis). Thereafter, soybean trypsin inhibitor (Sigma) was added to a 10-fold excess concentration, and coverslips were finally rinsed in PBS.

In other experiments fibronectin-coated or explanted coverslips were treated before the adherence assay with one of the three immunoglobulin fractions prepared from antiserum to fibronectin. Both types of coverslips were incubated for 30 min at 37 C with solutions of immunoglobulins at various concentrations in PBS supplemented with 5

mg of serum albumin/ml (PBS-HSA). Control coverslips, whether coated with fibronectin or explanted, were incubated in parallel in PBS-HSA in the absence of immunoglobulins. Control and immunoglobulin-treated coverslips were rinsed in PBS before being tested in the adherence assay.

Immunofluorescence microscopy. Coverslips freshly explanted from guinea pigs were rinsed and directly incubated for 30 min at 20 C with 32 µg of anti-FN IgG/ml of PBS-HSA. After extensive rinsing in PBS-HSA, coverslips were mounted on glass slides in Eukitt (Gribi, Bern, Switzerland) and were incubated for a further 30 min with fluorescein-conjugated goat antibody to rabbit IgG (Miles Yeda, Rehovot, Israel) diluted 1:20 in PBS. After extensive rinsing in PBS, coverslips were examined with a microscope (Photomicroscope III®; Carl Zeiss Instruments, New York). Photographs were taken with Plus-X® film (Eastman Kodak, Rochester, NY) that was developed at ASA 400 with Diafine® (Eastman Kodak).

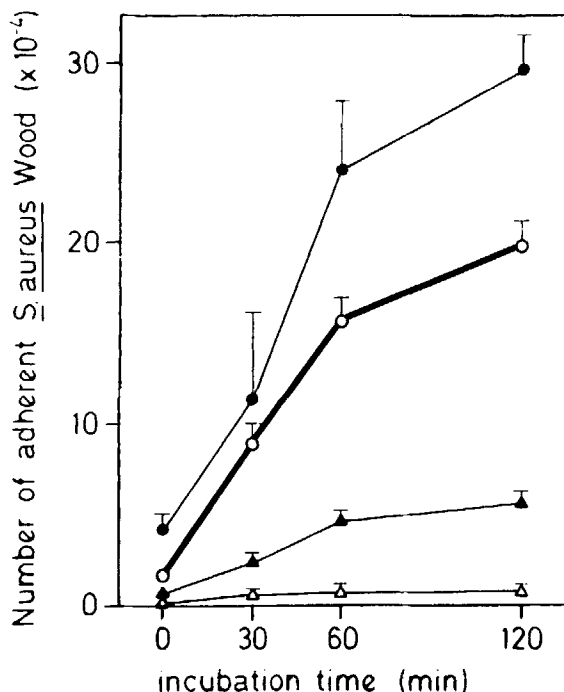


Figure 2. Adherence of *S. aureus* strain Wood 46 to unimplanted MTA coverslips. Concentrations of 10⁵ cfu/ml (Δ), 10⁶ cfu/ml (▲), 4 × 10⁶ cfu/ml (○), and 10⁷ cfu/ml (●) were incubated with coverslips for periods of 30 sec to 120 min in 1 ml of D-PBS (attachment phase), and the adherence assay was performed as described in Materials and Methods. The values shown are means (± SE) obtained in four experiments.

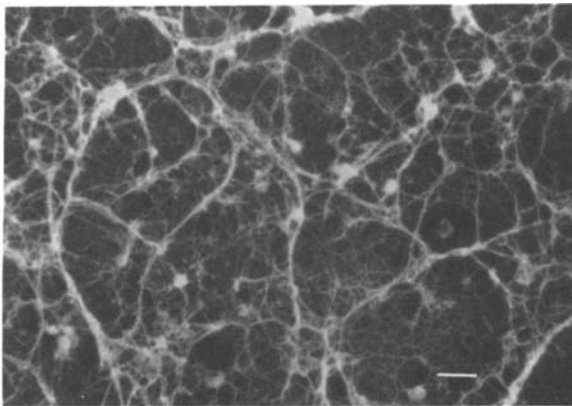


Figure 3. Immunofluorescent staining of explanted MTA coverslips for fibronectin with affinity-purified anti-FN IgG (32 µg/ml). The staining procedure is detailed in Materials and Methods.

