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Role of fibronectin in adherance of bacteria to a fibrin matrix

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ROLE OF FIBRONECTIN IN ADHERENCE OF BACTERIA TO A FIBRIN MATRIX

by

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THESIS

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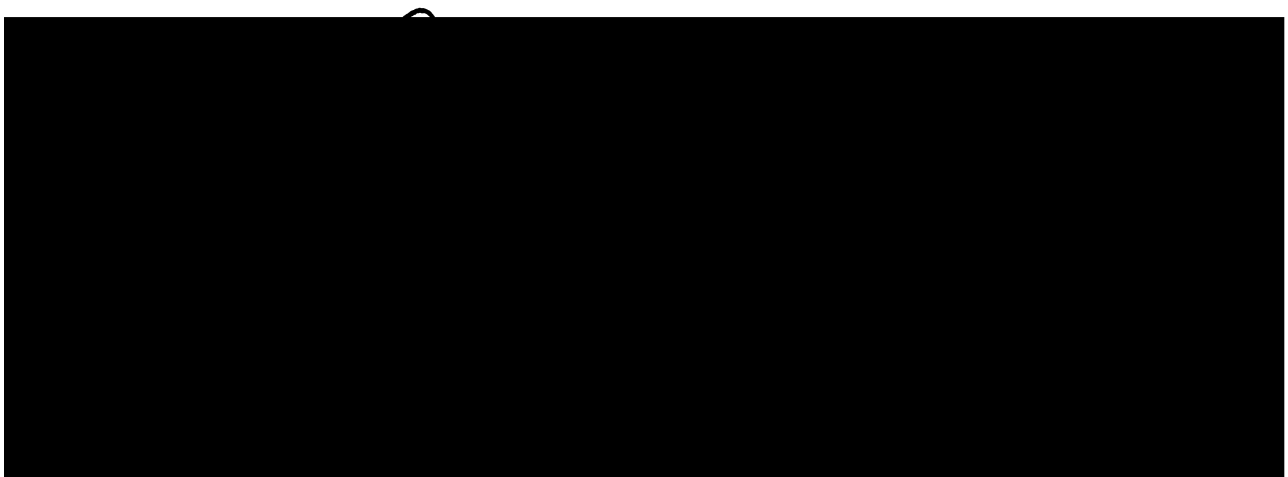

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To my father Dr. Kwan-Long Lai

mother Yong-Shei Lai

&

husband Yeong-Hau Lien

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ABSTRACT

Bacterial adherence to tissue is the initial event that can lead to tissue infection. The adherence of some bacteria to injured tissue may be enhanced by fibrin clots which form immediately following injury. Clinically, Staphylococcus aureus is one of the most common isolates from wound infections. Plasma fibronectin which is present in the fibrin clot is known to interact with S. aureus specifically. We therefore postulate that plasma fibronectin may enhance the adherence of S. aureus to injured tissues.

To test this hypothesis, We established an in vitro assay to measure the adherence of bacteria to fibrin matrices. Fibrin matrices were formed from plasma in the presence and absence of fibronectin to investigate the effect of fibronectin on bacterial binding. We made the following observations:

1) The adherence of S. aureus to fibrin matrix was 100-500 fold that of E. coli and S. epidermidis.
2) On removal of fibronectin from fibrin matrix, the adherence ratio decreased 40-60 % in some strains of S. aureus while the adherence ratio did not change in E. coli and S. epidermidis.
3) The effect of fibronectin on S. aureus adherence to the fibrin matrix had a dose-response relationship.

4) Substances which inhibit the binding between fibronectin and S. aureus also inhibited the adherence of S. aureus to the fibrin matrix.

In conclusion, plasma fibronectin enhances the binding of S. aureus to the fibrin matrix. This effect of fibronectin may play an important role in the pathogenesis of S. aureus infection.

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INTRODUCTION

Bacterial adherence to tissue is the initial event that can lead to tissue infection. The adherence of some bacteria to injured tissue may be enhanced by fibrin clots which form immediately following injury. Clinically, S. aureus is one of the most common species of micro-organisms isolated from wound infections (1). S. aureus is also the leading cause of bacterial endocarditis in patients without cardiac valvular deformity (2). S. aureus endocarditis is believed to be secondary to infection of injury-induced thrombi on cardiac valves (3). The high affinity of S. aureus to the fibrin clot may be important in the pathogenesis of S. aureus infections.

Recently, S. aureus has been shown to bind specifically to fibronectin (4,5). Fibronectin is a large glycoprotein found in a soluble form in plasma and various body fluids, and in an insoluble form in the extracellular matrix (6-10). Fibronectin has been implicated in a wide variety of cellular properties including cell adhesion, migration, differentiation, phagocytosis and hemostasis (6-10). Corresponding to its multi-functional characteristics, fibronectin has been shown to have multiple

binding sites. It has been shown to bind cells, bacteria, fibrin, factor XIII, collagens, glycosaminoglycans, actin, and DNA (6-10). Since fibronectin interacts with both S. aureus and fibrin, we postulate that fibronectin which is present in fibrin thrombi may enhance the adherence of S. aureus to injured sites.

To test this hypothesis, we have established an in vitro assay to measure the adherence of bacteria to fibrin matrices. Fibrin matrices were formed from plasma with or without fibronectin to investigate the effect of fibronectin on bacterial binding. Several inhibitors which are known to inhibit S. aureus-fibronectin binding (4,11) were tested. Finally, the morphology of bacteria-fibrin interaction was studied with scanning electronmicroscopy.

BACKGROUND

Properties of fibronectin

There are at least two types of fibronectin, plasma and cellular fibronectin. Plasma fibronectin exists as a soluble protein. The range of fibronectin concentration in human plasma is 180-720 ug/ml in males and 150-540 ug /ml in females (6). The major source of plasma fibronectin appears to be hepatocytes (12,53), although endothelial cells (13-15) and macrophages (16-18) may also contribute. Cellular fibronectin appears as a fibrillar form on cell surfaces and in the extracellular matrix (19,20). A wide variety of cells synthesize fibronectin in vitro. Fibroblasts and endothelial cells are major producers, but many other cell types, including some epithelial cells, synthesize fibronectin at lower levels (21). Cellular and plasma fibronectins, although distinguishable with monoclonal antibodies (22), are very similar in structure (23). The fibronectin molecule is asymmetric and consists of two similar subunits of molecular weight 220,000 $\pm$ 20,000 daltons held together by disulfide-bonds near their carboxyl termini (24,25). Fibronectin is a glycoprotein containing 5-9% asparagine-linked complex oligosaccharides (24). Biophysical measurements indicate that, although the

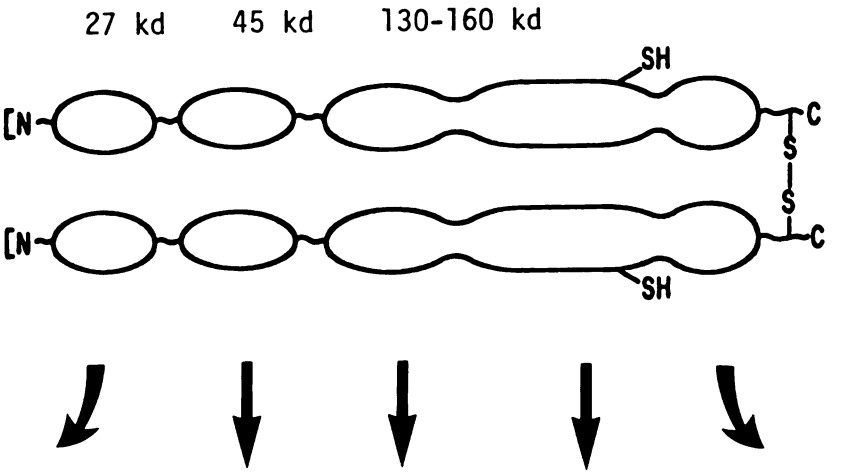
molecule as a whole is flexible, it contains compact globular domains (26,27).

Functional domains of fibronectin

Spectrophotometric and ultracentrifugation experiments indicate that fibronectin is an elongated molecule composed of structured domains separated by flexible, extendable regions of polypeptide chains (26,28). The flexible peptide regions are particularly susceptible to attack by a variety of proteases. Under appropriate conditions, the enzymes cleave fibronectin into protease-resistant domains which then can be isolated and characterized according to their affinity to various ligands. Fig. 1 shows the current model based on these results (9).

In brief, the 27kd amino-terminal domain (domain I) mediates binding to fibrin, bacteria, factor $XIII_a$, heparin, and actin. This domain is the substrate for factor $XIII_a$ transglutaminase which mediates the cross-linking of a glutamine residue of fibronectin to a lysine residue of fibrin (29,30), collagen (31-33), and a bacterial membrane component (5). The 45kd domain (domain II) near the amino-terminus is a collagen-binding domain. The remainder of fibronectin is less well-analyzed, but appears to contain regions that are relatively sensitive

to proteolysis and other less sensitive regions. A 15kd domain (a part of domain IV) located in the center of fibronectin mediates cell binding activity (34). The domain near the carboxyl terminus (domain IV) is the second binding site of heparin (30,35); the carboxyl-terminal domain (domain V) is the second binding site of fibrin (29,35,37).



