

# Attachment of Staphylococci and Streptococci on Fibronectin, Fibronectin Fragments, and Fibrinogen Bound to a Solid Phase

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The attachment of *Staphylococcus aureus* (Cowan I) and two strains of group A and G streptococci on glass cover slips coated with fibronectin, fibronectin fragments, or fibrinogen was studied. The attachment was quantitated by counting the attached bacteria on glass surfaces coated with a similar molarity of the proteins. Fibronectin was a more effective attachment factor than fibrinogen for staphylococci, while group G streptococci attached better on fibrinogen- than on fibronectin-coated cover slips. In this system, group A streptococci bound almost exclusively to substrate-bound fibrinogen. Attachment experiments involving the use of staphylococci pretreated with soluble fibronectin or fibrinogen revealed that bacterium-bound fibronectin and fibrinogen were able to enhance the adherence on cover slips coated with fibronectin. The 30-kilodalton NH<sub>2</sub>-terminal and the 120- to 140-kilodalton COOH-terminal fragments of fibronectin, both of which contain bacterial binding sites, mediated the staphylococcal attachment, suggesting that both parts of the molecule are involved in the attachment mediated by fibronectin.

Fibronectin is a high-molecular-weight glycoprotein present in many physiological fluids such as plasma (17), amniotic fluid (2, 13), seminal plasma (35), cerebrospinal fluid (14), and synovial fluid (32). It is composed of two disulfide-bonded 210- to 250-kilodalton (kDa) subunit polypeptides (11, 16, 38) and displays interactions with macromolecules, cells, and bacteria (for reviews, see references 18, 23, and 29). The interactions with bacteria include binding to surface components of *Staphylococcus aureus* and group A, C, and G streptococci (12, 21, 27). The bacterial binding takes place via two binding sites in fibronectin, one located at the NH<sub>2</sub>-terminal end (19, 27) and another located at the COOH-terminal region of the molecule (15).

In addition to fibronectin, staphylococci and group A, C, and G streptococci are able to bind to several soluble plasma proteins such as fibrinogen (9, 22), immunoglobulin (4, 7, 10),  $\beta_2$ -microglobulin (9), and albumin (8). However, this type of binding does not necessarily mean that a protein (although the bacterial surface can interact with soluble molecules) could mediate the attachment of bacteria on the tissues. To study the role of fibronectin and fibrinogen in bacterial attachment we quantitatively investigated the attachment of *S. aureus* and group A and G streptococci on glass cover slips coated with these proteins as well as with purified fragments of fibronectin.

## MATERIALS AND METHODS

**Purified proteins.** Human plasma fibronectin, purified as described previously (3, 36), human fibrinogen (Kabi Diagnostica, Stockholm, Sweden), and bovine serum albumin (BSA; Sigma Chemical Co., St. Louis, Mo.) were diluted in 0.001 M sodium phosphate-0.14 M sodium chloride, pH 7.4 (PBS). Isolation of cathepsin G fragments of fibronectin was performed as described previously (30, 31). For certain experiments fibrinogen was depleted of fibronectin by being passed through the Sepharose-gelatin column.

**Coating of cover slips.** Glass cover slips with ground edges (76 by 26 mm; Menzel-Gläser, Braunschweig, Federal Republic of Germany) were coated with fibronectin and fibrinogen solutions (10  $\mu$ g of PBS per ml) by incubation in a moistened chamber at 22°C overnight. The cover slips were further saturated with 1% BSA in PBS (PBS-BSA) by incubation for 3 h at 37°C. The coatings were made similarly by using the purified NH<sub>2</sub>-terminal 30-kDa fragment, the gelatin-binding 40-kDa fragment, and the COOH-terminal 120- to 140-kDa fragment of fibronectin. For experiments involving the use of bacteria pretreated with fibronectin or fibrinogen, glass cover slips (diameter, 12 mm) were coated with fibronectin solution (10  $\mu$ g/ml) as described above.

