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Peptide Analogs to a Fibronectin Receptor Inhibit Attachment of *Staphylococcus aureus* to Fibronectin-Containing Substrates

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Binding of cells of *Staphylococcus aureus* to fibronectin has been proposed as a mechanism of bacterial adhesion to host tissues. In this study, we have attempted to define the role of a recently identified fibronectin receptor in the adhesion of staphylococcal cells to fibronectin-containing substrates by using different receptor analogs as potential inhibitors of bacterial adherence. The results showed that synthetic peptides D1, D2, and D3, corresponding to variations of a repeated unit in the fibronectin-binding domain of the receptor, and ZZ-FR, a chimeric protein containing the fibronectin-binding domain of the receptor with the D1, D2, and D3 sequences, inhibited the attachment of staphylococcal cells to microtiter wells coated with intact fibronectin or with the 29-kilodalton amino-terminal fragment of fibronectin. The chimeric protein ZZ-FR also partially inhibited the adherence of staphylococci to human plasma clots formed in vitro but had no effect on bacterial adhesion to clots formed from fibronectin-depleted plasma. These data confirm previous reports suggesting that fibronectin may serve as a substrate for adhesion of staphylococcal cells and indicate that bacterial adhesion is mediated by the identified fibronectin receptor. Furthermore, analogs to the fibronectin receptor can be used to inhibit the adhesion of bacterial cells to these model substrates, and these analogs may be of clinical use.

Bacterial adherence to host tissues is the first step in the development of many bacterial infections. In most cases, proteins present at the surface of bacterial cells (so-called adhesins) recognize and bind specific ligands in the host tissue. These interactions have been identified as targets in new pharmacological strategies to prevent and treat microbial infections. Analogs to the active sites of the adhesins or the tissue ligands may be useful in preventing infections by interfering with bacterial adherence. Furthermore, adhesin analogs may be important vaccine components, since generated antibodies may not only lead to an immunological recognition of the microorganisms but also prevent attachment to host tissues. Consequently, the molecular mechanisms of microbial adhesion have attracted substantial research interest during the past decade.

Earlier studies on bacterial adhesion focused primarily on lectins present on the fimbriae or pili of gram-negative bacteria, which were shown to mediate adhesion of the microorganism to host cells. The carbohydrate structures recognized by these bacterial surface lectins range from simple monosaccharides such as mannose, fucose, galactose, *N*-acetylglucosamine, and sialic acid to complex glycolipids and glycoproteins (for reviews, see references 2 and 21). During the past years, the recognition and specific binding of adhesive extracellular matrix proteins by different pathogenic bacteria have been demonstrated. Both gram-positive and gram-negative bacteria as well as yeast cells and other eucaryotic parasites have been shown to bind matrix proteins such as fibronectin, fibrinogen, collagen, laminin (for a review, see reference 9), vitronectin (1), and osteopontin (14a) with high degrees of specificity and affinity. These interactions have been proposed to potentially mediate microbial adherence to host tissues where the tissue location of the matrix protein may impose tissue specificity of the infection. A few in vitro studies (10, 11, 13, 17) have demonstrated that staphylococci and streptococci can bind fibronectin or adhere to fibronectin adsorbed on glass or plastic or present in plasma clots. It has also been suggested that staphylococcal colonization of polymethylmethacrylate prosthetic implants in vivo is mediated by bacterial adhesion to fibronectin that is deposited on the implanted material (19, 20), although other adsorbed proteins such as fibrinogen and laminin may also play a role in mediating microbial adhesion (8).

In previous studies, a fibronectin-binding protein (FNBP) with a molecular mass of ~200 kilodaltons (kDa) has been isolated (3, 6) from *Staphylococcus aureus* Newman. Recently the gene for the FNBP from *S. aureus* 8325-4 was cloned in *Escherichia coli* (4) and the nucleotide sequence of the gene was determined (15). The fibronectin-binding domain was localized to a 38-amino-acid unit repeated three times and partly a fourth time. Three synthetic peptides, D1, D2, and D3, which mimic the deduced amino acid sequences of the three 38-amino-acid repeats, were shown to express fibronectin-binding activity, as indicated by their ability to inhibit fibronectin binding to staphylococcal cells (15). A genetically constructed chimeric protein, ZZ-FR (molecular mass, 63 kDa), containing two immunoglobulin G (IgG)-binding domains of protein A and three fibronectin-binding repeats (D1, D2, and D3) per molecule, was found to have a much higher affinity for fibronectin than the individual peptides did. The objective of the present study was to evaluate analogs of the active site of the FNBP as inhibitors of the adhesion of *S. aureus* cells to fibronectin-containing substrates.