I	II	III	IV	V
Fibrin I	Gelatin	?	Cell	Fibrin II
Bacteria	Collagen		Heparin II	
$XIII_a$				
Heparin I				
Actin				

Fig. 1. A current model for the molecular structure of fibronectin. The molecule is composed of elongated subunits linked by disulfide bonds near the carboxyl termini. Some regions are extremely sensitive to proteolysis and are depicted as extended chains (thin lines). Other regions are compact and globular and contain specific binding sites for other molecules. The locations of the different binding sites on fibronectin are indicated. The estimated molecular weight of each domain is also indicated (9).

Interactions of fibronectin with fibrin and factor XIIIa

Plasma fibronectin has been shown to be incorporated into the fibrin clot. The serum concentration of fibronectin is 20-50% lower than the plasma concentration (38). Grinnell and Feld, using an immunofluorescence technique on blood clots prepared on a plastic surface, have demonstrated that fibrin matrix is covered with a uniform coating of fibronectin (39). In wound tissues, fibronectin is found to be incorporated into fibrin meshwork, as well as absorbed onto the surface of fibrin thrombi (40).

Fibronectin binds to fibrin by either non-covalent binding or covalent cross-linking. The non-covalent binding occurs at the carboxyl-terminal domain of fibronectin, while the covalent cross-linking occurs at the 27kd amino-terminal domain (29,30,36,37). The cross-linking of fibronectin to the fibrin-chain is via an ε-(γ-glutamyl) lysine linkage, mediated by factor $XIII_a$ (42). At 22-37·C, only covalent cross-linking occurs, while at 0-4·C, both covalent cross-linking and non-covalent binding occur (41,42). This explains why serum fibronectin concentration is lower when clotting is carried out at 0-4·C than at 22-37·C. The components of a fibrin clot are 94.9 percent fibrin and 4.4 percent fibronectin, assuming that the concentration of

fibronectin is 320 ug/ml, of which 35% is incorporated into clot; the concentration of fibrinogen is 2400 ug/ml, of which 100% is incorporated (6).

Interaction of fibronectin with bacteria

The interaction of fibronectin with bacteria is reviewed in two aspects: A. the binding of fibronectin to bacteria; B. the role of fibronectin as an opsonin in the clearance of bacteria.

A. Fibronectin-bacteria binding

The binding of fibronectin to microorganisms was first described by Kuusela (4). When normal human plasma is incubated with *S. aureus* or *Mycobacterium butyricum*, the fibronectin content is enriched in the eluates of *S. aureus* but not in the eluates of *M. butyricum*. Subsequent studies demonstrated that fibronectin binds also to streptococci group A, C and G (43). The binding ability of various bacteria to fibronectin is summarized in Table 1.

The binding of fibronectin to *S. aureus* has been studied in detail. Proctor *et al* have demonstrated that when *S. aureus* suspension is incubated with purified plasma ^{125}I-labeled fibronectin, the binding is

Table 1 Ability of different bacterial species to bind to human fibronectin

Bacteria	Ability to bind fibronectin	References
Staphylococcus aureus	Yes	51,58,4,5,43
Staph. epidermidis	Yes	58
	No	51,43
Staph. saprophyticus	No	43
Streptococci		
Group A, C, G	Yes	59,60,43
Group B, D, M, N, P, U	No	43
Strep. uberis	No	43
Strep. pneumoniae	No	43
Neisseria meningitis	No	43
Neisseria gonorrhoeae	No	43
Haemophilus influenzae	No	43
Escherichia coli	No	57,43
Candida albicans	No	43
Mycobacterium butyricum	No	4
Pseudomonas aeruginosa	No	43

saturable, irreversible and non-covalent (44). The binding site of fibronectin to S. aureus is located on the 27kd amino-terminal domain which can be cross-linked to S. aureus by factor $XIII_a$ (5). However, the nature of the binding site of S. aureus to fibronectin remains unclear. S. aureus binds to plasma proteins other than fibronectin. It binds to IgG through protein A (45) and to fibrinogen through fibrinogen clumping factor (46-48). Since IgG and fibrinogen do not interfere with fibronectin binding to S. aureus, the fibronectin binding site may be distinct from that of protein A and fibrinogen clumping factor (4,44,49). This concept is further confirmed by studies using mutants of S. aureus which have protein A or fibrinogen clumping factor but cannot bind to fibronectin (49,50,58). Recently, the fibronectin binding site on S. aureus has been further characterized by Ryden et al (49).

Fibronectin-nonbinding mutants of S. aureus have been isolated. After external labeling with $Na^{125}I$, the bacteria have been solubilized and the lysate passed through a fibronectin-Sepharose affinity column and eluted with 5% SDS. The eluate has been subjected to SDS polyacrylamide gel electrophoresis. On the gel, one band with a molecular weight of 18,000 daltons is absent in the eluate of the fibronectin-nonbinding mutant in comparison with the fibronectin-binding strain. This component is tentatively identified as a fibronectin

receptor. However, further purification and kinetics studies are required to confirm this observation. Since fibronectin is able to serve as the bridge between bacteria and host tissue, the binding between bacteria and fibronectin may affect the pathogenesis of bacterial infections. The binding sites of fibronectin on clinical isolates and laboratory strains of S. aureus have been studied. Although the number of strains studied is small, more invasive clinical strains, such as those isolated from patients with prosthetic valve endocarditis, have more binding sites for fibronectin than strains isolated from healthy skin (44). This observation suggests that fibronectin may play an important role in S. aureus adherence and invasiveness.

B. Fibronectin as an opsonin

Fibronectin has been shown to promote the phagocytosis of gelatin-conjugated latex by rat peritoneal macrophages (51,52) and cultured macrophage-like cells (53). Clinically, fibronectin replacement has been reported to have a favorable effect in septic patients (54,55). Since fibronectin binds to certain strains of bacteria, whether it functions as an opsonin in clearance of bacteria has aroused

considerable interest. In 1981, Lanser and Saba (56) reported that complete serum is 1.5 to 2 times as effective as fibronectin-depleted serum in functioning as opsonin. However, more recently, Van De Water *et al* reexamined the same system using various bacteria-cell pairs including *S. aureus*-neutrophils which was used by Lanser and Saba. They find that neither plasma nor cellular fibronectin promote the uptake of bacteria in any combination they tested (11). Similar results have been reported by other groups (57,58). Taken together, these results suggest that if fibronectin plays a role in the opsonization of *S. aureus*, its role is minor.

MATERIALS AND METHODS

Purification of fibronectin

A. Starting material

Acid citrate dextrose anticoagulated human plasma was used as starting material for isolating fibronectin.

B. Affinity chromatography with gelatin-Sepharose 4B

Plasma fibronectin was purified by a gelatin affinity column according to a procedure described by Ruoslahti et al (61). Gelatin Sepharose-4B resin (Pharmacia) in 0.15M NaCl and 0.02% sodium azide was packed into a 1.5x10 cm column (Bio-Rad). The column was washed with 10 column volumes of 0.15 M NaCl and equilibrated with the same solution at room temperature. Human plasma was freed of particulate material by centrifugation at 3,000 xg for 10 minutes. Two column volumes (30ml) of plasma were applied to the column. The unbound material was washed with 0.15, 1.0 and 0.15M NaCl, 6 column volumes each, in sequence. The bound material was eluted with 4M urea and 0.05M Tris HCl at pH 7.5, followed by 8M urea in the same buffer. Elution rate was 1ml/minute and 3.4ml fractions were collected with the aid of a fraction collector. Fractions were tested for protein content

by measuring absorbance at 280nm. The protein-rich fractions were pooled, sterilized through a 0.45u Falcon 7102 filter and concentrated 15-20 fold with ultrafilter 30X (Millipore).

Identification of fibronectin by SDS polyacrylamide gel electrophoresis

Discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed according to Laemmli (62). Sample buffer containing 2% SDS, 10% glycerol, 0.001% bromophenol blue and 0.05M Tris was added to concentrated fibronectin (2-10mg/ml) obtained from the purification procedure. Reduction of disulfides, where indicated, was achieved by treating proteins with 2% 2-mercaptoethanol (v/v) in sample buffer (100·C, 3 minutes). Molecular weights were estimated by using the following proteins as standards: skeletal muscle myosin, 200,000; B-galactosidase, 116,000; phosphorylase B, 92,500; bovine serum albumin, 66,200 and ovalumin, 45,000 daltons (obtained from Bio-rad, Richmond, CA.).

Slab gels with 5% polyacrylamide were prepared. Separating gels (1.5mmx140mmx177mm) were 5% acrylamide, 0.19% N, N-methylene-bis-acrylamide (Bio-rad, Richmond, CA.) and 0.05% SDS in 0.375M Tris-HCl buffer pH 8.8. To

this solution, 0.3mg of ammonium persulfate per ml was added and degassed for 20 minutes and then mixed with 2ul of N, N, N, N-tetramethylethylenediamine (TEMED) (Bio-rad, Richmond, CA.) per ml of gel solution. The solution was poured into space sandwiched by two glass plates on a Hoefer SE 500 electrophoresis cell. The gel was overlaid with 1% SDS in distilled water and allowed to polymerize at room temperature overnight. On the next day, 3.5% stacking gels were prepared in the same way and allowed to polymerize for 30 minutes. Samples were applied to the gel in a volume of 50-100 ul. The gels were electrophoresed in 0.025M Tris, 0.192M glycine, 0.05% SDS pH 8.3 at a constant current of 12.5 mA per gel for 4 hours. The protein bands were visualized with Coomassie brilliant blue staining.