**Quantitative coating of glass tubes.** For quantitative coating, 5- to 6- $\mu$ g samples of proteins were iodinated with carrier-free Na<sup>125</sup>I by the chloramine-T method (5). The specific radioactivities for fibronectin and fibrinogen were  $1.5 \times 10^7$  and  $1.8 \times 10^7$  cpm/ $\mu$ g, respectively, and those for the 30-, 40-, and 120- to 140-kDa fragments of fibronectin were  $1.4 \times 10^4$ ,  $8.1 \times 10^5$ , and  $1.4 \times 10^6$  cpm/ $\mu$ g, respectively. The radioactive proteins were mixed with their unlabeled forms to give specific radioactivities ranging from  $2.0 \times 10^3$  to  $1.6 \times 10^6$  cpm/ $\mu$ g, and 0.5-ml solutions containing 0.1 to 30  $\mu$ g of each protein per ml were incubated in glass tubes overnight at 22°C. The supernatant was discarded, and the tubes were washed twice with 1.0 ml of PBS-BSA. The radioactivities associated with the tube walls were counted, and the molar concentrations of bound fibronectin and fibrinogen were calculated by using molecular weights of 420,000 and 340,000, respectively; for the 30-, 40-, and 120- to 140-kDa fragments, molecular weights of 30,000, 40,000 and 130,000, respectively, were used.

**Bacterial strains.** *S. aureus* (strain Cowan I) and two strains of group G streptococci (G-18 and G-148) have been described in more detail elsewhere (21). The two group A streptococcal strains included in the study (7991/81, T-type 12; 5473/82, non-T-typable) were from blood culture samples at the Department of Clinical Microbiology, University Hospital, Lund, Sweden. The capability of each strain to bind various radiolabeled proteins (Table 1) was determined

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TABLE 1. Binding of soluble serum proteins to the bacterial test strains

Species	Strain	% Uptake <sup>a</sup> of:		
		Human fibronectin	Human fibrinogen	BSA
<i>S. aureus</i>	Cowan I	35	12	5
Group A streptococci	7991/81	20	64	7
	5473/82	25	60	6
Group G streptococci	G-18	32	60	8
	G-148	40	6	5

<sup>a</sup> Binding levels are expressed as the percent uptake of 0.1  $\mu$ g of radiolabeled proteins to  $2 \times 10^8$  bacterial organisms.

as previously described (9, 20, 21). Bacteria were stored on sealed blood agar plates and grown for experiments for 16 h in Todd-Hewitt broth, washed with PBS containing 0.02% sodium azide, stabilized by heating to 80°C for 5 min, rewashed, and finally suspended in PBS. The concentrations of bacteria were determined by a microhematocrit analysis, assuming that a 0.5% solution contained  $10^9$  bacteria per ml. Stock suspensions of bacteria were diluted in PBS-BSA for the experiments.

**Attachment assay.** A previously described assay method (34) was used in a slightly modified form. Briefly, cover slips coated with various proteins were placed on the bottom of a plastic vessel (3 by 11 by 2 cm) and overlaid with 9 ml of bacterial suspension ( $1 \times 10^8$  to  $2 \times 10^8$  bacteria per ml) in PBS-BSA. After 2 h of incubation at 22°C, the bacterial suspension was removed and the cover slips were washed three times with 10 ml of PBS-BSA. The cover slips were then air dried, fixed by being warmed gently, and finally Gram stained. The number of attached bacteria was calculated by counting the bacteria in the photographed microscopic picture of the cover slips.

**Time dependence of attachment.** To find the optimal incubation time for attachment, bacterial suspensions were incubated for various periods. After incubation the cover slips were processed as described above.

