Binding of *Staphylococcus aureus* by Human Serum Spreading Factor in an In Vitro Assay

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Human serum spreading factor, an animal cell adhesion-promoting glycoprotein, bound *Staphylococcus aureus* in an assay in which association of bacteria to protein-precoated microtiter wells was quantitated. Interaction of human serum spreading factor with *S. aureus* suggests that this protein may play a role in host defenses and in the invasiveness and pathogenicity of microbial infections.

Fibronectin and laminin, two glycoproteins that mediate animal cell attachment and spreading in cultures (6, 9, 19), have been reported to bind *Staphylococcus aureus* (11, 12, 14, 18). Human serum spreading factor (SF) is another animal cell spreading-promoting glycoprotein that is present in circulating form in plasma and platelets and, like other attachment factors, is a component of the extracellular matrix (1-5, 7, 10, 15-17). Here we report that human serum SF, like fibronectin and laminin, is capable of binding *S. aureus*.

Difficulties in obtaining radioactively labeled SF in a biologically active form led us to develop for quantitative purposes a staphylococcus-binding assay in which bacteria associated with microtiter wells precoated with attachment-promoting proteins were assayed by using enzyme-linked probes. The staphylococcus-binding assay is rapid, sensitive, inexpensive, and adaptable to determination of microorganism-binding activity of other complex mixtures or purified substances. It may be particularly useful in situations in which the preparations to be tested are not easily radiolabeled or are available in limited amounts.

MATERIALS AND METHODS

Materials. The following bacterial strains were used in this study: *S. aureus* Cowan I (ATCC 12598, NCTC 8530), *S. aureus* Wood (ATCC 10832), *Staphylococcus epidermidis* Fussel (ATCC 14990), and *Escherichia coli* FDA Seattle 1946 (ATCC 25922). Also tested were laboratory strains of *S. epidermidis* and *E. coli* obtained from the Microbiology Department, Oregon State University, Corvallis, and a second sample of *S. aureus* Cowan I strain obtained from Sigma Chemical Co., St. Louis, Mo. Strains were stored lyophilized or maintained at 4°C on slants of brain heart infusion agar.

Fixed *S. aureus* (Cowan 50758 and Wood 557601) was obtained from Calbiochem-Behring, La Jolla, Calif. A detailed description of the procedure for the preparation of the bacteria used has been published (8). Briefly, bacteria were grown at 37°C in a rotary shaker to the stationary phase in fortified Penassay broth (Difco Laboratories, Detroit, Mich.) containing 10 mg of Casitone (Difco) per ml and 5 mg of β -glycerophosphate per ml, harvested by centrifugation, suspended and washed twice in 150 mM NaCl-40 mM

phosphate (pH 7.2) containing 0.05% sodium azide, resuspended to 10% (vol/vol) concentration, and stirred rapidly at room temperature for 1.5 h after adding Formalin to give a 1.5% formaldehyde concentration. Bacteria were then centrifuged and resuspended to the same concentration, heated rapidly to 80°C in a water bath for 5 min, chilled quickly, washed twice more in the buffer, made to a final 10% (vol/vol) suspension for stocks, and stored at 4°C. Identical results were obtained with fixed or live bacteria, and the use of fixed bacteria in these experiments eliminated potential artifacts due to bacteria-associated proteolytic activities.

Mouse and rabbit immunoglobulin G (IgG), peroxidase-conjugated goat IgG, and peroxidase-conjugated goat anti-IgG was obtained from Cappel Laboratories, Cochranville, Pa. Human α_2 -macroglobulin and anti-ribitol teichoic acid were obtained from Calbiochem-Behring. Medium components were obtained from Difco. Other chemicals were obtained from J. T. Baker Chemical Co., Phillipsburg, N.J. Rabbit anti-staphylococcus enterotoxin B and bovine serum albumin (BSA) were obtained from Sigma. Microtiter plates (96-well flat-bottom plates for tissue culture, no. 3596) were obtained from Costar, Cambridge, Mass. The microtiter reader was a Bio-Tek model EL310 Autoreader, Bio-Tek Instruments, Burlington, Vt.