Quantification of fibronectin by Immunoturbidimetric assay

Fibronectin concentrations were determined by an immunoturbidimetric assay (Boehringer-Mannheim, IN) as described by Saba et al (63). Briefly, 10ul of sample solution was added to 0.7ml of diluted rabbit anti-human-fibronectin antiserum in a 1cm light path cuvette. After mixing thoroughly, absorbance at 365nm was read at 1 and 10 minutes. The observed absorbance difference was obtained by substracting the one-minute

reading from the ten-minute reading. The standard concentrations of fibronectin included 125, 250, 750 and 1000ug/ml. The calibration curve was obtained by plotting the observed absorbance differences against the standard concentrations. The sample concentrations were calculated from the calibration curve by their observed absorbance differences.

Bacterial sources

Bacteria were obtained from the following sources: S. aureus ATCC 29213, E. coli ATCC 25922 and S. epidermidis ATCC 27626 from the American Type Culture Collection (Rockville, MD.) and the rest, isolated from patients' specimens, provided by Dr. M.A. Sande (San Francisco General Hospital, San Francisco, CA.). S. aureus strains 76, 106, and E. coli strains 139, 140, 198 were isolated from blood; S. aureus 68 from skin graft; S. aureus 69 from sputum; S. aureus 66 from peritoneal fluid; S. aureus 70 from urine specimens. Bacterial strains were stored at -70•C suspended in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD.).

Bacterial growth

Just before use, bacteria were grown in unbuffered trypticase soy broth and harvested in log phase (at 12-16 hours). Bacteria were collected by centrifugation at 3000xg for 20 minutes. Bacterial pellets were washed twice with o.15 M NaCl and resuspended in 0.15 M NaCl buffered with 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanosulfonic acid (HEPES) pH 7.4. Clumped bacteria were dispersed by a high speed Vortex mixer. Wet mounts of the bacterial suspensions were examined microscopically to comfirm that the suspensions contained primarily single bacterial cells. The concentrations of bacteria were adjusted spectrophotometrically to an absorbance at 595nm from 0.3 to 0.4 of optical density units per ml. The bacteria suspensions were further diluted to $2\text{-}8\text{x}10^3$ colony forming units (CFU) per 5 ml (10^4 dilution) in the same buffer. The exact final concentration was confirmed by colony counting.

Preparation of fibrin matrix

Fibrin matrices were prepared with the method modified from the procedure described by Scheld et al (64). Fibronectin depleted plasma was obtained from the fibronectin purification procedure. As indicated,

various amounts of fibronectin were added back to the fibronectin depleted plasma. Original plasma or fibronectin depleted or repleted plasma in the volume of 1 ml was mixed with 0.8ml of clotting reagent in a 60mm Petri dish (Falcon 1007 plactics, Oxnard, CA.). The clotting reagent contained 2.5 units/ml bovine thrombin (Parke-Davis, Morris Plans, NJ.) and 0.125M $CaCl_2$ in 0.15M NaCl. After mixing, the plasma was allowed to clot for a few minutes at room temperature. The fibrin matrix was then incubated at 37°C for 30 minutes, washed three times with 5 ml of 0.15M NaCl , and was then ready for the bacteria adhesion assay. It should be noted that the experimental surface used in this system was termed "FIBRIN MATRIX" because it contained at least 95% fibrin. In whole plasma derived fibrin matrix, the components of a fibrin matrix are 95% fibrin and 4.5% fibronectin.

Bacterial adhesion assay

The bacterial inoculum in a volume of 5 ml in HEPES buffer was poured onto the fibrin matrix and agitated on a rotatory shaker at the speed of 120 cycles per minute in a warm room at 37°C for 15 minutes. The unbound bacteria were removed and washed off with 0.15M NaCl three times. All the procedures were done aseptically. After washing, the fibrin matrix was overlaid with trypticase soy agar and incubated for 24 hours at 37°C.

The number of adherent organisms was determined by colony counting. The adherence ratio was defined as the number of organisms which adhered to the fibrin matrix (CFU) divided by the number of organisms in the initial inoculum (CFU). Results were expressed as mean ± standard deviation. Comparisons were made only with concurrent sets of experiments and were analyzed by the unpaired Student's t-test.

Inhibition tests

Inhibition of adherence was tested by supplementing the bacterial suspensions with various substances: L-lysine (0.2M), L-arginine (0.2M) D-glucuronic acid lactone (0.2M) and heparin (0.2mg/ml, 169 USP/mg). All these substances were purchased from Sigma, St. Louis, MO. The reactions were performed at 37·C for one hour. Inhibition percentages were calculated by the following formula:

$$\frac{\text{(adherence ratio without inhibitor-adherence ratio with inhibitor)}}{\text{adherence ratio without inhibitor}} \times 100$$

Scanning electronmicroscopy

Glass coverslips, 12 mm in diameter (Carolina Biological Supply, Gladstone, OR.), were placed onto Petri dishes and the fibrin matrix was prepared as described previously. The bacterial suspension was added to the Petri dish. The unbound bacteria were washed off with 0.15M NaCl three times. The sample was then fixed by immersion in a fixation solution of 2% glutaraldehyde (Polysciences, Warrington, PA.) in 0.15M cacodylate buffer pH 7.2 at room temperature for one hour. After fixation, the coverslips were carefully removed with the help of fine forceps from the Petri dishes and rinsed with normal saline. Dehydration was accomplished in ascending concentrations of ethanol: 20%, 40%, 60%, 80%, 95%, 100%, and 100%. Each dehydration step was 5 minutes. The specimens were dried by the method of critical point drying in liquid CO_2. The coverslips were then mounted on stubs using silver paste and allowed to dry thoroughly in a 40°C oven for at least one hour. The specimens were coated with a layer of gold palladium, 200 Å in thickness, and examined with a Cambridge S-150 scanning electron microscope.

RESULTS

Purification of fibronectin

Fibronectin was purified from outdated human plasma by gelatin-Sepharose 4B affinity chromatography at room temperature. Complete elution was achieved with 4M urea in 50mM Tris-HCl pH 7.5 (Fig.2). Subsequent elution with 8M urea did not cause further elution of protein. The purity of the fibronectin preparation was assessed by SDS polyacrylamide gel electrophoresis (Fig. 3). Without reduction, a single band was present near the top of the separating gel. After treatment with 2-mercaptoethanol as a reducing agent, only one band was present with an apparent molecular weight of 220kd. These patterns were comparable to the previous observations using pure fibronectin (6). The yield of fibronectin was 120-160 ug/ml human plasma.

Quantification of fibronectin

The concentrations of fibronectin in whole plasma, fibronectin-depleted plasma and fibronectin-repleted plasma were determined by immunoturbidimetric assay (range 100-1000ug/ml) (63). The standard curve is shown in Fig. 4. The range of fibroncetin concentration in whole plasma

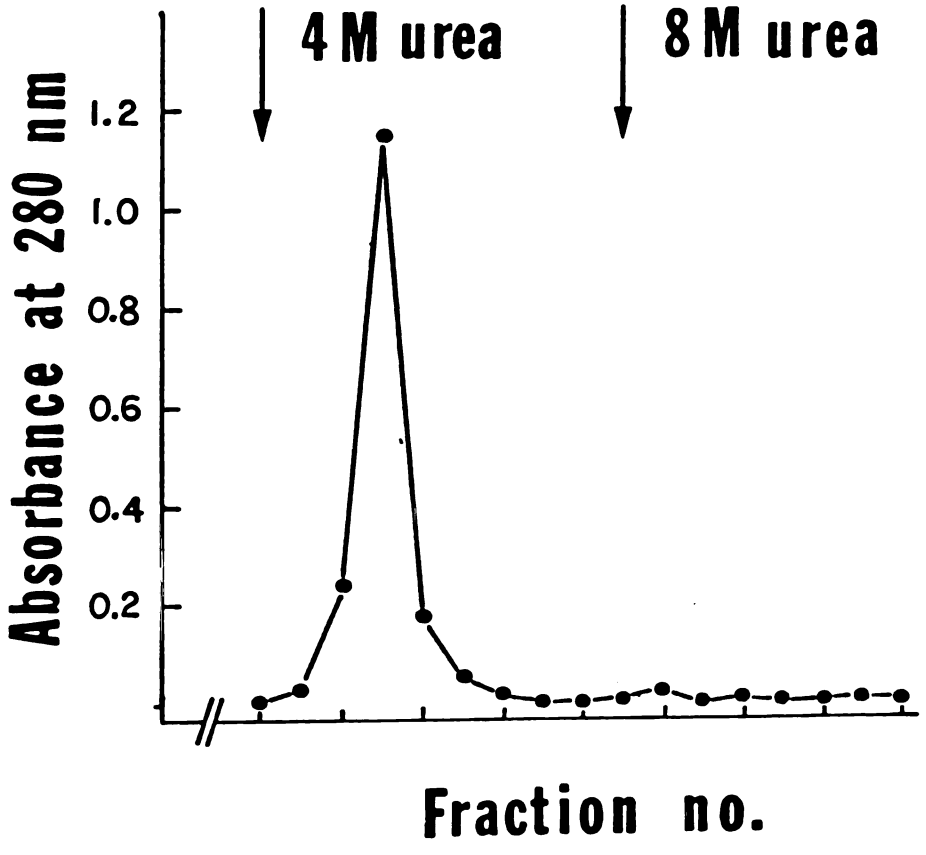


Fig. 2 Profile of gelatin-Sepharose 4B affinity chromatography. Elution of fibronectin bound to gelatin-Sepharose 4B was accomplished with a solution containing 4M urea and 0.05M Tris-HCl, pH 7.5. The eluant was collected in 3.4 ml fractions. Subsequent elution with 8M urea did not cause additional elution of proteins.