**Attachment of bacteria pretreated with soluble protein.** Cover slips coated with fibronectin were placed at the bottom of the wells of a tissue culture multiwell plate (Linbro; Flow Laboratories) and overlaid with 0.4 ml of bacterial suspension ( $10^8$  bacteria per ml) which was preincubated for 2 h, at room temperature with various amounts of fibronectin and fibrinogen. After 2 h of incubation the bacterial suspension was removed, the cover slips were washed three times with 1.0 ml of PBS-BSA, and the attached bacteria were quantitated as described above.

## RESULTS

**Quantitative coating of glass tubes.** Glass tubes were coated with radioactive proteins, and the amounts of the bound proteins were calculated as described in Materials and Methods. Figure 1A shows that fibronectin and fibrinogen were approximately equally effective in coating the glass surfaces. A coating concentration of 30  $\mu$ g of each protein per ml produced 9 to 12 pmol of protein attached on the glass. The purified fragments of fibronectin, on the other hand, behaved differently. A 10-pmol coat was achieved at a concentration of 1.5  $\mu$ g/ml for the 30-kDa fragment, 3  $\mu$ g/ml for the 40-kDa fragment, and 10  $\mu$ g/ml for the 120- to 140-kDa fragment (Fig. 1B).

**Time dependence of attachment.** To find the optimal time

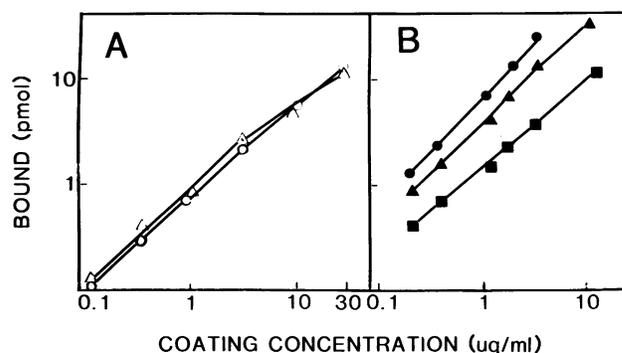


FIG. 1. Quantitative coating of glass surfaces with various proteins (A) and fibronectin fragments (B). Symbols:  $\circ$ , fibronectin;  $\Delta$ , fibrinogen;  $\bullet$ , 30-kDa  $\text{NH}_2$ -terminal fragment;  $\blacktriangle$ , 40-kDa gelatin-binding fragment;  $\blacksquare$ , 120- to 140-kDa COOH-terminal fragment. For experimental details, see Materials and Methods.

for the attachment, cover slips were coated with fibronectin and incubated for various periods with a suspension containing  $2 \times 10^8$  staphylococci per ml. The attachment curve reached its maximum in approximately 1 h (Fig. 2).

**Attachment of bacteria on coated cover slips.** When cover slips were coated with a 10- $\mu$ g/ml solution of fibronectin, fibrinogen, or BSA, staphylococci attached well on those coated with fibronectin and fibrinogen (Fig. 3). Attachment could be observed at bacterial concentration ranging from  $7 \times 10^7$  to  $2 \times 10^8$  bacteria per ml (Fig. 3). Only a low background binding was seen with the negative control protein BSA. The comparative attachment assay performed with the bacterial concentration of  $10^8$ /ml showed that fibronectin was more effective than fibrinogen in binding the *S. aureus* organisms to the glass surface (Table 2). The number of bacteria attached on the fibronectin-coated cover slips was twice that on the fibrinogen-coated surfaces. All four streptococcal strains showed a strong attachment on sur-

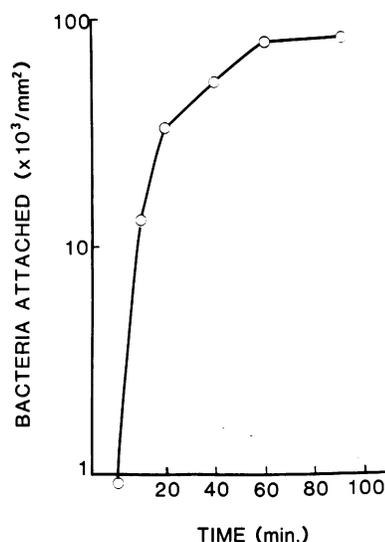


FIG. 2. Time dependence for staphylococcal attachment. Cover slips coated with fibronectin (10  $\mu$ g/ml) were overlaid with a suspension of *S. aureus* cells ( $2 \times 10^8$  bacteria per ml), and the bacterial attachment was recorded after the indicated intervals. Each point is the mean of two determinations.

