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Human Fibronectin Binding to Staphylococcal Surface Protein and Its Relative Inefficiency in Promoting Phagocytosis by Human Polymorphonuclear Leukocytes, Monocytes, and Alveolar Macrophages

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The interaction between human fibronectin and 17 strains of staphylococci was studied in an attempt to elucidate the staphylococcal cell wall component(s) involved in fibronectin binding and to determine the influence of fibronectin upon phagocytosis by three types of phagocytic cells. Purified, radiolabeled fibronectin bound to a similar degree to six laboratory strains and three fresh clinical isolates of *Staphylococcus aureus*; similar binding of fibronectin was found with *S. aureus* strains deficient in cell wall teichoic acid or clumping factor and coagulase, as well as with three strains of *S. epidermidis*. There was minimal binding of fibronectin to encapsulated *S. aureus* and to *Escherichia coli*. Fibronectin bound to intact cells and to a crude cell wall preparation of *S. aureus* H, but not to purified cell walls or peptidoglycan. Trypsinization of staphylococci prevented subsequent fibronectin binding, but binding did not correlate well with the protein A content in *S. aureus* cell walls. At physiological concentrations, fibronectin binding to staphylococci did not promote phagocytosis of bacteria by human polymorphonuclear leukocytes, monocytes, or alveolar macrophages. Also, depletion of fibronectin from normal human serum did not result in a measurable loss of opsonic activity for staphylococci. It is concluded that fibronectin binding to staphylococci involves a surface protein shared among strains of *S. aureus* and *S. epidermidis*, and that in comparison to C3b and IgG, fibronectin plays a relatively minor role as an opsonin for staphylococci.

Efficient recognition and ingestion of staphylococci by human phagocytic cells is dependent upon opsonic factors in serum and other body fluids (14, 30, 33). Well-established staphylococcal opsonins are a fragment of the third component of complement (C3b) and immunoglobulin G class immunoglobulins. The opsonins' most important interaction is with the peptidoglycan moiety of the staphylococcal cell wall (22, 29). Recently, fibronectin, a major glycoprotein of blood (300 ± 100 μg per ml of plasma), has been shown to bind to *Staphylococcus aureus* but not to *Mycobacterium butyricum* or *Escherichia coli* (12, 16; R. A. Proctor, E. Prendergast, and D. F. Mosher, Clin. Res. **27**:650A, 1979), and it has been reported that fibronectin enhances the chemiluminescence response and staphylocidal activity of human polymorphonuclear leukocytes (PMN) challenged with otherwise unopsonized staphylococci (Proctor et al., Clin. Res. **27**:650A, 1979). Together, these effects were taken to indicate that fibronectin acts as a staphylococcal opsonin (Proctor et al., Clin. Res. **27**: 650A, 1979). The binding site for fibronectin in the cell wall of staphylococci remains unknown to date. In this study, we have attempted to further characterize the fibronectin binding site within the mosaic of the staphylococcal cell wall and have compared the opsonic activity of fibronectin with that of immunoglobulin G and C3b by using an assay that directly quantitates bacterial uptake by phagocytes.

MATERIALS AND METHODS

Bacterial strains and cultural conditions. Previously described laboratory strains of *S. aureus* were Cowan I, EMS (a protein A-deficient mutant of Cowan I provided by A. Forsgren, Malmö University, Malmö, Sweden), 502A, H, HSmR (a spontaneous streptomycin-resistant mutant of strain H), 52A5 (a teichoic

acid-deficient mutant of HSmR provided by J. T. Park, Tufts University, Boston, Mass.), Wood 46, and the M (encapsulated) and M variant (unencapsulated) strains, both provided by A. Melly, Vanderbilt University, Nashville, Tenn. (22, 29, 30, 32). One strain of *S. aureus* that was coagulase and clumping factor negative was kindly provided by C. P. van Boven (University of Limburg, Maastricht, The Netherlands).

In addition, fresh blood isolates of *S. aureus* were obtained from five patients with staphylococcal bacteremia. Two strains of *S. epidermidis* were likewise cultured from bacteremic patients, and *S. epidermidis* ATCC 14990 was from the American Type Culture Collection (Rockville, Md.). *E. coli* ON2 (serotype O22:H16) is a serum-resistant strain kindly provided by B. Björksten (University of Umeå, Umeå, Sweden). All strains were maintained on blood agar plates at 4°C. For each experiment, bacteria were freshly grown in Mueller-Hinton broth in a 37°C shaking incubator for 16 h, washed three times with phosphate-buffered saline, pH 7.4, containing 0.30 mg of bovine serum albumin per ml (Reheis Chemical Corp., Phoenix, Ariz.) (PBS-BSA), and resuspended in PBS-BSA to indicated concentrations. Initially, bacterial counts were obtained microscopically in a Petroff-Hausser chamber and used to calibrate a Coleman junior spectrophotometer (Coleman Instruments, Maywood, Ill.). Spectrophotometric adjustments were used thereafter. For studies of staphylococcal opsonization and phagocytosis, bacteria were radioactively labeled by adding [^{3}H]adenine (specific activity, 34 Ci/mmol; ICN, Irving, Calif.) to Mueller-Hinton broth as previously described (21).

Isolation of cell wall fragments of *S. aureus* H. Lyophilized preparations of crude cell walls, containing proteins as well as peptidoglycan and teichoic acid, purified cell walls, consisting of teichoic acid covalently linked to peptidoglycan, and purified peptidoglycan were isolated from *S. aureus* H. The isolation and analysis of these cell wall preparations have been previously described in detail (22, 31).