Results

Figure 2 shows how various incubation periods and bacterial concentrations influenced the adherence of *S. aureus* strain Wood 46 to unimplanted MTA coverslips in D-PBS medium. On the basis of these data, we selected a single bacterial concentration (4×10^6 cfu/ml) and incubation period (60 min) for use in all further assays involving unimplanted and explanted coverslips. An increase in coverslip area by a factor of two, three, or four resulted in an increase in the adherence of *S. aureus* strain Wood 46 by a factor of 2.09 ± 0.08 , 3.04 ± 0.19 , or 4.45 ± 0.34 , respectively (mean \pm SE; three experiments). All further adherence assays were performed with a single coverslip area (1×1 cm).

The presence of serum proteins at a concentration of 5 mg/ml had a striking effect on the adherence of *S. aureus* strain Wood 46 to unimplanted coverslips. Table 1 shows that $<10^3$ bacteria attached to MTA in the presence of human or guinea pig serum, human plasma, purified human serum albumin, or guinea pig "tissue-cage" fluid [15]. These results represented inhibition of adherence by $>99\%$ in comparison with adherence to unimplanted coverslips in buffer alone. Lower serum or albumin concentrations also inhibited the adherence of *S. aureus* strain Wood 46, with inhibition by 50% at a concentration of 0.02 mg of protein/ml. Preincubation of MTA coverslips with serum, albumin, or tissue-cage fluid at a protein concentration of ≥ 0.05 mg/ml, followed by washing

in D-PBS, inhibited the adherence of *S. aureus* strain Wood 46 by 90%. Thus, virtually no binding sites for *S. aureus* were left on unimplanted MTA coverslips after exposure to serum, tissue-cage fluid, or albumin.

Explanted coverslips showed modified adherence properties with regard to *S. aureus* strain Wood 46 (table 1). In the presence of human albumin or of serum or tissue-cage fluid from guinea pigs, explanted coverslips bound 15–20 times more bacteria than did unimplanted coverslips ($P < .001$). This enhanced adherence to explanted coverslips was reduced by 84% when the coverslips were pretreated with trypsin.

Microscopic examination revealed that explanted coverslips were coated with fibers and cellular materials that stained intensely with 32 µg of anti-FN IgG/ml (figure 3); staining was not observed after incubation of coverslips with dIgG or with preimmune rabbit IgG at concentrations as high as 500 µg/ml (data not shown). In addition, no staining was observed after incubation with purified fluorescein isothiocyanate-labeled antibody

Table 1. Influence of serum, plasma, interstitial "tissue-cage" fluid, purified albumin, and coverslip trypsinization on the adherence of *S. aureus* strain Wood 46 to unimplanted and explanted MTA coverslips.

Treatment of incubation mixture	No. of staphylococci (cfu $\times 10^2$) adherent to indicated type of coverslip*	
	Unimplanted	Explanted
None	1,590 \pm 110 (46)	ND
Human serum	4 \pm 2 (6)	ND
Human plasma	6 \pm 3 (4)	ND
Guinea pig serum	10 \pm 4 (5)	210 \pm 35† (4)
Guinea pig tissue-cage fluid	8 \pm 2 (15)	190 \pm 40† (7)
Human albumin		
Untrypsinized coverslips	9 \pm 2 (16)	155 \pm 70† (7)
Trypsinized coverslips	ND	25 \pm 10‡ (3)

NOTE. Each adherence assay was performed with a single inoculum of 4×10^6 cfu of *S. aureus* strain Wood 46/ml of D-PBS supplemented with the indicated protein sources (protein concentration, 5 mg/ml).