Human fibronectin was purified from human plasma by gelatin-Sepharose affinity chromatography (19) and was obtained from Meloy Laboratories, Springfield, Va. Mouse laminin from the EHS sarcoma grown intramuscularly in C57BL/6 mice was purified by neutral 0.5 M salt extraction, 1.7 M salt precipitation, and chromatography on DEAE-cellulose and A5 M agarose (9) and was a gift of Hynda Kleinman and George Martin, National Institute of Dental Research.

Human serum SF was prepared by sequential chromatography on glass microbeads, concanavalin A-Sepharose, DEAE-agarose, and Heparin-agarose and described previously (12). Mouse monoclonal antibody to human SF was derived and purified from serum-free hybridoma-conditioned culture medium by protein A-Sepharose affinity chromatography as described previously (11, 13). Fibronectin, laminin, and SF each were judged to be greater than 97% pure and free of contamination with the others by polyacrylamide gel electrophoresis, immunoblot, and enzyme-linked immunosorbent assay as described previously (11-13).

Assay of staphylococcus-binding activity. Microtiter wells were incubated for 1 h with 100 μ l of solutions of proteins to

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FIG. 1. *S. aureus* binding to microtiter wells precoated with the following: BSA, 10 $\mu\text{g/ml}$ (A); human serum SF, 10 $\mu\text{g/ml}$ (B); fibronectin, 10 $\mu\text{g/ml}$ (C); laminin, 10 $\mu\text{g/ml}$ (D). Magnification, $\times 448$.

be tested for staphylococcus-binding activity at the indicated concentrations in phosphate-buffered saline (PBS) consisting of (per liter) 0.2 g of KCl, 0.2 g of KH_2PO_4 , 8.0 g of NaCl, and 2.16 g of $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (pH 7.4). These solutions were aspirated, and the wells were washed three times with 200 μl of PBS containing 1 mg of BSA per ml. Immunoassay of precoating solutions after incubation on the microtiter wells and direct immunoassay of material bound to the microtiter wells by procedures described previously (5, 15) indicated that greater than 85% of the protein initially present in the precoating solutions had become associated with the wells under the conditions described.

After the precoating, wells were incubated for 1 h with 1% (vol/vol) suspensions of fixed *S. aureus* in PBS with BSA, followed by three washes as described above. Washing of the wells was performed gently from this point to prevent removal of microtiter-associated bacteria by fluid shear. *S. aureus* bound to the wells was quantitated by further incubations described below. All incubations were carried out at room temperature. Individual determinations of replicate samples in this assay did not vary more than 15% from the average. Results identical to those obtained with fixed bacteria were obtained with live bacteria.

For detection of the Cowan strain (protein A positive), wells were incubated for 30 min with 100 μl of peroxidase-conjugated goat IgG (1:1,000 in PBS with 1 mg of BSA per ml). This incubation was followed by three washes and the addition of peroxidase-dependent chromogen 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (Sigma) in phosphate-citrate buffer containing hydrogen peroxide as described previously (11-13). Extent of the enzyme-dependent reac-

tion was determined by spectrophotometric measurement of A_{405} of the reaction mixture after inhibition of the reaction by addition of 1 mg of NaF per ml.

Determination of dose response of the number of bacteria found at increasing bacterial numbers in the incubation mixture indicated that in a range between 0.25 and 2.5% (vol/vol) of the bacterial suspension a linear relationship existed between the bacterial concentration in the incubation suspension and the number of bacteria remaining associated with the microtiter well after the washes, and within this range the number of microtiter-associated bacteria represented approximately 0.01% of the total bacteria in the incubation suspension.

For detection of the Wood strain (protein A negative), wells were incubated for 1 h with rabbit anti-staphylococcus enterotoxin B (0.5 mg/ml in PBS) followed by three washes, incubation with peroxidase-conjugated anti-rabbit IgG (1:1,000), subsequent washes, and addition of 2,2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) as described above. Data presented have been corrected for background levels due to nonspecific binding of rabbit IgG. Backgrounds were determined by replacing rabbit anti-staphylococcus enterotoxin with an equal amount of nonspecific rabbit IgG.