Quantitation of cell wall protein A. The amount of protein A present on the surface of intact *S. aureus* strains was quantitated by indirect hemagglutination, using a modification of previously described methods (20). Briefly, a 1% suspension of sheep erythrocytes (SRBC), sensitized with a subagglutinating dose of immunoglobulin G antibody to sheep erythrocytes (Cappel Laboratories, Cochranville, Pa.), was incubated with serial twofold dilutions of the bacterial suspension (10^9/ml). The hemagglutinating titer of each strain was compared with the titer of a simultaneously run solution containing 5 μg of purified protein A per ml (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.), and the protein A content of each strain was expressed as picograms per 10^6 cocci. All strains were tested in duplicate on 3 separate days.

Trypsin treatment of staphylococci. To remove cell surface proteins, staphylococci (10^{10}/ml in PBS without BSA) were incubated with indicated concentrations of trypsin (type IX, Sigma Chemical Co., St. Louis, Mo.) for 30 min at 37°C. After incubation, trypsin activity was neutralized by adding a twofold excess of chicken egg white trypsin inhibitor (type III-O; Sigma), and the bacteria were washed three times with PBS-BSA. The final pellet was resuspended with PBS-BSA to the original concentration.

Fibronectin purification and labeling. Fibronectin was purified from whole human plasma by a two-step chromatographic procedure. First, plasma was chromatographed on a gelatin-agarose (Bio-Rad) affinity column in PBS, with 1 mM ethylenediaminetetraacetic acid, pH 8 (5). Bound fibronectin was eluted with 20 mM sodium acetate-1 M sodium bromide, pH 5, and dialyzed against 10 mM sodium phosphate-50 mM sodium chloride, pH 8.5. Affinity-purified fibronectin was next chromatographed on a diethylaminoethyl-cellulose (DE-52; Whatman, Clifton, N.J.) ion-exchange column equilibrated with 10 mM sodium phosphate-50 mM sodium chloride, pH 8.5, and eluted with a linear gradient of sodium chloride to 0.7 M. Fibronectin was concentrated by precipitation with 50% ammonium sulfate adjusted to 3 mg/ml in PBS, pH 7.4, and dialyzed against the same. ^{3}H-fibronectin was prepared by reductive alkylation (24). Fibronectin, 60 mg, in 10 ml of 50 mM sodium borate, pH 9, was treated with 5 mCi of ^{3}H-formaldehyde (New England Nuclear Corp., Boston, Mass.) for 30 min at 4°C. The reaction was stopped by three successive additions of 0.2 ml of 0.34 M $NaBH_4$. The solution was adjusted to 2 mg/ml with PBS, pH 8, and dialyzed against the same at 4°C. Functionally active ^{3}H-fibronectin was isolated by affinity chromatography on gelatin-agarose as described above, concentrated by 50% ammonium sulfate precipitation, dialyzed against PBS, pH 8, and stored at −70°C at 1 mg/ml until used in binding assays. The specific activity of ^{3}H-fibronectin prepared under these conditions was 1 mCi/μmol. The purity of fibronectin and ^{3}H-fibronectin was confirmed by two-dimensional immunoelectrophoresis, as described previously (6).

Serum sources and preparation of fibronectin-depleted serum. Serum from 10 healthy donors was pooled and stored in 1-ml portions at −70°C. Serum was also obtained from a recently described patient with homozygous C3 deficiency (H. A. Verbrugh, J. Roord, W. C. van Dijk, M. R. Daha, and J. Verhoef, Program Abstr. Intersci. Conf. Antimicrob. Agents Chemother. 20th, New Orleans, La., abstr. no 144, 1980). This serum contained normal levels of fibronectin (182 μg/ml), but no detectable C3. Purified human C3 was kindly provided by M. R. Daha (University of Leiden, Leiden, The Netherlands). Fibronectin-depleted serum was prepared by affinity chromatography of whole serum on gelatin-agarose as described above. Fibronectin-depleted serum was concentrated back to the original volume by ultrafiltration in a membrane filter equipped with a 10,000-molecular-weight cut-off filter, dialyzed against Hanks balanced salt solution (HBSS) at 4°C, and frozen at −70°C until use. Control serum was treated in an identical fashion except that the chromatography was done on agarose. The fibronectin levels in normal pooled serum, fibronectin-depleted serum, and control-treated serum were 152, <15, and 160 μg/ml, respectively. Just before use, sera were thawed and diluted to the desired concentration in HBSS containing 0.30 mg of BSA per ml (HBSS-BSA).

Measurement of fibronectin binding to intact

bacteria and cell wall fragments. Suspensions of bacteria or cell wall components were washed once with PBS-BSA containing 0.7 mM Ca^{2+} and 0.5 mM Mg^{2+} ions (5 min, 1,600 × *g* for intact cells; 10,000 × *g* for cell wall fragments). Duplicate 400-μl samples of bacteria (containing 4 × 10^9 microorganisms) or cell wall components (1 mg) were added to glass tubes (10 by 75 mm) and mixed with varying amounts of ^{3}H-fibronectin in a total volume of 0.56 ml. After 30 min of incubation at room temperature, the particles were washed two times with PBS-BSA, transferred to a second glass tube, washed a third time, transferred to scintillation vials, and dried (overnight at 60°C). Seven milliliters of scintillation liquid (Liquiflour; New England Nuclear) was then added, and the particle-associated radioactivity was determined in a Beckman LS-230 liquid scintillation counter (Beckman Instruments, Inc., Chicago, Ill.). In some experiments, surface-bound fibronectin was visualized by using fluorescein-conjugated goat antibody [F(ab')$_2$ fragment] against human fibronectin (Cappel Laboratories) by previously described methods (19).