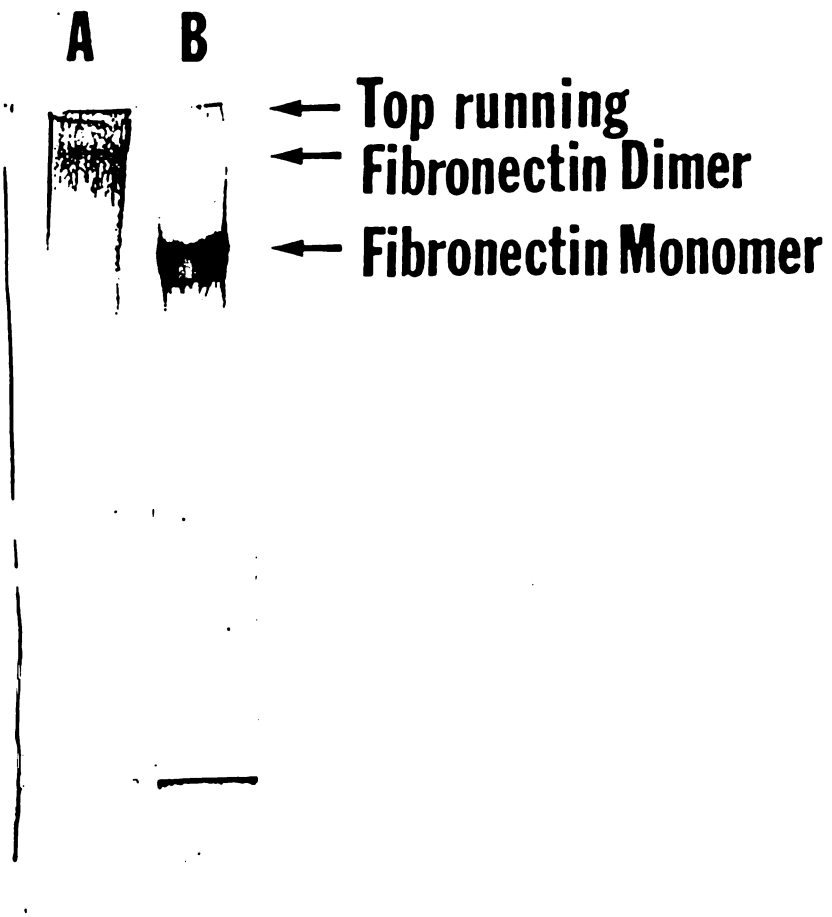


Fig. 3 SDS polyacrylamide gel electrophoresis of purified fibronectin. Purified plasma fibronectin was analyzed on a slab with 3.5 % stacking gel and a 5 % running gel without (lane A) or with (lane B) reduction. Proteins were stained with Coomassie brilliant blue.

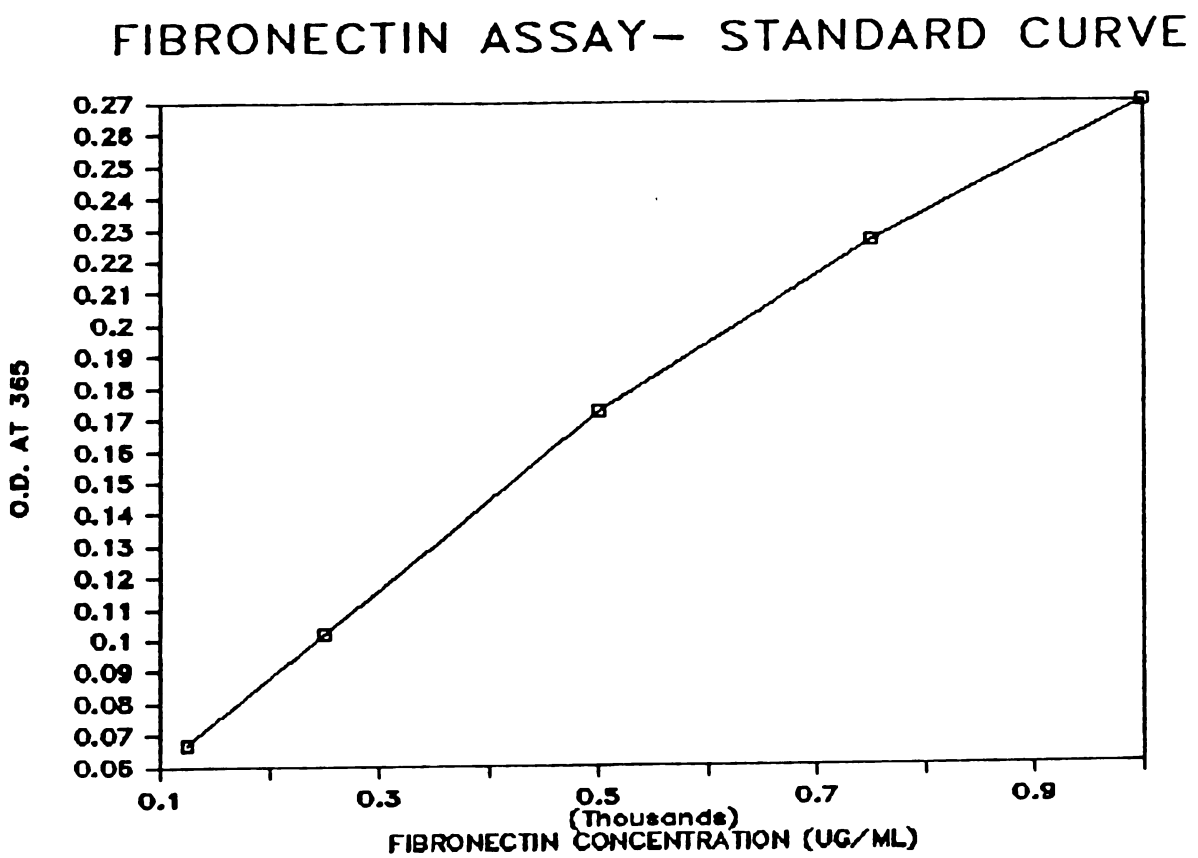


Fig. 4 Standard curve of immunoturbidimetric assay for quantification of human fibronectin. Abscissa represents the standard concentrations of fibronectin (ug/ml). Ordinate represents the observed absorbance differences at 365 nm (ΔA).
ΔA=O.D. at 10 min.—O.D. at 1 min.

was 200-300ug/ml. In fibronectin depleted plasma, no fibronectin was detectable.

Standardization of the bacterial adhesion assays

Bacterial adherence to the fibrin matrix can be influenced by the following factors: the number of bacteria added, the growth phase of bacteria, the buffer system, incubation temperature and incubation time. Initial studies were therefore conducted to standardize the assay system.

A. Number of bacteria per inoculum

The number of S. aureus (strain 66) which adhered to the fibrin matrix was proportional to the inoculum number of bacteria over the range of $5x10^2$ to $5x10^4$ (Fig. 5). An inoculum of approximately $5x10^3$ was chosen for subsequent experiments.

B. Growth phases of bacteria

The affinity of S. aureus to the fibrin matrix was affected by the growth phase of bacteria. S. aureus (strain 66) in log phase had higher affinity than that in stationary phase (Table 2). In subsequent experiments, bacteria harvested in log phase (at 12-16 hour in unbuffered trypticase soy broth) were used.

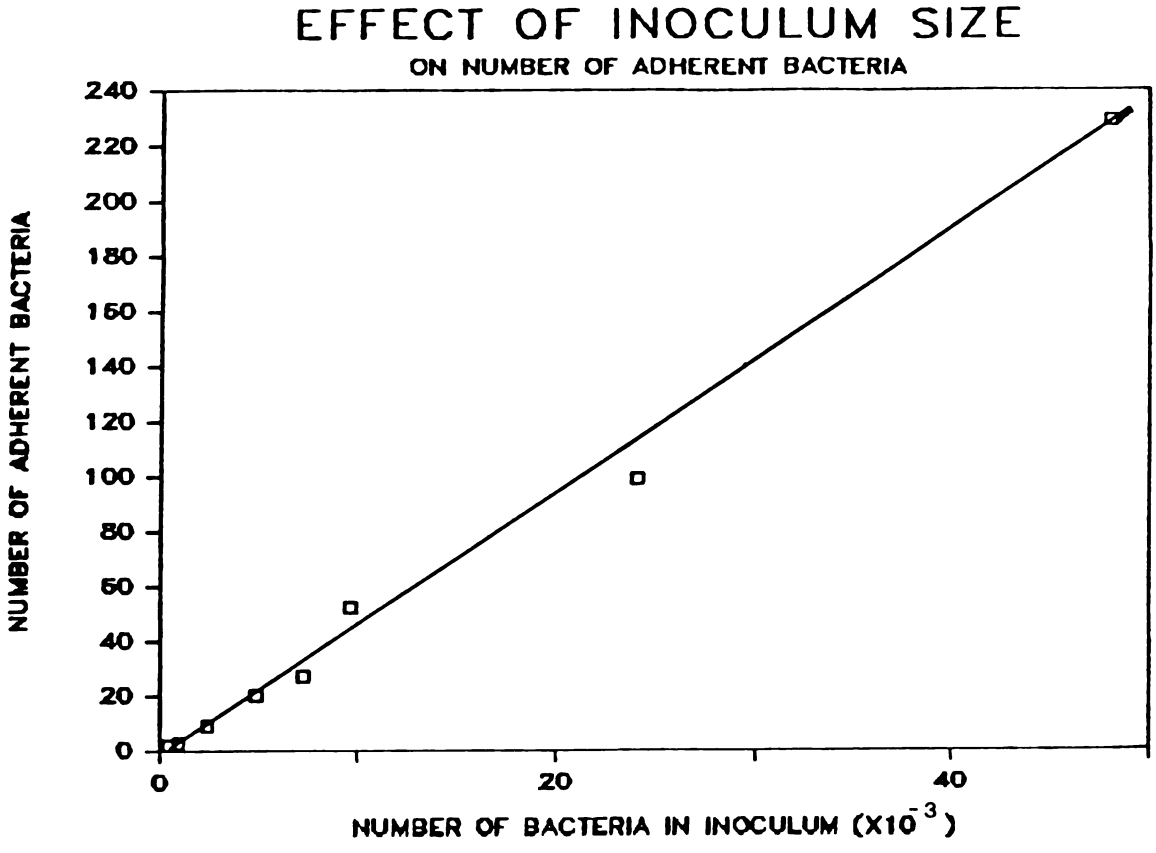


Fig. 5 Relationship between the amount of S. aureus inoculated and the number of adherent bacteria. Reactions were performed at 37°C for 20 min. in 0.025 M HEPES buffered normal saline, pH 7.4, containing $5x10^2$-$5x10^4$ CFU of S. aureus strain 66.