MATERIALS AND METHODS

Reagents. Human fibronectin was obtained from the New York Blood Center, New York, N.Y. Fibronectins isolated from bovine and porcine plasma were a gift from Richard LeBaron and Sherry Miles, Department of Biochemistry,

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University of Alabama, Birmingham. Human fibrinogen obtained from Kabi, Stockholm, Sweden, was further purified as previously described (12). The laminin-nidogen complex was a generous gift from Rupert Timpl, Max-Planck Institut für Biochemie, Martinsried, Federal Republic of Germany. Bovine serum albumin (BSA), gelatin, ovalbumin, IgG, fetuin, and hemoglobin were from Sigma Chemical Co., St. Louis, Mo. Iodogen was from Pierce Chemical Co., Rockford, Ill. Brain heart infusion medium was supplied by Difco Laboratories, Detroit, Mich. $Na^{125}I$ was obtained from Amersham Corp., Arlington Heights, Ill., and thrombin was obtained from Parke Davis, Morris Plains, N.J. The 29-kDa amino-terminal domain of fibronectin was generated by digestion of intact protein with thermolysin.

Fibronectin receptor analogs. A chimeric protein consisting of a truncated form of protein A fused with an ~200-amino-acid segment of the staphylococcal fibronectin receptor containing the active site was described elsewhere (4). The active site of the receptor consists of a 38-amino-acid motif repeated three times and partly a fourth time. Synthetic peptides D1, D2, and D3, which mimic the three 38-amino-acid units, were produced by the protein core facility of the Cancer Center at the University of Alabama at Birmingham as described previously (15).

Bacterial cultures. The source of strains of *S. aureus* used in this study (Newman and strain 8325-4) have been described previously (4, 6). Bacterial cultures were grown in brain heart infusion broth overnight. After being harvested, bacteria were incubated at 88°C for 20 min to inactivate proteases, and the bacterial suspension was stored at −20°C.

Radiolabeling of bacteria. Bacteria (10^{10} cells in 0.5 ml of phosphate-buffered saline [PBS]) were mixed with 0.2 mCi of $Na^{125}I$ and incubated for 10 min in glass tubes (12 by 75 mm) coated with iodogen. The glass tubes were coated with iodogen by adding 25 μl of a 0.5-mg/ml solution of iodogen in chloroform or dichloromethane to the bottom of the tubes and gently rotating the tubes until evaporation of the solvent was complete. The iodinated bacterial suspension was diluted with 3 ml of PBS and then centrifuged at 1,350 × *g* for 15 min. The supernatant containing unincorporated iodine was aspirated, and the pellet of iodinated bacteria was suspended in 3 ml of PBS. The radiolabeled bacteria were washed three additional times with PBS, suspended in 2 ml of PBS with 0.1% BSA, 5 mM phenylmethylsulfonyl fluoride, and 5 mM *N*-ethylmaleimide, and stored at 4°C. Radiolabeled bacteria were used within 8 to 10 days after iodination. In experiments with freshly iodinated bacteria, about 15 to 20% of the added bacteria bound to the wells. However, the ability of the bacteria to adhere to fibronectin gradually declined by about 50% in a week.

Coating of plastic surfaces. Adhesion experiments were performed by using microtiter plates with detachable wells (Immulon 2; Dynatech Industries, Inc., Chantilly, Va.). The wells were coated by incubation with 50 μl of a protein solution at the indicated concentrations overnight at 4°C. The microtiter plates were shaken at 50 rpm during the incubation period and then washed four times with 200 μl of PBS to remove unbound proteins. The wells were subsequently incubated with 50 μl of 1% BSA at 4°C for 25 min to block additional protein-binding sites and again washed four times with 200 μl of PBS. The specific activity of the bacterial preparation was about 10^4 cpm/10^6 bacteria.