Preparation of phagocytes. The isolation of PMN and monocytes (MN) from fresh heparinized donor blood by dextran sedimentation and separation over a Ficoll-Isopaque gradient as well as the subsegmental saline lavage of the lingula or middle lobe of the right lung of normal donors to obtain alveolar macrophages (AM) have been described previously (10, 21). The PMN and AM suspensions were ≥90% pure, whereas purity of MN suspensions varied from 25 to 45%. Viability of PMN, MN, and AM was >95% as assessed by trypan blue exclusion.

Assessment of bacterial opsonization. Opsonization of bacteria was assessed by quantitating the uptake of radiolabeled bacteria by human PMN, MN, and AM. The assay used has been described in detail previously (22, 29, 30). Briefly, 0.1 ml of bacteria (5 × 10^8/ml in HBSS-BSA) was incubated with 0.9 ml of opsinin-containing solutions for 30 to 60 min at 37°C in a shaking incubator. Then 100 μl of the opsonized bacterial suspension was mixed with 0.1 ml of either PMN, MN, or AM (5 × 10^6 cells/ml in HBSS-BSA) in polypropylene vials (Bio-vials; Beckman), and phagocytosis was allowed to proceed for 60 min at 37°C with shaking. The final bacteria-to-phagocyte ratio was about 10:1. Phagocytosis was interrupted by adding 3 ml of ice-cold PBS to the mixture. Non-phagocyte-associated bacteria were removed by three cycles of differential centrifugation (5 min, 160 × *g*), and the phagocyte-associated radioactivity in the final pellet was determined by liquid scintillation counting. Phagocytosis was expressed as percentage of uptake of total added radioactivity, determined in a separate vial. The percentage of uptake was taken as a measure of bacterial opsonization (29).

RESULTS

Fibronectin binding to *S. aureus* Cowan I. Initial experiments with *S. aureus* Cowan I were performed to establish the characteristics of fibronectin binding to staphylococci. The binding of radiolabeled fibronectin to this strain was directly proportional to the amount of fibronectin added to the incubation mixtures (Fig. 1). A linear correlation ($r = 0.99$) was maintained over a final fibronectin concentration range of 50 to 1,000 μg/ml. At lower concentrations, the amount of fibronectin bound rapidly dropped to undetectable levels. At physiological levels of fibronectin, 300 ± 100 μg/ml (13), saturation of the surface of *S. aureus* did not occur, and binding of radiolabeled fibronectin was inhibitable by adding excess nonradiolabeled fibronectin. From the experiments, it is estimated that 200 to 1,000 molecules of fibronectin became fixed to the surface of a staphylococcal cell at these fibronectin concentrations (using 450,000 as the molecular weight of intact dimeric plasma fibronectin and 6.023 × 10^{23} as Avogadro's number). Fibronectin fixation to the surface of *S. aureus* Cowan I, incubated with purified fibronectin or serum, was also confirmed by immunofluorescent microscopy. The reproducibility of the fibronectin binding assay was calculated from six separate experiments, performed on different days; the coefficient of variation was 11.0%. In all subsequent experiments, the fibronectin binding capacity of other bacterial strains was compared with that of simultaneously run *S. aureus* Cowan I. The results are presented as percentage of fibronectin bound relative to Cowan I.

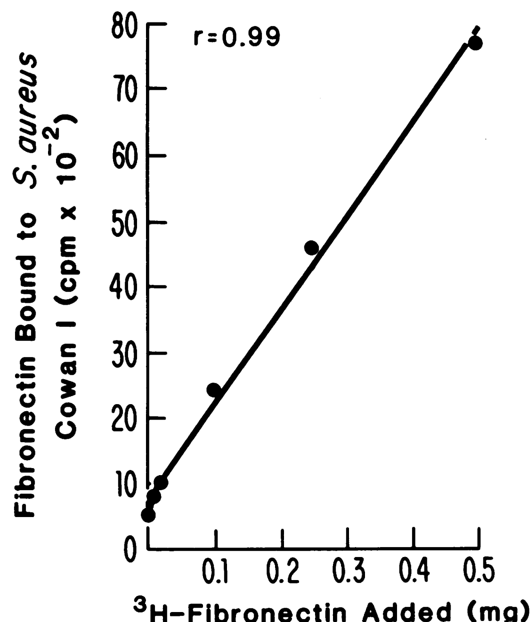

FIG. 1. *Dose-dependent binding of ^{3}H-fibronectin to S. aureus Cowan I. Four hundred microliters of S. aureus (4 × 10^9 bacteria) was mixed with indicated amounts of ^{3}H-fibronectin, and the binding of fibronectin to S. aureus Cowan I was determined. r = correlation coefficient.*

Fibronectin binding by other staphylococcal strains. A total of 15 strains of staphylococci were tested for their ability to bind human fibronectin (Fig. 2). Six strains of *S. aureus* that had been maintained in the laboratory for several years bound fibronectin relatively well; their mean fibronectin binding compared with *S. aureus* Cowan I was 82 ± 9%. Three fresh clinical isolates of *S. aureus* bound fibronectin to a degree that was comparable to that of the laboratory strains (66 ± 6% binding). This finding suggests that the ability of *S. aureus* to bind human fibronectin is a relatively stable characteristic that is not influenced by prolonged cultivation in vitro. The presence of cell wall teichoic acid or of clumping factor or the ability to produce extracellular coagulase was not required for efficient binding of fibronectin to staphylococci, as is evidenced by the binding capacities of strains completely devoid of these characteristics. In contrast, encapsulation of *S. aureus* almost abolished the ability of staphylococcus to bind fibronectin. Binding of fibronectin to the encapsulted M strain was only 12 ± 4%, compared with 45 ± 2% for the unencapsulated M variant strain. Three isolates of *S. epidermidis* bound human fibronectin as well as the *S. aureus* strains (Fig. 2), indicating that fibronectin binding sites of similar affinity are present in both staphylococcal species. Minimal binding (10 ± 2%) was observed with the one strain of *E. coli* tested.