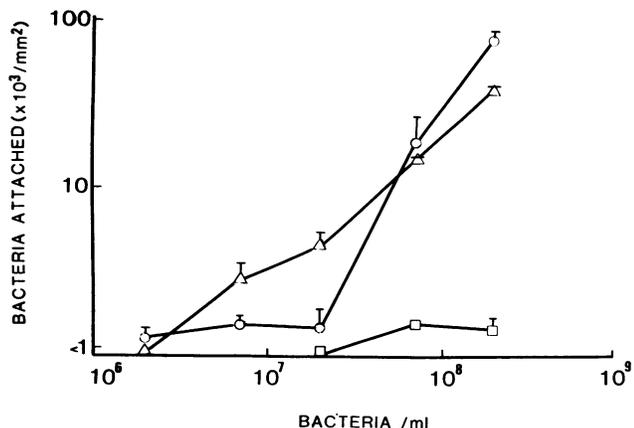


FIG. 3. Attachment of *S. aureus* on cover slips coated with fibronectin (O), fibrinogen (Δ), and BSA (□). Each point represents the mean of two determinations. For experimental details, see Materials and Methods.

faces coated with fibrinogen. The group G streptococcus strain G-148 exhibited a strong fibronectin-mediated attachment, whereas another strain, G-18, showed only a weak binding on fibronectin-coated surfaces (Table 2). Group A streptococci seemed to attach exclusively only on fibrinogen-coated cover slips (Table 2).

**Attachment of bacteria pretreated with soluble proteins.** To study the effect of soluble ligands on the attachment of bacteria, staphylococci were incubated with various amounts of fibronectin and fibrinogen before application of the bacterial suspension to fibronectin-coated cover slips. Fibronectin concentrations ranging from 10 to 100 μg/ml failed to inhibit but rather enhanced the attachment (Fig. 4). Lower fibronectin concentrations had no effect on the binding. A fibrinogen concentration of 100 μg/ml showed a slight inhibition of attachment, while concentrations of 10 to 30 μg/ml slightly enhanced the adherence to fibronectin-coated surfaces (Fig. 4). In some experiments, high fibronectin concentrations caused clumping of bacteria. For counting, however, such areas were selected on cover slips where bacteria existed in unclumped form.

**Attachment of bacteria on cover slips coated with fragments of fibronectin.** To study the role of different domains of fibronectin in the attachment of bacteria, cover slips were coated with purified NH<sub>2</sub>-terminal 30-kDa, gelatin-binding