RESULTS

Precoating of microtiter wells with fibronectin, laminin, or human serum SF resulted in binding of *S. aureus*, while binding was not observed in wells precoated with BSA or α_2 -macroglobulin (Fig. 1 and 2). Microscopic examination indicated that all three attachment factors promoted binding of Cowan or Wood strains of *S. aureus*, but only fibronectin

was capable of binding *S. epidermidis*, and none of the attachment factors bound *E. coli* (not shown). Binding of fibronectin and laminin to *S. aureus* and failure of these two attachment factors to bind *S. epidermidis* and *E. coli* have been observed with other methods, and the interaction of these proteins with *S. aureus* has been suggested as playing a role in host defenses and in invasiveness and pathogenicity of microbial infections (11, 12).

Previously we reported the development of a mouse IgG monoclonal antibody to human serum SF that inhibits the animal cell spreading-promoting activity of the protein (5). The monoclonal anti-SF antibody inhibited binding of bacteria to SF-coated microtiter wells but did not inhibit binding of bacteria to fibronectin-coated wells, confirming the specificity of the assay (Table 1). In this experiment the protein A-negative Wood strain was used as the indicator microorganism to avoid complicating interactions between bacterial surface-associated protein A and the added IgG. Binding of bacteria to the microtiter wells in this experiment was quantitated by peroxidase-linked immunoassay with anti-staphylococcus enterotoxin and was confirmed by microscopic examination. Identical results also were obtained when the immunoassay was carried out with anti-staphylococcus ribitol teichoic acid followed by anti-IgG peroxidase conjugate.

In experiments in which we determined the staphylococcus-binding specificity of soluble versus plastic bound protein, we found that soluble SF (88 $\mu\text{g/ml}$) was capable of inhibiting by about 40% bacterial binding to wells precoated with SF (10 $\mu\text{g/ml}$), but soluble SF did not significantly inhibit bacterial binding to wells precoated with fibronectin. In analogous experiments in which soluble fibronectin was incubated with staphylococcus in microtiter wells, aggregation of the bacteria occurred as a result of bacterial binding

TABLE 1. Inhibition of staphylococcus-binding activity of human serum SF by anti-SF monoclonal antibody

Incubation with IgG	A_{405}	
	SF precoat	Fibronectin precoat
PBS with 1 mg of BSA per ml ^a	0.456	0.574
Mouse monoclonal anti-SF (30 $\mu\text{g/ml}$ in PBS/BSA)	0.147	0.592
Nonspecific mouse IgG (30 $\mu\text{g/ml}$ in PBS/BSA)	0.474	0.644
Mouse monoclonal anti-SF (50 $\mu\text{g/ml}$ in PBS/BSA)	0.064	0.508
Nonspecific mouse IgG (50 $\mu\text{g/ml}$ in PBS/BSA)	0.507	0.514

^a No IgG or antibody.

to the dimeric fibronectin in solution, and the degree of inhibition by soluble fibronectin of bacterial binding to the plastic could not be estimated because of settling of the aggregated clumps of bacteria. Synthetic peptides derived from the amino acid sequence of the cell-binding site of fibronectin and capable of inhibiting both fibronectin-promoted and SF-promoted animal cell spreading (16) had no effect on the staphylococcus-binding activity of either fibronectin or SF (not shown).

DISCUSSION

Bacterial interactions with animal cell adhesion proteins have been examined by other investigators, because binding of microorganisms by these proteins may indicate a potential functional involvement in phagocytosis, invasion, or colonization during bacterial infection (11, 12, 14, 18). Like fibronectin, SF coated on latex beads promotes phagocytosis of the beads by animal cells (13), and our observation that human serum SF bound bacteria suggests that this protein also may mediate microbial disease processes or host responses in vivo. In addition, our results together with the previous reports regarding bacterial binding by fibronectin and laminin raise the possibility that invasion and colonization of the host may be influenced by independent interactions of bacteria with multiple glycoprotein components of the extracellular matrix.

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FIG. 2. Concentration dependence of *S. aureus*-binding activity of human serum SF, human fibronectin, and mouse laminin in microtiter assay. Binding of bacteria to wells that were not preincubated with the proteins represented an A_{405} of 0.18. Symbols: ○, human serum SF; ▲, human fibronectin; □, mouse laminin; △, human α_2 -macroglobulin; ●, BSA.

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