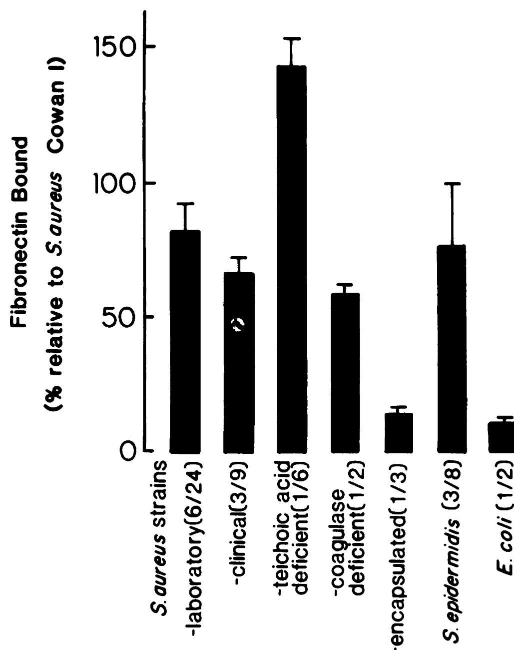

FIG. 2. *Binding of ^{3}H-fibronectin to strains of S. aureus, S. epidermidis, and E. coli. Binding of fibronectin is expressed as mean ± standard error of the mean percentage of the binding to simultaneously run S. aureus Cowan I. Results were obtained with a 0.2-mg/ml final concentration of ^{3}H-fibronectin. Numbers in parentheses indicate number of strains per number of separate tests.*

Fibronectin binding to *S. aureus* cell wall components. To further delineate the receptor site for human fibronectin in the wall of staphylococci, binding of ^{3}H-fibronectin to intact cells and cell wall components of *S. aureus* H was determined. Neither teichoic acid nor peptidoglycan was involved in the binding of fibronectin since, in contrast to crude cell walls from *S. aureus* H, neither purified cell walls (teichoic acid covalently linked to peptidoglycan) nor peptidoglycan bound fibronectin (Table 1). Because teichoic acid and peptidoglycan together represent about 90% of the cell wall of *S. aureus* H (22), binding of fibronectin probably occurs to a minor component (by weight) of the staphylococcal cell wall that is present in isolated crude cell walls but which is removed in subsequent purification steps (22). The amounts of cell wall materials in these tests were chosen on the basis of the estimation by Chatterjee (4) that 10^{8} whole microorganisms yield approximately 25 μg of cell walls.

Effect of trypsin on fibronectin binding. Prior treatment of *S. aureus* Cowan I and *S. epidermidis* ATCC 14990 with varying concentrations of trypsin reduced their ability to bind fibronectin (Fig. 3). Half-maximal inhibition was found with 2 mg of trypsin per ml for *S. epidermidis* and with 20 mg/ml for *S. aureus*. In addition, two clinical isolates of *S. aureus* showed 55 and 65% inhibition of fibronectin binding after treatment with 2 mg of trypsin per ml (data not shown). These data indicate that fibronectin binding to staphylococci involves a protein-containing structure(s) of the bacterial cell wall. This is also in keeping with the binding

TABLE 1. *Binding of ^{3}H-fibronectin to S. aureus H intact cells, crude cell walls, purified cell walls, and peptidoglycan*

Test material[a]	% binding[b] (mean + SEM)	No. of tests
Intact cells	46.0 ± 2.0	4
Crude cell walls	174.5 ± 12.9	2
Purified cell walls	15.4 ± 0.1	2
Peptidoglycan	14.1 ± 1.8	2

[a] Intact cells tested at 10^{10} bacteria/ml; all cell wall components tested at 2.5 mg/ml (see text).

[b] Percentage of fibronectin bound relative to simultaneously run *S. aureus* Cowan I. SEM, Standard error of the mean.

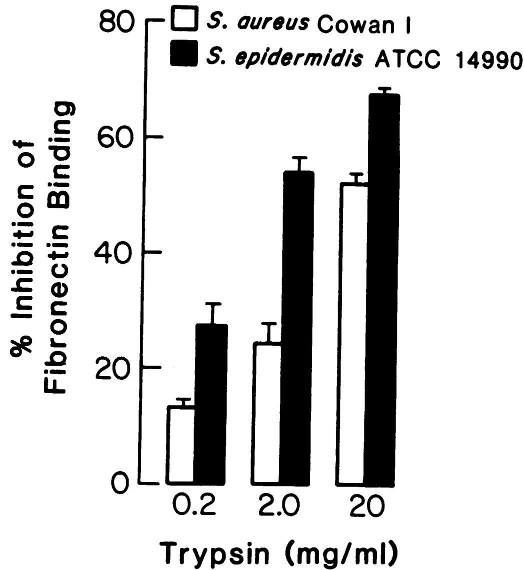

FIG. 3. *Inhibitory effect of trypsin pretreatment of S. aureus Cowan I and S. epidermidis ATCC 14990 on subsequent binding of ³H-fibronectin. Bacteria (10^{10}/ml in PBS) were incubated with indicated concentrations of trypsin for 30 min and washed, and their binding of fibronectin was determined. Inhibition is expressed as mean ± standard error of the mean percentage compared with simultaneously run control bacteria incubated without trypsin.*

of fibronectin by the crude cell wall preparation of *S. aureus* H which contains protein constituents (31).