Table 2 Effect of growth phase on adherence of S. aureus to fibrin matrix

growth phase	time of culture growth (h)	adherence ratio $x10^4$ (mean ± S.D.)
log	12	247±37
stationary	24	142±32

1. S. aureus strain 66 was used in this experiment.
2. Data represent mean values from 3 experiments, each consisting of 4 identical preparations.

C. Buffer systems

Originally, bacteria were suspended in phosphate buffered saline over the surface of the fibrin matrix. The precipitation interfered with subsequent colony counting. Therefore, a HEPES buffer system was used in subsequent experiments.

D. Incubation temperatures

The effect of incubation temperature on the adherence of S. aureus to fibrin matrix is shown in Table 3. The adherence ratio increased when the assays were performed at 37·C in comparison with room temperature. Subsequent assays were performed at 37·C.

E. Incubation times

The time course of adherence of S. aureus to fibrin matrix is shown in Fig. 6. The time required to reach the maximal adherence was 15-30 minutes. Based on this observation in subsequent experiments bacteria were allowed to interact with the fibrin matrix for 20 minutes.

Table 3 Effect of incubation temperature on adherence of *S. aureus* to fibrin matrix

temperature	adherence ratio $x10^4$ (mean±S.D.)
37^oC	270±36
22^oC	157±28

1. *S. aureus* strain 66 was used in this experiment.
2. Data represent mean values from 3 experiments, each consisting of 4 identical preparations.

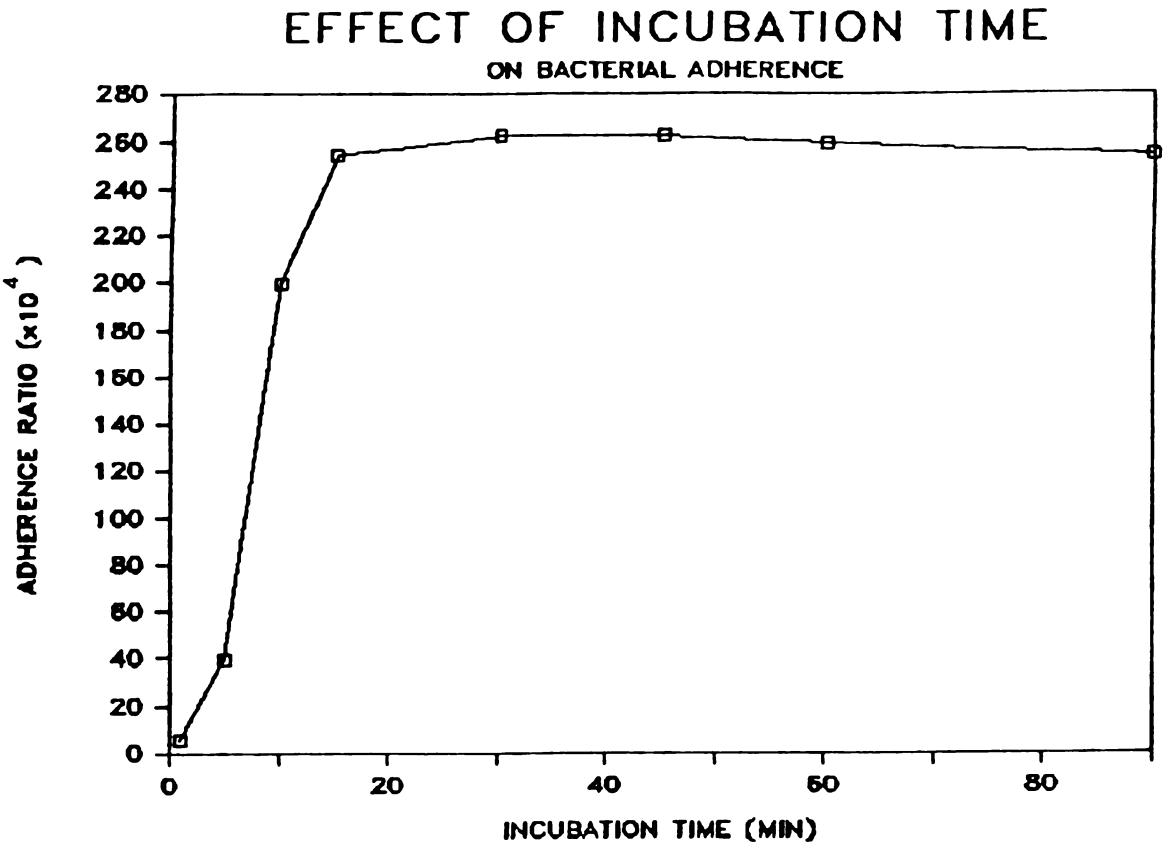


Fig. 6 Time course of adherence of S. aureus strain 66 to fibronectin-containing fibrin matrix. Reactions were performed at 37°C in 0.025 M HEPES buffered normal saline pH 7.4, containing 4,800 CFU of organisms. Adherence ratios were calculated as described in Methods.

Effect of fibronectin on bacterial adherence to the fibrin matrix

Several strains of *S. aureus*, *S. epidermidis*, as well as *E. coli* were tested for their adherence to a fibrin matrix with or without fibronectin. The results are shown in Table 4 and 5 and Fig. 7 and 8. The major findings are summarized as follows:

1) According to the adherence ratio, the bacteria tested can be classified in three groups:

   Group I included *S. aureus* 66, 106 and ATCC 29213. The adherence ratio was in the range of $200\text{-}500\times10^{-4}$.

   Group II included *S. aureus* 68, 69, 70 and 76. The adherence ratio was in the range of $100\text{-}150\times10^{-4}$.

   Group III included *S. epidermidis* ATCC 27626 and *E. coli* 139, 140, 198 and ATCC 25922. The adherence was less than 3×10^{-4}.

2) In the absence of fibronectin, the adherence ratio decreased significantly in Group I ($p<0.001$ except in

Table 4 Effect of fibronectin on adherence of *S. aureus* to fibrin matrix

Bacteria	Inoculum Number CFU	Adherence ratio $\times 10^4$ Fn(+)	Fn(-)	Fn(-)/Fn(+) %	n*	p#
Group I						
S.aureus 66						
Experiment 1	4800	384±54	194±35	50	8	<0.001
Experiment 2	6720	209±38	106±15	50	8	<0.001
Experiment 3	6320	248±36	152±17	61	8	<0.001
Experiment 4	5120	284±46	168±38	59	8	<0.001
S.aureus 106						
Experiment 1	9500	305±24	169±26	55	4	<0.001
Experiment 2	7200	285±23	128±16	45	4	<0.001
Experiment 3	5333	331±25	209± 7	63	4	<0.001
Experiment 4	5783	278±25	203±22	73	4	0.001<p<0.01
Experiment 5	5300	220±16	126±35	57	4	0.001<p<0.01
Experiment 6	9125	171±12	72±16	42	4	<0.001
Experiment 7	3933	442±38	248±35	56	4	<0.001
S.aureus ATCC 29213	5500	340±42	220±24	64	4	<0.001
Group II						
S.aureus 69	4960	116±28	76±26	65	8	0.001<p<0.01
S.aureus 70	4250	100± 8	74±11	74	4	0.001<p<0.01
S.aureus 68	4200	135±23	116±28	86	4	0.1<p<0.5
S.aureus 76	6780	102±26	96±22	94	8	0.1<p<0.5

*;#: See Table 5

Table 5 Effect of fibronectin on adherence of *E. coli* and *S. epidermidis*

Bacteria	Inoculum number CFU	Adherence ratio$\times 10^4$ Fn (+)	Fn (−)	n*	p#
Group III					
E. coli 139	6,730	0.31	0.28	8	>0.5
E. coli 149	14,100	2.4	1.8	8	>0.5
E. coli 198	14,460	2.5	3.4	8	>0.5
E. coli ATCC 25922	20,000	0.78	1.1	8	>0.5
S. epidermidis ATCC 27626	8,200	0.28	0.32	8	>0.5

* n= number of plates tested in each set of matrix in one experiment.

p values were calculated according to unpaired Student's t-test.