Preparation of human plasma clots. Clotting of plasma was induced by mixing 20 μl of a thrombin solution (320 U of thrombin per ml in water containing 0.15 M NaCl and 0.125 mM $CaCl_2$) with 20 μl of human plasma (American Red Cross) in microtiter wells. The mixture was incubated at 37°C for 30 min, and the clot formed was washed five times with 200 μl of PBS. Control wells were set up by mixing plasma with clotting buffer in the absence of thrombin. Fibronectin-depleted clots were prepared by using plasma that had been passed through a gelatin-Sepharose column containing 0.2 mg of gelatin per milliliter of Sepharose. Microtiter wells used for preparation of plasma clots had been precoated with 100 μl of 1% BSA at 4°C for 25 min and then washed four times with 200 μl of PBS.

Attachment assays. Suspensions of radiolabeled bacteria (2 × 10^7 cells in 50 μl of PBS containing 0.1% BSA) were incubated with the protein-coated wells for 2 h or with plasma clots for 25 min at 37°C in the presence or absence of potential inhibitors. When potential inhibitors were tested, the wells were preincubated with 25 μl of inhibitor at 37°C. After 1 h, 25 μl of bacterial suspension (containing 2 × 10^7 cells) was added, and the incubation was continued for 2 h at 37°C. Unbound bacteria were removed by washing the wells five times with PBS, and the number of labeled bacteria attached per well was quantified with a gamma counter.

Scanning electron microscopy. Tissue culture cover slips (13-mm diameter, Miles Scientific, Div. Miles Laboratories, Inc., Naperville, Ill.) were placed in 24-well dishes (Linbro) containing 0.05 mg of human fibronectin per ml. Coating was carried out at 4°C overnight with gentle shaking (about 50 rpm) on a gyratory platform shaker. At the end of the incubation period, the fibronectin solution was aspirated and the cover slips were washed four times with 2 ml of PBS. The fibronectin-coated cover slips were further treated with 0.5 ml of PBS containing 1% BSA at 4°C with gentle shaking for 20 min and then washed again four times with 2 ml of PBS.

Protein-coated cover slips were placed in six-well tissue culture dishes (Costar, Cambridge, Mass.), and 50 μl of the bacterial suspension (10^9 cells per ml) was gently spread over each cover slip. Bacteria were incubated with cover slips coated with fibronectin or with BSA alone (negative controls) at 37°C for 2 h. In attempts to inhibit bacterial attachment, the cover slips were preincubated with 50 μl of the fusion protein solution (1 mg/ml) at 37°C for 1 h, and subsequent incubations with bacteria were continued in the presence of the fusion protein. Unbound bacteria were removed by washing the cover slips five times with 2 ml of PBS. The samples were fixed with 0.1% glutaraldehyde at 4°C overnight and then treated successively with 2 ml each of 50, 70, 80, and 90% ethanol and twice with 2 ml of absolute ethanol. Each ethanol treatment was at room temperature for at least 30 min. The specimens were next subjected to critical-point drying as previously described (14). The dried specimens were fixed on stalks with silver paint, coated with a 20-nm-thick gold plating, and examined with an ISI 100B scanning electron microscope.

RESULTS

Attachment of staphylococci to substrates composed of different isolated proteins. Different proteins were adsorbed in microtiter wells, and the abilities of the proteins to serve as substrates for the adhesion of staphylococcal cells was evaluated. The proteins tested included fibronectin (human, bovine, or porcine), the 29-kDa amino-terminal fragment of fibronectin, fibrinogen, albumin, ovalbumin, gelatin, IgG, fetuin, laminin-nidogen complex, and hemoglobin at concentrations of 0.05 or 0.5 mg/ml, as described in Materials and Methods. Radiolabeled bacteria (2 × 10^7 cells in 50 μl) were