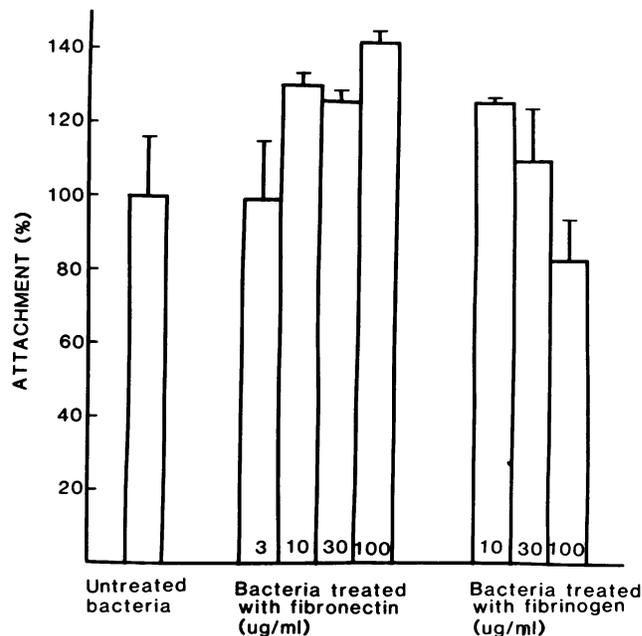


FIG. 4. Attachment of *S. aureus* organisms (10<sup>8</sup> bacteria per ml) pretreated with fibronectin and fibrinogen on fibronectin-coated cover slips. The attachment measured with untreated organisms is shown as 100%. Each bar is the mean of two determinations. For experimental details, see Materials and Methods.

40-kDa, or COOH-terminal 120- to 140-kDa fragments of the molecule. The 30- and 120- to 140-kDa fragments, containing the bacterial binding sites (21), were good mediators of staphylococcal attachment, whereas the 40-kDa gelatin-binding fragment, which lacks bacterial binding sites, was inactive (Fig. 5).

DISCUSSION

Previous studies have clearly shown that *S. aureus* and group A, C, and G streptococci have specific high-affinity

TABLE 2. Attachment of staphylococci and streptococci on cover slips coated with various proteins

Species	Strain	No. of bacteria <sup>a</sup> attached on cover slips coated with:		
		Fibronectin	Fibrinogen	Albumin <sup>b</sup>
<i>S. aureus</i>	Cowan I	142.2 ± 4.2	67.6 ± 6.8	20.0 ± 0.4
Group A streptococci	7991/81	18.9 ± 1.7	85.6 ± 6.3	15.4 ± 5.1
	5473/82	15.8 ± 2.5	80.9 ± 7.3	5.2 ± 0.4
Group G streptococci	G-18	34.9 ± 7.0	110.3 ± 22.1	10.1 ± 1.4
	G-148	81.7 ± 13.1	74.0 ± 8.9	19.2 ± 2.3

<sup>a</sup> Number of attached bacteria expressed as the mean ± standard deviation of three determinations as counted in a photographed microscopic picture.  
<sup>b</sup> Coating with albumin includes coating with plain buffer and saturation with PBS-BSA.

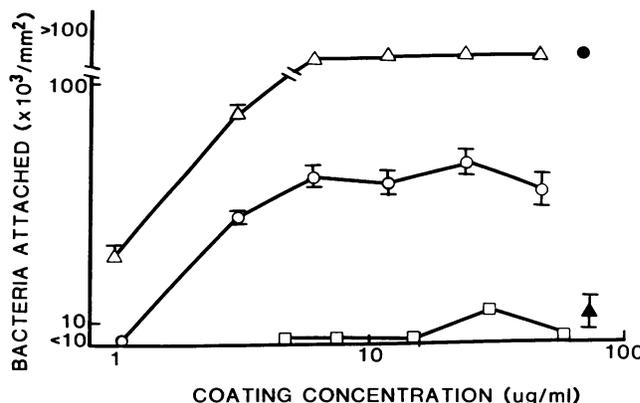


FIG. 5. Attachment of *S. aureus* on glass cover slips coated with the 30-kDa NH<sub>2</sub>-terminal fragment (O), the 40-kDa gelatin-binding fragment (□), and the 120- to 140-kDa COOH-terminal fragment of fibronectin (Δ). The attachment on cover slips coated with intact fibronectin (10 μg/ml) and with BSA (10 μg/ml) is indicated as single symbols (● and ▲, respectively). Each point is the mean of two determinations. For experimental details, see Materials and Methods.

binding sites for soluble fibronectin and fibrinogen. It is, however, not known whether these binding sites exposed on the bacterial cell surface can actually mediate the attachment of bacteria to insoluble protein as found on cell surfaces, extracellular matrices, or blood clots. To explore this possibility a standardized comparative assay system was established. The major merit of this assay is that uniform test conditions can be used, thereby allowing valid comparisons to be made between various proteins and their fragments as well as between defined bacterial strains.