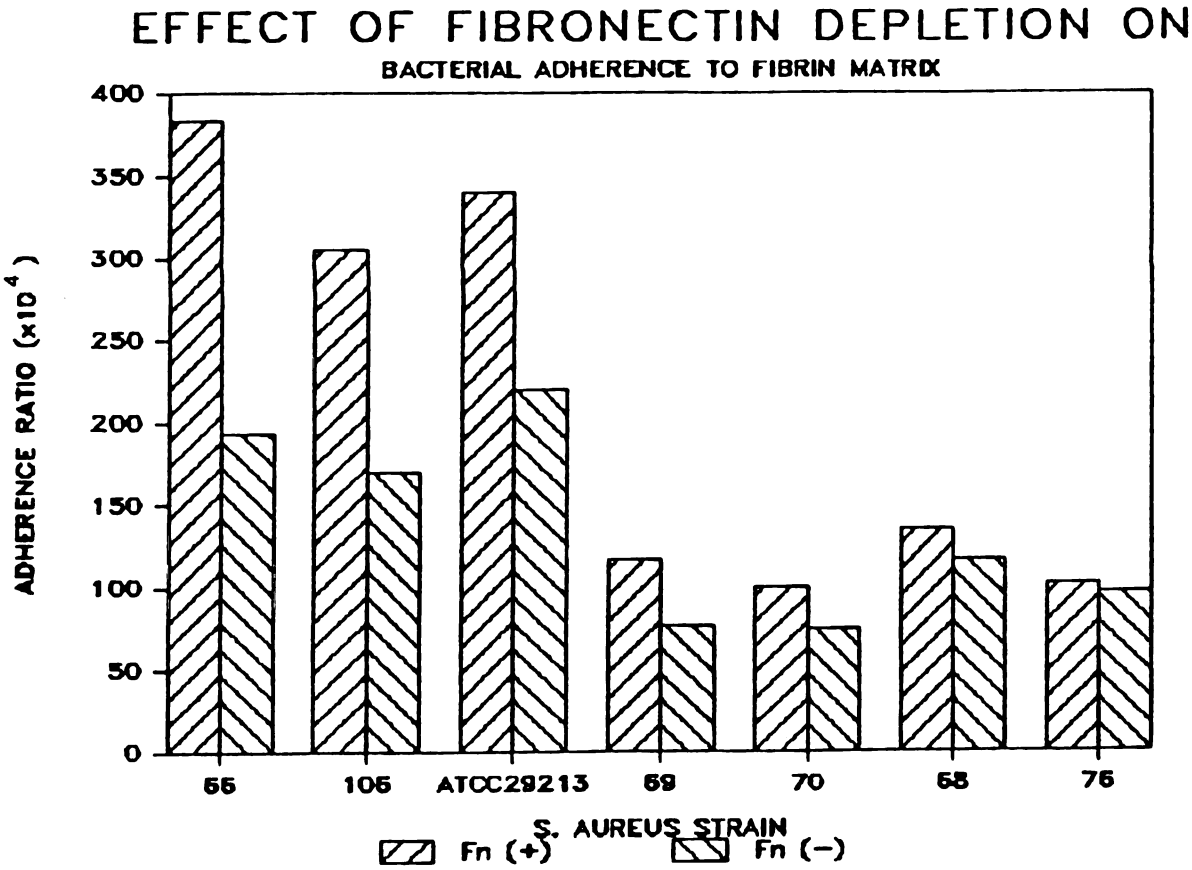


Fig. 7 Effect of fibronectin depletion on adherence of S. aureus. Data shown are as presented in Table 4. In S.aureus strains 66 and 106, experiment 1 was chosen to represent all the other experiments.

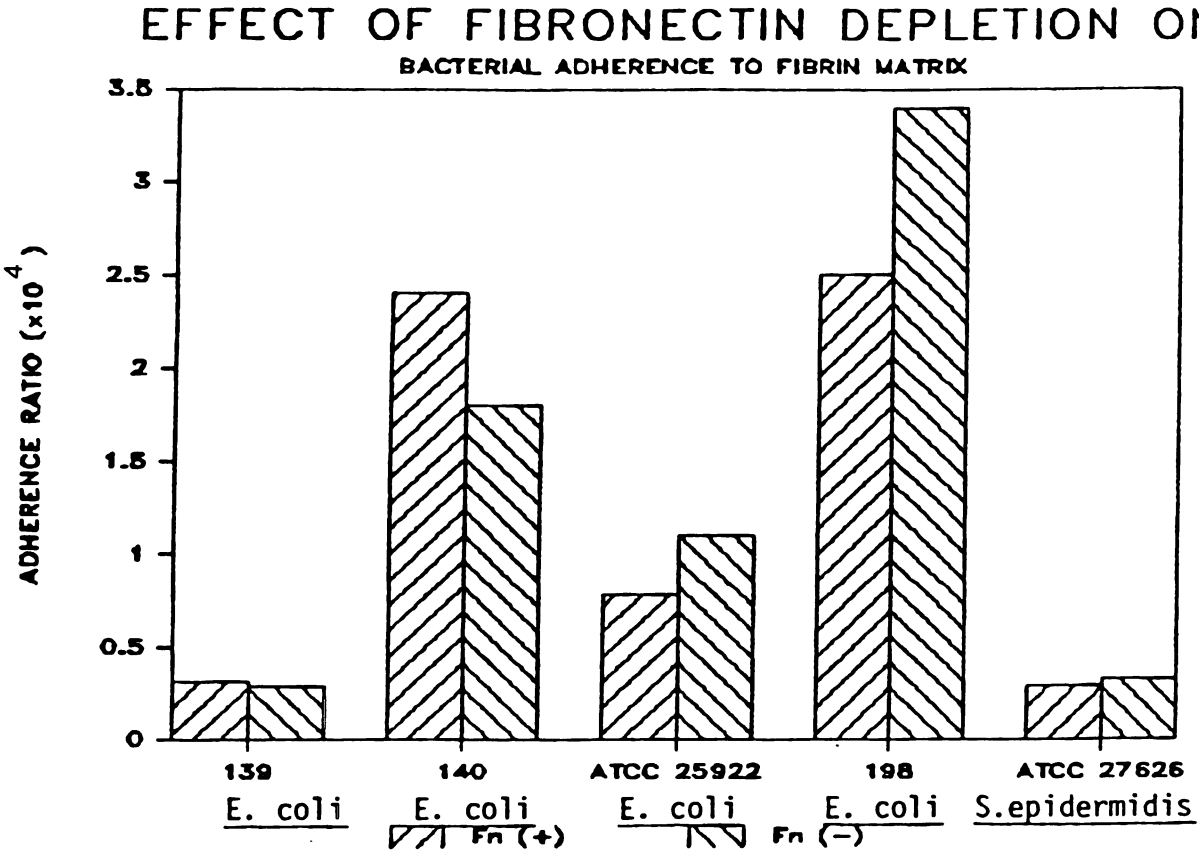


Fig. 8 Effect of fibronectin depletion on adherence of E. coli and S. epidermidis. Data shown are as presented in Table 5.

experiments 4 and 5 of S. aureus 106). There was a 40-60% decrease in comparison with the adherence to a fibronectin containing fibrin matrix. The adherence ratio of Group II decreased slightly on removal of fibronectin, however there was no statistical significance in this difference. In Group III, no changes were observed on removal of fibronectin.

The effect of fibronectin on the adherence of S. aureus was further investigated by adding back purified fibronectin to the fibronectin-depleted plasma. The dose-response relationship is shown in Fig. 9.

Inhibition of S. aureus adherence to the fibrin matrix

Substances which inhibit the binding of fibronectin to S. aureus were tested in our system. The results are shown in Table 6. In the presence of fibronectin, L-lysine inhibited 45%, L-arginine inhibited 55%, D-glucuronic acid lactone inhibited 65%, and heparin inhibited 40% of the adherence of S. aureus 66. In the absence of fibroncetin, no inhibition was observed.