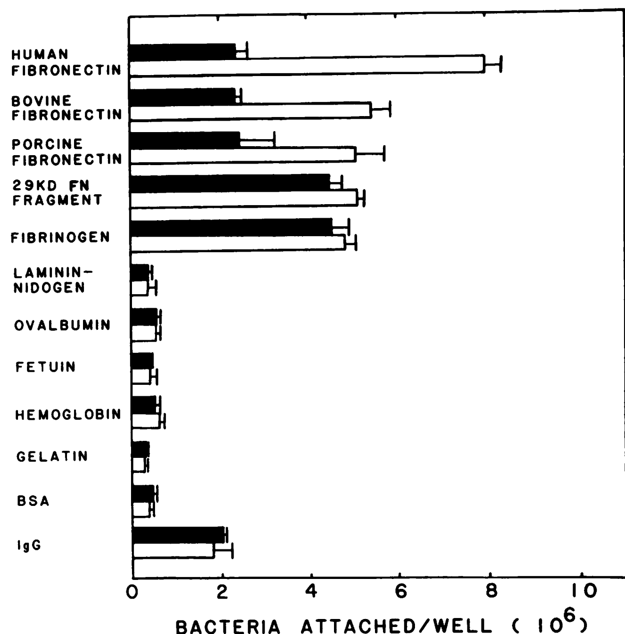

FIG. 1. Attachment of ^{125}I-labeled cells of *S. aureus* Newman to different proteins. ^{125}I-labeled bacteria (2×10^7 cells in 50 μl of PBS–0.1% BSA) were incubated at 37°C in microtiter wells precoated with 50 μl of solutions of different proteins at 0.05 (■) or 0.5 (□) mg/ml. After 2 h, the wells were washed and bacterial attachment was quantitated as described in Materials and Methods. Bars represent standard deviations from the means of quadruplicate determinations.

added to the protein-coated wells, and the microtiter plate was incubated for 2 h at 37°C. Subsequently, unattached cells were removed, the wells were washed, and the number of radiolabeled cells attached to the wells was determined. The results (Fig. 1) indicate that staphylococci adhered to wells coated with fibronectin regardless of whether the source was human, bovine, or porcine plasma; fibrinogen; or the 29-kDa amino-terminal fragment of fibronectin. These results are consistent with results from previous studies (11, 13), which showed that staphylococci attach effectively to surfaces coated with human fibronectin and fibrinogen. Of the other proteins tested as substrates for the adherence of staphylococcal cells, only IgG supported bacterial adherence, but at a low level. The relatively low efficiency of bacterial adherence to IgG substrates is surprising in view of the very high concentration of the IgG-binding protein A present on the staphylococcal surface.

Inhibition of bacterial adherence by analogs to the FNBP. Synthetic analogs of the FNBP from *S. aureus* have been shown to effectively inhibit binding of soluble ^{125}I-labeled fibronectin to staphylococci (15). In the current study, three synthetic peptide analogs of the active site of the receptor, designated D1, D2, and D3, and the chimeric protein ZZ-FR, which contains the active site of the FNBP, were tested as potential inhibitors of the adhesion of cells of two strains of *S. aureus* to fibronectin-containing substrata. The results show that synthetic peptides D1, D2, and D3 and the fusion protein ZZ-FR effectively inhibit adherence of strain 8325-4 to substrates composed of the amino-terminal domain (Fig. 2B). Also, the attachment of cells of strain Newman to substrates of the 29-kDa fragment was inhibited by the FNBP analogs, although in this case D1 was somewhat less effective than the other FNBP analogs (Fig. 2A).