Role of cell wall protein A in fibronectin binding. Although fibronectin binding to strains of *S. epidermidis* suggested that cell wall protein A is not required for the interaction between staphylococci and fibronectin, a role for protein A in fibronectin binding to *S. aureus* strains was not yet excluded. We therefore correlated the amount of protein A available on the surface of intact *S. aureus* strains with their capacity to bind human fibronectin. A relatively poor correlation ($r = 0.28$, $P > 0.1$) was found between the protein A content of *S. aureus* and fibronectin binding (Fig. 4), indicating that protein A is not significantly involved in fibronectin fixation by *S. aureus*.

Capacity of fibronectin to promote staphylococcal phagocytosis. The opsonic activity of fibronectin was determined in an assay system that directly measures the uptake of radiolabeled bacteria by phagocytic cells. Table 2 provides data that compare the capacities of purified fibronectin and normal human serum (10%) to induce phagocytosis of *S. aureus* Cowan I and five clinical isolates of *S. aureus* by PMN. Bacteria opsonized with 10% normal human serum were readily taken up by the PMN; the mean uptake for *S. aureus* Cowan I and the five clinical isolates was 72.5 and 90.6%, respectively. In contrast, purified human fibronectin in concentrations up to 700 μg/ml failed to induce significant uptake of any of these strains. Phagocytosis

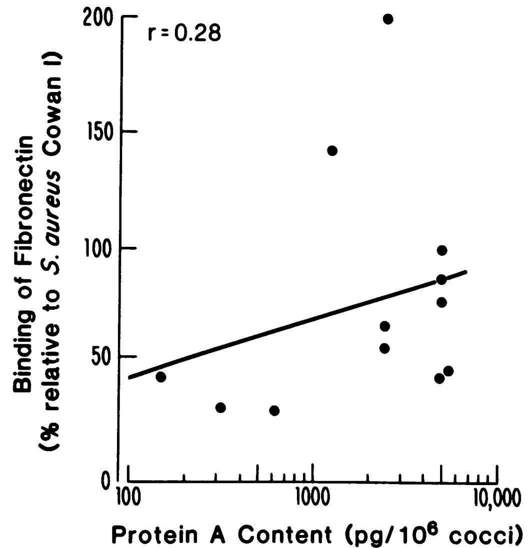

FIG. 4. *Correlation between cell wall protein A content of S. aureus strains and their capacity to bind ³H-fibronectin. r = correlation coefficient.*

TABLE 2. *Opsonic activity of purified human fibronectin for S. aureus Cowan I and five clinical isolates of S. aureus*

S. aureus strain	Opsonic source[a]	% phagocytosis[b] by PMN	No. of tests
Cowan I	Normal serum (10%)	72.5 ± 2.1	6
	C3-deficient serum (10%)	19.1 ± 1.5	3
	C3-deficient serum (10%) + C3 (100 μg/ml)	81.0	1
	Fibronectin (700 μg/ml)	6.4 ± 0.3	7
	Fibronectin (700 μg/ml) + heparin (10 U/ml)	8.0 ± 0.5	2
	HBSS-BSA	6.4 ± 0.4	6
Clinical isolates (n = 5)	Normal serum (10%)	90.6 ± 3.6	1
	Fibronectin (700 μg/ml)	3.0 ± 0.4	1
	HBSS-BSA	5.0 ± 0.4	1

[a] Number in parentheses indicates concentration of opsonin used.

[b] ³H-labeled bacteria were incubated with indicated opsonins for 30 to 60 min, and then mixed with PMN; percentage of phagocytosis is percentage of uptake of total added radioactivity after 60 min of incubation.

was always less than 10% and did not differ from that of control bacteria incubated with HBSS-BSA alone. Addition of heparin, which has been reported to enhance the uptake of gelatin-coated latex particles by monolayers of macrophages (9), did not improve the ability of fibronectin to promote phagocytosis. The treatment of bacteria with fibronectin, either before or after opsonization with 10% serum, did not affect the rapid uptake of bacteria by PMN (data by shown). The binding of fibronectin, thus, did not influence either the binding or function of serum opsonins. The crucial role of complement in serum opsonization was evidenced by the much reduced opsonic capacity of serum devoid of complement factor C3 but with normal levels of fibronectin. The opsonic defect in the patient's serum was corrected by the addition of purified C3 (Table 2).

Because fibronectin may possibly express its opsonic effect only when present in normal body fluids, e.g., serum or plasma, we next compared the opsonic activity of normal serum with that of serum depleted of fibronectin. In these experiments, *S. epidermidis* ATCC 14990 was used and three types of phagocytes were studied. No significant differences were found in the opsonic titers of normal serum versus the same serum depleted of its fibronectin, and similar results were obtained with PMN, MN, and AM (Fig. 5). Likewise, for *S. aureus* Cowan I, fibronectin-depleted serum and control serum had similar opsonic activities (data not shown).