* Data are means \pm SE values, with the number of experiments indicated in parentheses. ND = not done.

† $P < .001$ vs. results obtained with identically treated, unimplanted coverslips (Student's two-tailed *t* test).

‡ $P < .01$ vs. results obtained with albumin-treated, untrypsinized coverslips (Student's two-tailed *t* test).

to fibrinogen at a concentration of 500 $\mu\text{g/ml}$ (Nordic Immunological Laboratory, Tilburg, The Netherlands; data not shown).

To demonstrate that fibronectin deposited on MTA coverslips during *in vivo* exposure was a major factor mediating the adherence of *S. aureus* strain Wood 46 to the coverslips, we tried to block binding sites with specific antibodies to fibronectin. This approach is feasible with *S. aureus* strain Wood 46, which is devoid of protein A and does not attach nonspecifically to IgG by its Fc portion [16]. Inhibition of the adherence of *S. aureus* strain Wood 46 by antibodies to fibronectin was studied first on unimplanted MTA coverslips coated *in vitro* with fibronectin. Recent studies by other investigators [23] and by our group [23a] have shown that either fibronectin-coated tissue-culture wells [23] or fibronectin-coated MTA coverslips can promote the adherence of *S. aureus* in the presence of 5 mg of human albumin/ml of buffer solution. Under our experimental conditions fibronectin-coated coverslips bound $7.72 \times 10^4 \pm 1.58 \times 10^4$ cfu (mean \pm SE; four experiments) of *S. aureus* strain Wood 46 from an initial suspension of 4×10^6 cfu/ml during incubation for 60 min at 37 C. No adherence ($<10^3$ cfu/ml) was observed after either heating of the fibronectin (5 min, 90 C) or trypsinization of the fibronectin-coated coverslips (authors' unpublished observations). When fibronectin-coated coverslips were treated with antibodies to fibronectin before the adherence assay, the adherence of *S. aureus* strain Wood 46 was inhibited in a dose-related fashion (figure 4, graph A). The specificity of this inhibitory effect was shown as follows. First, when antibodies to fibronectin were removed from the immunoglobulin fraction by affinity chromatography on fibronectin-Sepharose, the resulting dIgG did not inhibit the adherence of *S. aureus* (figure 4, graph A). Second, elution of antibody to fibronectin from the fibronectin-Sepharose column led to the recovery of the fraction inhibiting the adherence of *S. aureus* strain Wood 46 (anti-FN IgG), which showed an even higher specific activity (figure 4, graph A.)

When antibodies to fibronectin were added to explanted coverslips, the adherence of *S. aureus* strain Wood 46 was again inhibited in a dose-related manner (figure 4, graph B); the degree of inhibition reached 80% after pretreatment of explanted coverslips with 32 μg of anti-FN IgG/ml. The adherence of *S. aureus* strain Wood 46 to ex-