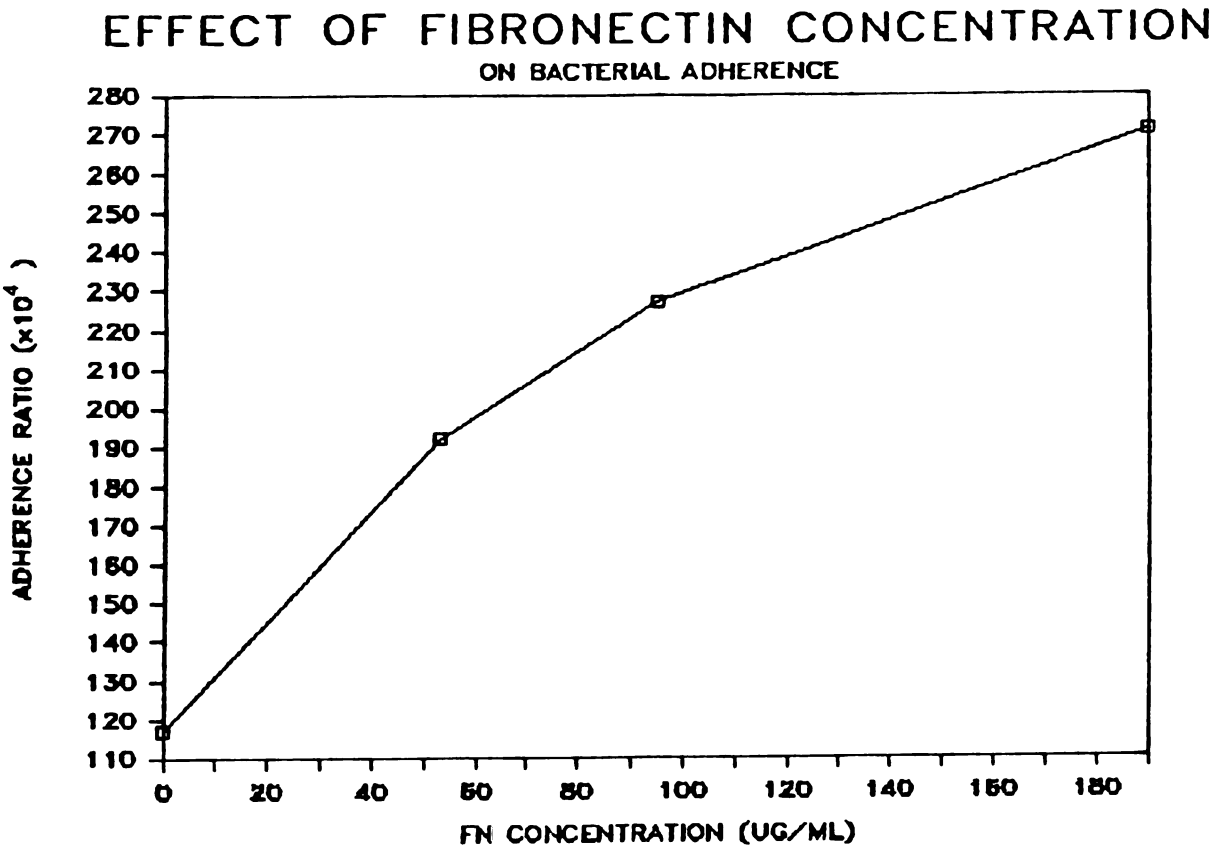


Fig. 9 Dose-response relationship of fibronectin concentration in fibrin matrix and S. aureus adherence. Reaction mixture contained 5,100 CFU of S. aureus strain 66. Data represent mean values of 8 identical preparations.

Table 6 Inhibition of adherence of S. aureus to fibrin matrix

inhibitor	adherence ratio $x10^4$ (mean ± S.D.)	% inhibition
L-lysine (0.2 M)	115 ± 28	45%
L-arginine (0.2 M)	93 ± 36	55%
D-glucuronic acid lactone (0.2 M)	60 ± 9	65%
Heparin (0.2 mg/ml)	120 ± 15	40%
Control	196 ± 22	

1. S aureus strain 66 was used in this experiment.
2. Data represent mean values from 2 experiments, each consisting of 6 identical preparations.

Scanning electronmicroscopy

Examined by the scanning electronmicroscopy, the surface of a fibrin matrix preparation demonstrated a coarse fibrin meshwork (Fig. 10) which was similar to the morphology of fibrin clot on wound tissue (64). When $5x10^8$ CFU of S. aureus strain 66 in 5 ml solution were added to the fibrin matrix, the bacteria were observed to adhere to the fibrin meshwork in large amounts. Due to the high concentration of bacteria, some aggregations of S. aurues could be seen (Fig.11). At the same concentration, adherence of E. coli was rarely observed (Fig. 12).

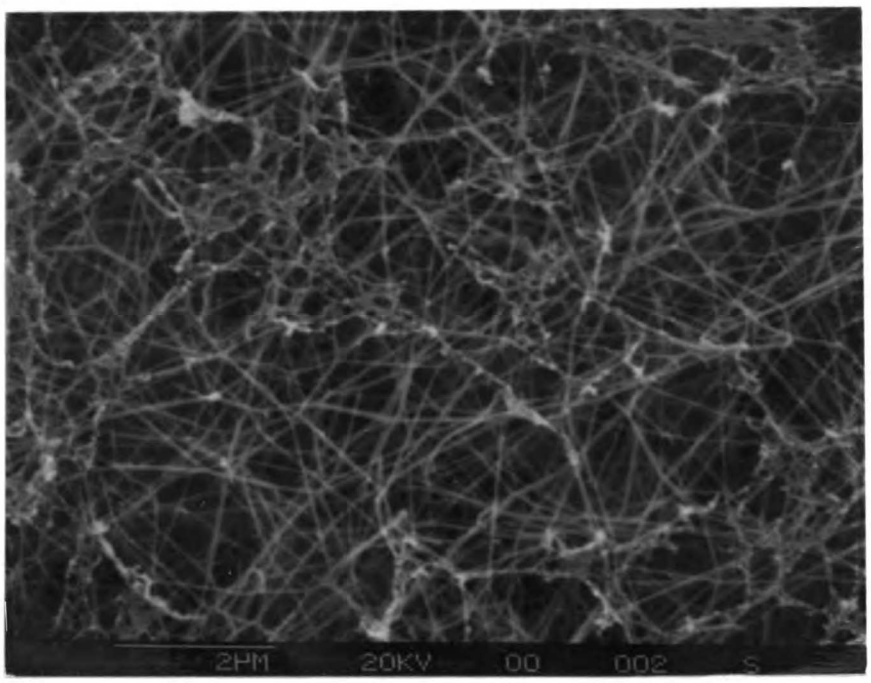


Fig. 10 Scanning electronmicrography of fibrin matrix surface.
(x8,000; Bar=2um)

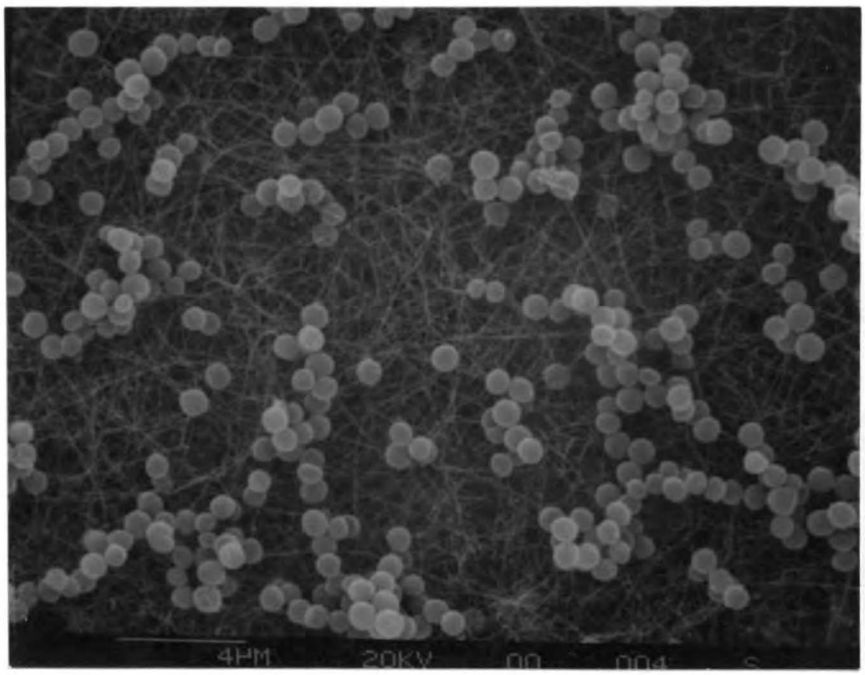


Fig. 11 Scanning electronmicrography of the fibrin matrix exposed to S. aureus ($5x10^8$ CFU). Reaction was performed at 37^oC for 30 min. (x4,250; Bar=4um)

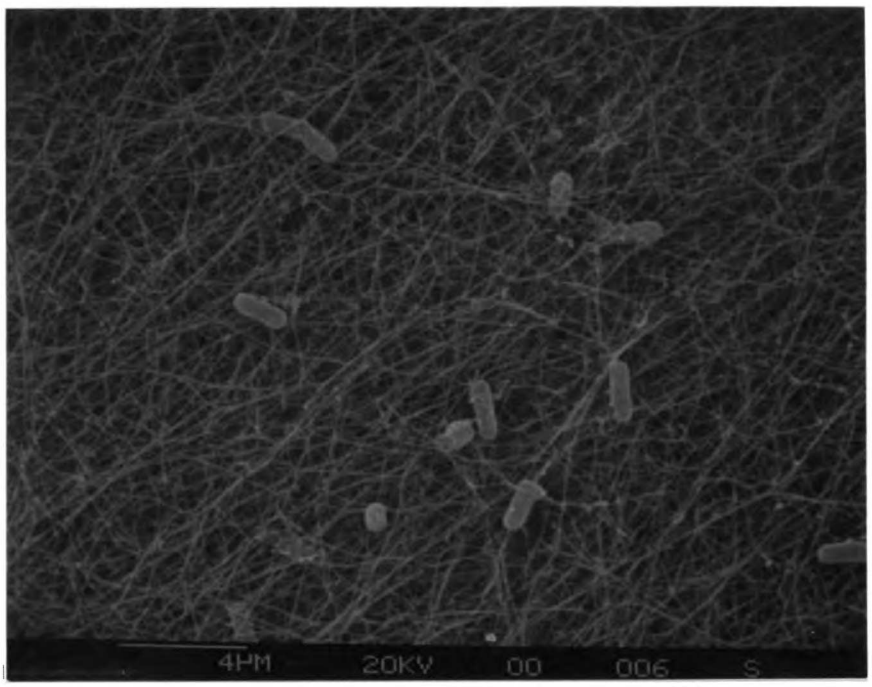


Fig. 12 Scanning electronmicrography of the fibrin matrix exposed to E. coli (5.5×10^8 CFU). Reaction was performed at $37^{o}C$ for 30 min. (x4,250; Bar=4um)

DISCUSSION

Standardization of bacterial adhesion assays

Studies conducted to standardize the assay system demonstrated that the optimum conditions for bacterial adherence to fibrin matrices were: log phase bacteria, an inoculum size of $5x10^3$, incubation period of 20 minutes and incubation temperature of 37·C. Factors which affected bacterial adherence are discussed as follows.