DISCUSSION

Results of this investigation indicate that human fibronectin binding to staphylococci involves a minor proteinaceous surface component of the staphylococcal cell wall, but point to a relative inefficiency of bound fibronectin, when studied at physiological concentrations, in promoting staphylococcal phagocytosis by human phagocytic cells. Fibronectin is a large glycoprotein present in blood and other body fluids and has been proposed to play an important role in the clearance of particles from the bloodstream (26). Decreased levels of fibronectin have been found in patients with disseminated intravascular coagulation and with certain trauma such as severe burns (17, 27). The decreased levels of fibronectin are thought to increase the host's susceptibility to invasion by microorganisms and to contribute to organ failure in such patients.

Trypsin-sensitive binding of human fibronectin to *S. aureus* has been reported previously (12, 16; Proctor et al., **27**:650A, 1979), but the binding site in the cell wall of this bacterial species was not further characterized. We studied binding of radiolabeled fibronectin in a total of 15 strains of staphylococci, some of which have well-characterized differences in cell surface composition. Fourteen of these strains showed significant fibronectin binding that was directly dependent upon the amount of fibronectin added. At physiological levels of fibronectin (300 ± 100 μg/ml [14]) between 200 and 1,000

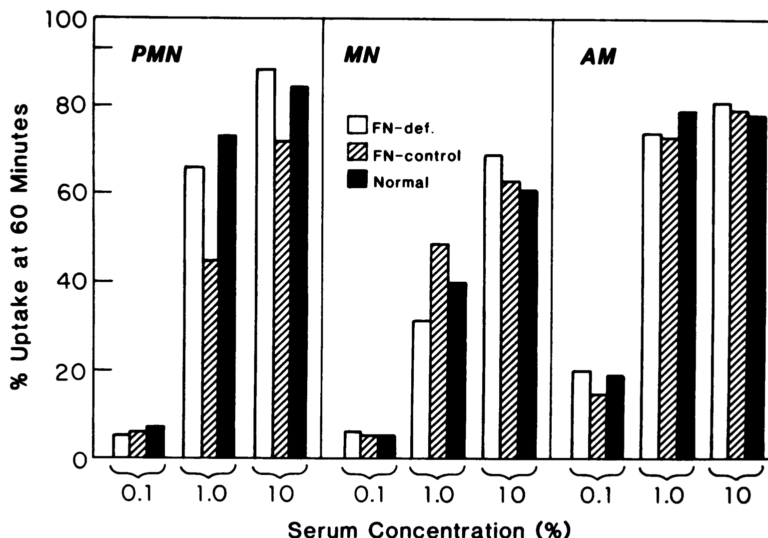

FIG. 5. *Opsonic activity of fibronectin-depleted, control-treated, and non-treated human serum for uptake of S. epidermidis ATCC 14990 by human PMN, MN, and AM. Staphylococci were incubated with indicated opsonic solutions for 30 min before mixing with the phagocytes. Phagocytosis was determined after 60 min of incubation at 37°C.*

molecules of fibronectin were calculated to become attached to the surface of a staphylococcus. Under these conditions, saturation of the bacteria did not occur, and this observation is consistent with the finding of Proctor et al. (R. A. Proctor, D. F. Mosher, and G. Christman, 20th ICAAC, abstr. no. 643), who have reported 5×10^3 fibronectin binding sites per cell for *S. aureus* ATCC 25923.

In the present study, binding occurred to a similar degree to *S. aureus* and *S. epidermidis* strains and was found not to be mediated by the major cell wall components of *S. aureus* (peptidoglycan, teichoic acid, or protein A). Also, purified peptidoglycan and complexes of peptidoglycan and teichoic acid, isolated from *S. aureus* H, failed to bind fibronectin. Crude cell wall preparations of this strain, however, which contain protein constituents, bound considerable amounts of fibronectin. The increased fibronectin binding of crude cell walls compared with intact cells suggests that in broken cells additional (inner) cell wall surfaces are exposed that may contain fibronectin binding sites. Similarly, an increased number of complement activation sites in crude cell walls as opposed to intact cells has been noted before (31).

Fibronectin binding to both *S. aureus* and *S. epidermidis* was trypsin sensitive. Together with the previously reported binding of fibronectin to *Micrococcus luteus* (Proctor et al., Clin. Res. **27**: 650A), these results indicate that the capacity to fix fibronectin is shared by both members of the family *Micrococcaceae*. The binding of fibronectin involves a minor protein-containing structure of the staphylococcal cell wall. Little is known about the minor proteins of the staphylococcal cell wall, but at least 30 different antigenic sites, mostly proteins, have been demonstrated in *S. aureus* by serological techniques (18).

Interestingly, fibronectin did not bind to the encapsulated M strain of *S. aureus*, suggesting that the acidic amino sugar polymers of staphylococcal capsules do not function as fibronectin binding sites and that they form a barrier for fibronectin binding to the cell wall protein beneath the capsule. The mechanism of this barrier is presently unknown but may relate to the relatively large size of the fibronectin dimers (molecular weight 450,000 ± 25,000). The capsule of this strain has previously been shown not to exclude other soluble proteins such as lysostaphin (molecular weight 28,000) (11) and complement factor C3 (molecular weight 180,000) (19, 32).