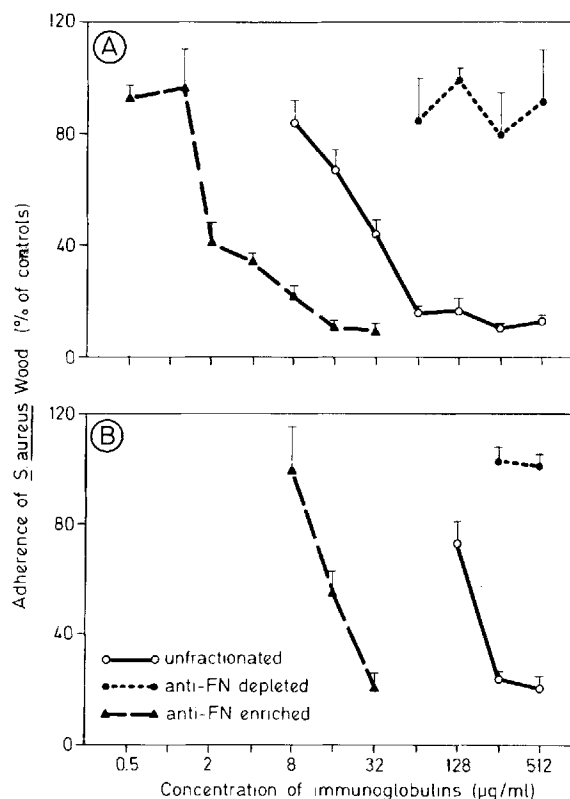


Figure 4. Inhibition of the adherence of *S. aureus* strain Wood 46 to fibronectin-coated, unimplanted MTA coverslips (A) and explanted coverslips (B) by antibodies to fibronectin. Immunoglobulins from sera of rabbits immunized against fibronectin (O), dIgG from the same sera (●), or anti-FN IgG (▲) was applied to the coverslips. After rinsing, the adherence assay was performed as described in Materials and Methods in the presence of 5 mg of human serum albumin/ml, which was added to D-PBS during the attachment phase. The values shown are means (\pm SE) obtained in four experiments.

planted coverslips was not inhibited by dIgG (figure 4, graph B). Thus, the fibronectin deposited on MTA coverslips during their implantation into guinea pigs appeared to be a major factor in the binding of *S. aureus* strain Wood 46 to this foreign material.

To exclude the possibility that traces of antibodies to an antigen other than fibronectin were contributing significantly to the inhibition of bacterial adherence, we did the following control experiments. (1) The preparation of anti-FN IgG was further purified of possible antibodies to fibrinogen in undetected trace amounts by chromatography on immobilized purified fibrinogen showing $<0.05\%$ contamination with fibronectin. After removal of the antibodies to fibrinogen by affinity

chromatography, anti-FN IgG was still inhibitory to *S. aureus* strain Wood 46 on unimplanted, fibronectin-coated coverslips or on explanted coverslips. Furthermore, the potency of anti-FN IgG was not modified after its incubation with immobilized fibrinogen, since the dose-response curves of the antibody's inhibitory effect on bacterial adherence could be superimposed on those shown in figure 4 (data not shown). (2) Unimplanted MTA coverslips coated with purified fibrinogen (from a solution containing 500 µg of protein/ml) were found to bind *S. aureus* strain Wood 46 rather poorly (6×10^3 cfu) in the presence of human albumin in the adherence assay. A similarly low level of adherence was recorded on MTA coverslips precoated with denatured collagen [23a]. (3) Unimplanted MTA coverslips coated with fibrinogen were found to be unaffected by the presence of anti-FN IgG in the adherence assay. (4) The presence of purified fibrinogen (500 µg/ml) in the assay of adherence to fibronectin-coated MTA coverslips was found to have no stimulatory or inhibitory effect on bacterial adherence [23a]. These data made unlikely the contribution of trace amounts of fibrinogen to the process of adherence to fibronectin-coated MTA coverslips.

Discussion

Fibronectin is a multifunctional glycoprotein found either in connective tissues and basement membranes or in a more soluble form in plasma and other body fluids [24, 25]. The binding of fibronectin to *S. aureus* has been described in a number of reports [26–28], although the functional significance of this event is still a matter of controversy [29–31]. Our studies suggest that fibronectin may be an important mediator of bacterial adherence and thus may contribute to the pathogenesis of foreign body infection.

A wide array of blood and tissue proteins may interact with either *S. aureus* [16, 23, 26–34] or implanted synthetic material [11, 12]. Our experimental approach, which involved the use of specific antibodies, allowed us to study the contribution of fibronectin to bacterial adherence under complex conditions that approximated the in vivo situation. Our study was also facilitated by the fact that bacterial adherence to MTA coverslips devoid of adsorbed fibronectin was negligible in albumin-containing media. Vercelloti et al. [23] recently reported similar findings with regard to a different

kind of plastic surface: hydrophilic tissue-culture wells.