The present results show that both fibronectin and fibrinogen can mediate the attachment of *S. aureus*. For streptococci the situation seems to be different. Although fibrinogen can cause the adherence of both group A and G streptococci, fibronectin in our test system appears to be a weak and variable attachment factor. Interestingly, there was no strict correlation between the adherence data and the capacity of bacteria to bind soluble proteins (Tables 1 and 2). For example, both group A streptococcal strains failed to bind to fibronectin-coated surfaces despite the uptake of the soluble fibronectin. This unexpected observation might be the result of differences in the receptor exposition on the bacterial surface so that the soluble ligand can reach binding sites located too deep to interact with substrate-bound ligand. This finding could also be due to the heat inactivation of organisms used in our experiments. Although the bacterial binding site for fibronectin is shown to be rather resistant to inactivation by heat (21), it may be that some surface modifications induced by heating could lead to diminished adherence of some streptococci. The opposite situation, i.e., positive attachment with only weak background binding of soluble protein, was seen with the group G streptococcus strain G-148 and fibrinogen. The discrepancy can be explained by multipoint attachment mediated by a multitude of low-affinity sites acting together to anchor the organisms to the solid-phase protein. Under our experimental conditions the fibronectin concentration on the cover slips was calculated to be 0.8  $\mu\text{g}/\text{cm}^2$ , indicating that fibronectin is packed on the cover slip as a multilayer (6, 33). This would increase the multipoint attachment of bacteria. The pretreatment of *S. aureus* with soluble fibronectin and fibrinogen enhanced rather than inhibited the attachment to solid-phase fibronectin. This event is most probably due to interactions of purified fibronectin with itself and fibrinogen (24, 37). The lack of inhibition in bacterial attachment by soluble fibronectin is difficult to explain. Grinnell and Feld (6) have proposed that fibronectin on various surfaces may have a different conformation. Further experiments on attachment inhibition indicate that denatured fibronectin is able to inhibit the staphylococcal attachment on fibronectin-coated microwells (Kuusela et al., unpublished results). Vaudaux et al. (34) were able to inhibit staphylococcal attachment on fibronectin-coated polymethylmethacrylate cover slips by using antibodies to fibronectin, although  $\text{F}(\text{ab}')_2$  fragments of fibronectin antibodies had no inhibitory effect on interaction between soluble fibronectin and staphylococci (12).

The clinical significance of the present observations remains to be determined, but it is reasonable to assume that our in vitro data to some extent can be extrapolated to the in vivo situation. Insoluble fibronectin, for example, is found on the surface of many human cell types and is also part of the extracellular matrix (18, 23, 29). It is possible that this cell and tissue component may mediate attachment of invading *S. aureus* organisms and some streptococcal strains. Such a mechanism would help pathogenic bacteria to establish themselves in the tissue and thereby initiate a local

infection. It has been demonstrated recently that the adherence of *Streptococcus pyogenes* on oral epithelial cells is mediated by fibronectin on the cellular surface and that this interaction can be inhibited by soluble fibronectin (25). The finding is in disagreement with our results, which indicate that fibronectin is a poor attachment mediator for group A streptococci. This discrepancy may reflect differences in fibronectin conformations on various cells and surfaces. The role of fibronectin in streptococcal adherence on various human cell lines is indicated by the correlation of surface fibronectin and the number of attached organisms on these cells (26). There is also evidence that the surface fibronectin on buccal cells plays an important role in colonization of the upper respiratory tract by gram-negative bacteria (1, 39).

A similar situation can be envisaged for fibrinogen. Studies conducted with fibrinogen fragments have shown that the bacterial binding site is also part of the fibrin molecule, which is an important component of blood clots as found in surgical wounds. Toy et al. have demonstrated that the staphylococcal adherence on a fibrin clot is better if fibronectin is present in the clot (28). Adherence to fibrin may therefore be an important mechanism for development of surgical wound infections with pyogenic organisms.

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