A. Growth phases

Previous studies have shown that more fibronectin binds to S. aureus harvested from log phase than from stationary phase (1000 versus 500 ng fibronectin per $4x10^8$ S. aureus) (44). In the present study, the adherence ratio of log phase S. aureus was higher than that of stationary phase (247 versus 142 $x10^{-4}$). Apparently, S. aureus in log phase have a higher capacity for fibronectin binding. It is known that the number of surface antigens expressed on S. aureus is greater in log phase cultures than in stationary phase cultures (65). The development of capsular material on stationary phase S. aureus may result in the blocking of surface receptors (66).

B. Number of bacteria per inoculum

The number of S. aureus that adhered to fibrin matrix was proportionally increased when inoculum size increased from $5x10^2$ to $5x10^4$ (Fig. 5). At a higher dose of $5x10^8$, S. aureus tend to aggregate together as demonstrated by scanning electronmicroscopy (Fig. 11). Therefore, using this assay system, it was difficult to investigate whether the adherence was saturable.

C. Incubation times

The incubation time required to reach the maximal adherence was 15-30 minutes (Fig. 6). This observation was consistent to other reports in which the specific binding of fibronection to S. aureus reaches a plateau at 20 minutes (44).

D. Incubation temperatures

The adherence ratio of S. aureus to fibrin matrix was higher at 37·C than at room temperature (270 versus $160x10^{-4}$). The mechanism of the temperature effect is unclear.

Adherence of different bacterial species to the fibrin matrix

The affinity of S. aureus to fibrin matrix was 100-500 fold that of E. coli and S. epidermidis (Table 4 & 5). In the absence of fibronectin, the adherence ratio was still much higher than in the other species. Only S. aureus of the bacteria tested here binds to both fibrinogen and fibronectin (44,46). The high affinity of S. aureus to a fibronectin-depleted fibrin matrix probably represent fibrinogen- S. aureus interaction. While in whole plasma derived fibrin matrix, fibronectin had an additive effect.

Effect of fibronectin on S. aureus adherence to the fibrin matrix

Fibronectin is known to have binding domains for S. aureus as well as fibrinogen (Fig. 1). The binding of fibronectin to S. aureus is saturable and irreversible (44). In the present studies, several pieces of evidence indicate that fibronectin enhanced the binding of S. aureus to the fibrin matrix.

1) On removal of fobronectin, the adherence ratio decreased 40-60% in three out of seven strains of S. aureus (strain 66, 106, and ATCC 29213) ($p<0.001$) (Table 4).

2) The four strains of S. aureus (strains 68, 69, 70, and 76) which were less affected by fibronectin ($p>0.001$) also had lower affinity for fibrin matrix derived from whole plasma (Table 4).

3) Using fibronectin-repleted fibrin matrix, the adherence of S. aureus strain 66 was found to be dose-dependent on the purified fibronectin added (Fig. 9).

4) Substances which inhibit the binding between fibronectin and S. aureus (4,11) also inhibited the adherence of S. aureus strain 66 to fibrin matrix (Table 6).

The variations of the adherence ratio of different strains of S. aureus to fibrin matrix as reported here were consistent with previous reports (44). The binding sites of fibronectin to different strains of S. aureus has a wide range from less than 10 to 7400 molecules per bacteria (44). It has been suggested that the invasiveness of S. aureus correlates with the number of surface fibronectin binding sites (44). In the present studies, no such correlation could be made because the number of clinical strains was too small and strains were not tested for invasiveness.

Inhibition of S. aureus adherence to the fibrin matrix

The results of inhibition tests in our system (Table 6) were comparable to previous studies. The same inhibitors were found to block the binding between fibronectin and S. aureus (L-lysine 80% inhibition at 0.2M; L-arginine 90% at 0.2M; D-glucuronic acid 80% at 0.2M; heparin 60% at 0.2mg/ml) (11). The mechanism of inhibition has been proposed to be related to the charges on fibronectin and on the surface of S. aureus (11). The S. aureus binding site of fibronectin is in the 27 kd N-terminal domain which is positive in charge (5); while the surface of S. aureus is negative in charge. Therefore basic amino acids such as lysine and arginine may compete with the basic binding domain of fibronectin. On the other hand, heparin and glucuronic acid which are acidic substances, may compete with the surface components of S. aureus. In addition, the 27 kd N-terminal domain of fibronectin is also one of the binding domains for heparin (Fig. 1; 35). Heparin may inhibit fibronectin - S. aureus interaction by competing for the same binding domain. Since the fibronectin receptor on S. aureus has been tentatively identified (49), the real mechanism of fibronectin - S. aureus interaction may be elucidated by investigating the inhibition on the interaction between the purified fibronectin receptor and the 27 kd N-terminal domain of fibronectin.

Interactions among fibrin, fibronectin and S. aureus

In our assay system, the main components were fibrin, fibronectin and S. aureus. These three components interact with one another. S. aureus has distinct binding sites for fibrinogen and fibronectin. S. aureus binds to fibrinogen through surface fibrinogen clumping factor (46). Fibronectin receptors on S. aureus have been tentatively identified as a protein with an apparent molecular weight of 18 kd (49). Fibrinogen also interacts with S. aureus and fibronectin through different domains. The carboxyl terminal segment of fibrinogen γ-chain has the major binding sites for staphylococcal clumping factor (46), while the fibrinogen α-chain is cross-linked to fibronectin by factor $XIII_a$ transglutaminase (29.30). As for fibronectin, there are two binding sites for fibrinogen and one for S. aureus. Fibronectin binds to fibrinogen covalently through the 27 kd N-terminal domain and noncovalently through the C-terminal domain (Fig. 1). The S. aureus binding site on fibronectin is also the 27 kd N-terminal domain. Our observations provided indirect evidence that fibrinogen may not block the binding between fibronectin and S. aureus. It has been demonstrated that normally the components of a fibrin clot are 94.9 percent fibrin and 4.4 percent fibronectin (6). If the binding

site on fibronectin for S. aureus were identical to that for fibrinogen, these binding sites would be occupied by the excessive amount of fibrin in the system. In that situation, the presence of preoccupied fibronectin would not affect the adherence of S. aureus to fibrin matrix. However, fibronectin in fact can enhance the adherence of S. aureus to fibrin matrix. Therefore, we conclude from these studies that fibronectin may have different binding sites for fibrinogen and S. aurues.

Clinical implications

Our observations have several implications in the pathogenesis of infectious diseases. First, the affinity of bacteria to the fibrin matrix correlated with the infectivity of bacteria in injured tissue. S. aureus, a common cause of wound infection and of endocarditis even in patients without cardiac valvular deformity, bound strongly to fibrin matrix; while E. coli which rarely causes skin infection or endocarditis (except in patients with prosthetic valves) bound to fibrin matrix poorly. Animal experiments show that to cause experimental endocarditis in 50% of rabbits, an inoculum of $10^{3.75}$ organisms is required for S. aureus, while $10^{6.29}$ is required for E. coli (2). In other words, the effective inoculum size of E. coli is about 300 times that of S. aureus. Using our adherence assay system, it was demonstrated that the affinity of S. aureus to the fibrin matrix was 100-500 times that of E. coli. This may

explain why more E. coli are needed to Induce endocarditis.

Secondly, fibronectin enhanced the adherence of several strains of S. aureus to fibrin matrix. Examined with scanning electronmicroscopy, the fibrin matrix prepared in vitro was similar to the fibrin clot in wound tissue (Fig.9). Therefore, fibronectin may play an important role in the bacterial adherence to injured tissue. For example, in the presence of fibronectin, S. aureus may easily adhere to the thrombi on the injured valve, which can then develop into endocarditis.

Thirdly, plasma fibronectin has been shown to be dimidished in several disease states, such as in disseminated intravascular coagulation, sepsis, trauma and burn (54, 67). Several mechanisms have been proposed for septic hypo-fibronectinemia including consumption of fibronection during clearance of bacteria, degradation of circulating fibronectin by proteolytic enzymes released after sepsis and decreased synthesis or release of fibronectin (68). Interestingly, in animal studies, the circulating fibronectin level decreases one hour following intraperitoneal inoculation of S. aureus in rats. The fibronectin level returns to base line between one and six hours and rises to levels 2-3 times normal over 12-24 hour period (69). However, E. coli bacteremia in rats does not

decrease the bioassayable fibronectin activity in plasma (70). Since fibronectin was required for optimal adherence of S. aureus, but not E. coli, to fibrin matrix in our system (Table 4 and 5), this difference could explain the different responses of fibronectin level to bacterial inoculations. Apparently, in animals with S. aureus inoculation, circulating fibronectin may accumulate in the tissue where bacteria deposit. In this way, plasma fibronectin might be consumed. While in E. coli bacteremia, fibronectin might not be consumed because of the low affinity between E. coli and fibronectin. Whether the initial hypofibronectinemia after S. aureus sepsis is due to consumption of fibronectin during S. aureus adherence to tissue remains to be determined.

Finally, the bacterial adhesion assay developed in this thesis can be used to investigate the invasiveness of bacteria. It has been suggested that the invasiveness of S. aureus is related to the affinity of S. aureus to fibronectin (44). Our assay system can be used to survey the affinity of bacteria isolated from patients to the fibrin matrix. The affinity may indicate the invasiveness of bacteria to the injured tissues.

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