In a separate study [23a] we defined the experimental conditions that allowed the adsorption of fibronectin onto MTA coverslips. Such adsorption did not occur on the smooth plastic surface in the presence of human serum proteins, as represented by a fibronectin-depleted pool. In contrast, precoating of MTA with denatured collagen resulted in a significant level of adsorption of fibronectin onto MTA coverslips, even in the presence of serum proteins.

In the present study the choice of *S. aureus* strain Wood 46, which is poorly virulent to humans, was motivated by the lack of protein A on the surface of this organism [16]; this characteristic prevents nonspecific binding to the various immunoglobulin fractions used in this study. The fact that this strain can cause infections when associated with a foreign body has been well documented [15]. As far as we know, this is the first report of inhibition of fibronectin-mediated binding of a microorganism by antibodies to fibronectin. A previous attempt to block the binding of fibronectin and a protein A-positive strain of *S. aureus* with F(ab')₂ fragments of antibodies to fibronectin was unsuccessful [26]. It should be stressed, however, that other fibronectin-mediated functions, such as fibroblast attachment to substrates [35] or fibronectin binding to gelatin [36], can be blocked by antibodies to fibronectin.

At least two types of fibronectin—plasma and cellular [24]—are structurally and functionally similar, although not identical. Monoclonal antibodies have been found that bind preferentially to cellular rather than to plasma fibronectin [24]. MTA coverslips explanted from guinea pigs may contain these different types of fibronectin, and both types may contribute to the adherence of *S. aureus* strain Wood 46, although the relative importance of their contributions remains unknown. Microscopic observation of *S. aureus* strain Wood 46 organisms stained with acridine orange after their attachment to explanted coverslips has shown these organisms to be preferentially located on fibronectin-positive fibers (authors' unpublished observations). That such a fibrillar network may include other macromolecular components (such as collagen and sulfated proteoglycans) codistributed with fibronectin is suggested by other studies of the pericellular matrix of connective tissue [37]. Further studies of the interaction of

S. aureus strain Wood 46 with fibronectin located on the fibrillar network of explanted coverslips will need to include a consideration of the possible contribution of collagen and proteoglycans to fibronectin-mediated attachment. The authors of recent reports [23, 32, 33] have stressed the role of tissue-bound fibronectin as an important ligand of *S. aureus* that promotes the development of various types of staphylococcal infections.

Although in the present report we have identified fibronectin as the surface protein involved in the adherence of *S. aureus* to explanted coverslips, the other component of such interactions—namely, the bacterial adhesin—has yet to be defined for staphylococcal strain Wood 46. We found no difference in bacterial adherence to explanted coverslips when *S. aureus* strain Wood 46 was incubated with liquids varying in their protein composition, such as serum, interstitial fluid, or purified albumin (table 1). Such data do not exclude the possibility that other purified blood fractions interacting mainly with the bacterial surface may modulate the subsequent adherence of *S. aureus* to explanted coverslips. The possible interference of soluble fibronectin with the adherence of *S. aureus* to surface-bound fibronectin was recently considered [23]. Surprisingly, *S. aureus* pretreated with purified soluble fibronectin showed an enhanced ability to adhere to the fibronectin of endothelial cells [23]. These data may be the result of bacterial clumping induced by fibronectin or may reflect other, more complex processes.

In conclusion, our studies have demonstrated that fibronectin is deposited on the surface of a foreign body implanted in an animal. This deposited fibronectin plays an important role in the attachment of *S. aureus* strain Wood 46 to the foreign material. It remains to be established whether our observation is restricted to the type of material studied and the experimental system selected or whether it applies to other types of prosthetic devices, including those widely used in various clinical situations.

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