In a recent communication, Proctor et al. (Clin. Res. **27**:650A, 1979) reported that fibronectin increased the chemiluminescence response of human PMN to other unopsonized *S. aureus* ATCC 25923 and to *M. luteus*, but not to *E. coli*. In addition, a significant loss of micrococcal colony-forming units occurred when fibronectin was present in an in vitro bactericidal assay. Together, these findings were taken to indicate that fibronectin promoted the uptake of the bacteria by PMN and that fibronectin therefore can act as an opsonin for micrococci. In this study, fibronectin had no detectable opsonic activity for seven strains of staphylococci and three types of human phagocytic cells (PMN, MN, and AM). Uptake of bacteria incubated in the presence of purified fibronectin was minimal and did not differ from that of bacteria incubated with buffer alone. In contrast, 10% human serum promoted brisk uptake of staphylococci. Numerous previous studies have shown that the complement system and immunoglobulin G are major opsonic proteins in human serum. The important role of complement in serum opsonic activity was demonstrated in this investigation by the much reduced uptake of bacteria incubated in serum from a patient with a complete C3 deficiency, but with normal levels of fibronectin. A similarly reduced opsonic activity can be found by heat inactivation of serum complement for 30 min at 56°C (30); however, fibronectin is not inactivated by such treatment (Proctor et al., Clin. Res. **27**:650A, 1979). Thus, fibronectin would not be anticipated to be part of the heat-labile serum opsonic system for staphylococci. The role of fibronectin as a serum opsonin was further delineated in experiments with fibronectin-depleted serum. No differences in opsonic titers appeared when normal and fibronectin-depleted human sera were compared.

These results do not support the conclusion of Proctor et al. (Clin. Res. **27**:650A, 1979) that human fibronectin is an important nonantibody, noncomplement opsonin for staphylococci. Most probably, these divergent results can be explained in part by differences in methodology and techniques. The assay system used in this study directly quantitates uptake of radiolabeled bacteria in suspension with phagocytic cells. Uptake is recorded only when bacteria are firmly attached to or ingested by the phagocytes and are not removed by the differential centrifugation cycles. On the other hand, Proctor et al. (Clin. Res. **27**:650A, 1979) used the chemiluminescence response of PMN and a fall in colony-forming units in their mixtures. Both of these latter assays have been used previously as indirect indicators of particle uptake, and thus opsonization of bacteria (8, 23). However, both PMN chemiluminescence and a fall in colony-forming units do not necessarily depend on true

particle ingestion. An oxidative metabolic response of PMN with concomitant generation of chemiluminescence can be elicited by soluble stimuli such as phorbol myristate acetate, a variety of ionophores, and the complement peptide C5a (1, 7). Assays measuring a fall in colony-forming units in phagocytic mixtures, on the other hand, are very susceptible to cell-cell, particle-particle, and cell-particle clumping and need correction for extracellular killing (28). Similar difficulties exist in the interpretation of results of the fibronectin-mediated uptake of gelatin-coated particles by slices of liver or monolayers of peritoneal macrophages (3, 9, 25).

Although fibronectin does not appear to be a true opsonin, promoting both attachment and ingestion, our evidence does not rule out that fibronectin enhances adherence of micrococci to certain phagocytic cells. It has recently been demonstrated that fibronectin mediates binding, but not ingestion, of gelatin-coated particles to surface-bound human monocytes (2). Our results suggest that the possible adherence of fibronectin-coated staphylococci to human PMN, MN, and AM is relatively weak and easily disrupted by washing the cells. The results of Proctor et al. (Clin. Res. 27:650A, 1979), on the other hand, suggest that bacterial adherence would be sufficient for activation of human PMN, resulting in increased oxidative metabolic rates, degranulation and, possibly, membrane-killing, i.e., extracellular killing of bacteria. Further investigations are needed to test these hypotheses.

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LITERATURE CITED

1. **Babior, B. M.** 1978. Oxygen-dependent microbial killing by phagocytes (first of two parts). N. Engl. J. Med. **298:** 659-668.
2. **Bevilacqua, M. P., D. Amrani, M. W. Mossesson, and C. Bianco.** 1981. Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. J. Exp. Med. **153:**42-60.
3. **Blumenstock, F. A., T. M. Saba, P. Weber and R. Laffin.** 1978. Biochemical and immunological characterization of human α2-SB glycoprotein: its identity with cold-insoluble globulin. J. Biol. Chem. **253:**4287-4291.
4. **Chatterjee, A. M.** 1969. Use of bacteriophage-resistant mutants to study the nature of the bacteriophage receptor site of *Staphylococcus aureus*. J. Bacteriol. **98:**519-527.
5. **Engvall, E., and E. Ruoslahti.** 1975. Binding of soluble form of fibroblast surface protein, fibronectin, to collagen. Int. J. Cancer **91:**79-99.
6. **Furcht, L. T., D. F. Mosher, and G. Wendelschafer-Crabb.** 1978. Immunocytochemical localization of fibronectin (LETS protein) on the surface of L6 myoblasts: light and electron microscopic studies. Cell **13:** 263-271.
7. **Goldstein, I. M., and G. Weissman.** 1979. Nonphagocytic stimulation of human polymorphonuclear leukocytes: role of the plasma membrane. Semin. Hematol. **16:**175-187.
8. **Grebner, J. V., E. L. Mills, B. H. Gray, and P. G. Quie.** 1977. Comparison of phagocytic and chemiluminescence response of human polymorphonuclear neutrophils. J. Lab. Clin. Med. **89:**153-159.
9. **Gudewicz, P. W., J. Molnar, M. Z. Lai, D. W. Beezhold, G. E. Siefring, Jr., R. B. Credo, and L. Lorand.** 1980. Fibronectin-mediated uptake of gelatin-coated Latex particles by peritoneal macrophages. J. Cell. Biol. **87:**427-433.
10. **Hoidal, J. R., J. G. White, and J. E. Rine.** 1979. Influence of cationic local anaesthetics on the metabolism and ultrastructure of human alveolar macrophages. J. Lab. Clin. Med. **93:**857-863.
11. **King, B. F., M. L. Biel, and B. J. Wilkinson.** 1980 Facile penetration of the *Staphylococcus aureus* capsule by lysostaphin. Infect. Immun. **29:**892-896.
12. **Kuusela, P.** 1978. Fibronectin binds to *Staphylococcus aureus*. Nature (London) **276:**718-720.
13. **Lauser, M. E., T. M. Saba, and W. A. Scovill.** 1980. Opsonic glycoprotein (plasma fibronectin) levels after burn injury. Relationship to extent of burn and development of sepsis. Ann. Surg. **192:**776-782.
14. **Li, I. W., and S. Mudd.** 1965. The heat-labile serum factor associated with intracellular killing of *Staphylococcus aureus*. J. Immunol. **94:**852-857.
15. **Mosesson, M. W., and R. A. Umfleet.** 1970. The cold-insoluble globulin of human plasma. J. Biol. Chem. **245:** 5728-5736.
16. **Mosher, D. F., and R. A. Proctor.** 1980. Binding and Factor $XIII_a$-mediated cross-linking of a 27-kilodalton fragment of fibronectin to *Staphylococcus aureus*. Science **209:**927-929.
17. **Mosher, D. F., and E. M. Williams.** 1978. Fibronectin concentration is decreased in plasma of severely ill patients with disseminated intravascular coagulation. J. Lab. Clin. Med. **91:**729-735.
18. **Oeding, P., and A. Grov.** 1972. Cellular antigens, p. 333-356. *In* J. O. Cohen (ed.), The staphylococci. John Wiley & Sons, Inc., New York.
19. **Peterson, P. K., Y. Kim, B. J. Wilkinson, D. Schmeling, A. F. Michael, and P. G. Quie.** 1978. Dichotomy between opsonization and serum complement activation by encapsulated staphylococci. Infect. Immun. **20:** 770-775.
20. **Peterson, P. K., J. Verhoef, L. D. Sabath, and P. G. Quie.** 1977. Effect of protein A on staphylococcal opsonization. Infect. Immun. **15:**760-764.
21. **Peterson, P. K., J. Verhoef, D. Schmeling, and P. G. Quie.** 1977. Kinetics of phagocytosis and bacterial killing by human polymorphonuclear leukocytes and monocytes. J. Infect. Dis. **136:**502-508.
22. **Peterson, P. K., B. J. Wilkinson, Y. Kim, D. Schmeling, S. D. Douglas, P. G. Quie, and J. Verhoef.** 1978. The key role of peptidoglycan in the opsonization of *Staphylococcus aureus*. J. Clin. Invest. **61:**597-609.
23. **Quie, P. G., R. P. Messner, and R. C. Williams, Jr.** 1968. Phagocytosis in subacute bacterial endocarditis: localization of the primary opsonic site to Fc fragment. J. Exp. Med. **128:**553-570.
24. **Rice, R. H., and G. E. Means.** 1971. Radioactive labeling of proteins in vitro. J. Biol. Chem. **248:**831-832.

25. **Ruoslahti, E., E. Engvall, and E. G. Hayman.** 1981. Fibronectin: current concepts of its structure and functions. Coll. Res. 1:95–128.
26. **Saba, T. M., and E. Jaffe.** 1980. Plasma fibronectin (opsonic glycoprotein): its synthesis by vascular endothelial cells and role in cardiopulmonary integrity after trauma as related to reticuloendothelial function. Am. J. Med. **68**:577–594.
27. **Scovill, W. A., S. J. Annest. T. M. Saba, F. A. Blumenstock, J. C. Newell, H. H. Stratton, and S. R. Powers.** 1979. Cardiovascular hemodynamics after opsonic alpha-2-surface binding glycoprotein therapy in injured patients. Surgery **86**:284–293.
28. **Stossel, T. P.** 1975. Phagocytosis: recognition and ingestion. Semin. Hematol. **12**:83–116.
29. **Verbrugh, H. A., W. C. van Dijk, M. E. van Erne, R. Peters, P. K. Peterson, and J. Verhoef.** 1980. Opsonic recognition of staphylococci mediated by cell wall peptidoglycan: antibody-independent activation of human complement and opsonic activity of peptidoglycan antibodies. J. Immunol. **124**:1167–1173.
30. **Verhoef, J., P. K. Peterson, Y. Kim, L. D. Sabath, and P. G. Quie.** 1977. Opsonic requirements for staphylococcal phagocytosis: heterogeneity among strains. Immunology **33**:191–197.
31. **Wilkinson, B. J., Y. Kim, P. K. Peterson, P. G. Quie, and A. F. Michael.** 1978. Activation of complement of cell surface components of *Staphylococcus aureus*. Infect. Immun. **20**:388–392.
32. **Wilkinson, B. J., S. P. Sisson, Y. Kim, and P. K. Peterson.** 1979. Localization of the third component of complement on the cell wall of encapsulated *Staphylococcus aureus* M: implications for the mechanism of resistance to phagocytosis. Infect. Immun. **26**:1159–1163.
33. **Wright, A. E., and S. R. Douglas.** 1904. An experimental investigation of the role of the body fluids in connection with phagocytosis. Proc. R. Soc. London **72**:357–370.