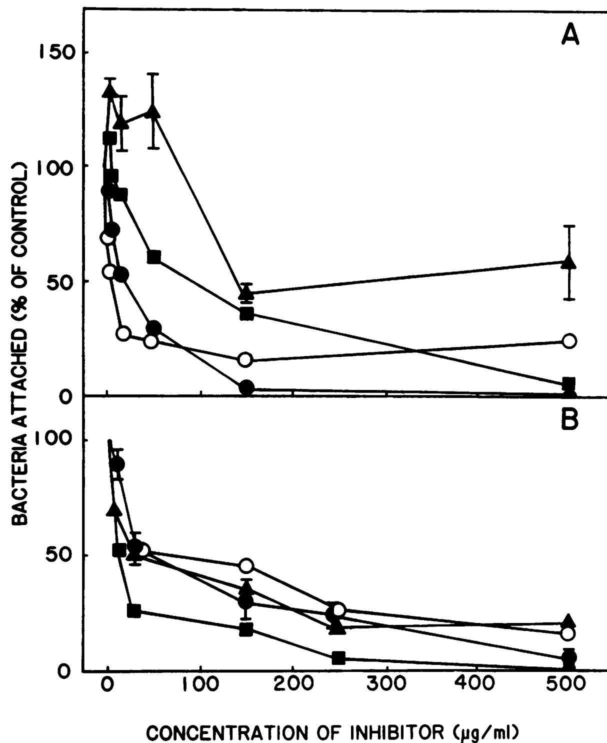

FIG. 2. Effect of synthetic peptides and fusion protein ZZ-FR on attachment of ^{125}I-labeled staphylococci to substrates composed of the 29-kDa amino-terminal fragment of human fibronectin. Microtiter wells coated with 25 μg of the 29-kDa amino-terminal fibronectin fragment per ml were incubated with 25 μl of the indicated concentrations of peptides D1 (▲), D2 (■), and D3 (●) and fusion protein ZZ-FR (○) or PBS alone (control) at 37°C for 1 h. ^{125}I-labeled bacteria (2×10^7 cells in 25 μl of PBS–0.1% BSA) were then added to each well, mixed well, and further incubated at 37°C for 2 h. The wells were then washed, and bacterial attachment was quantitated as described in Materials and Methods. Bars represent standard deviations from the means of quadruplicate determinations. (A) *S. aureus* Newman; (B) *S. aureus* 8325-4.

The FNBP analogs were also tried as potential inhibitors when intact fibronectin coating the microtiter wells was used as a substrate for bacterial adherence. The fusion protein efficiently inhibited the adherence of both strain Newman and strain 8325-4 (Fig. 3). Peptide D3 was essentially as effective as ZZ-FR, whereas D2 was somewhat less active and D1 only reduced the number of adhering bacteria by less than 50%.

The inhibitory effect of the fusion protein ZZ-FR on attachment of cells of *S. aureus* Newman to fibronectin-coated substrates was confirmed by using scanning electron microscopy (Fig. 4). The number of cells of *S. aureus* attaching to fibronectin-coated cover slips was greatly reduced in the presence of the fusion protein ZZ-FR (compare Fig. 4A and B) and was comparable to the number of bacteria adhering on BSA-coated cover slips (Fig. 4C).

Attachment of staphylococci to plasma clots. *S. aureus* is a primary wound pathogen. In the wound, the microorganism can attach to fibronectin in the extracellular matrix of the damaged tissue and plasma fibronectin incorporated into the blood clot (7). In addition, other adhesive mechanisms may also be of importance. The potential role of fibronectin in the blood clot as a substrate for bacterial adherence was examined in an in vitro model system in which radiolabeled

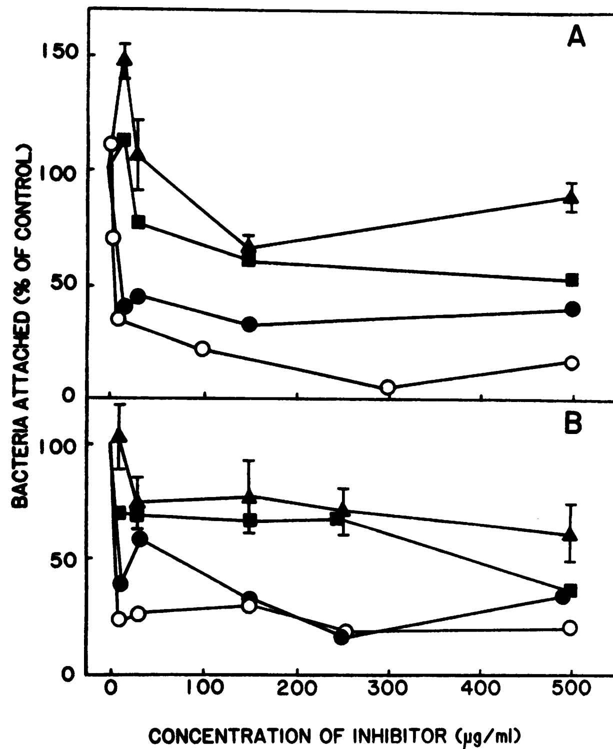

FIG. 3. Effect of synthetic peptides and fusion protein on attachment of cells of *S. aureus* to fibronectin-coated wells. Microtiter wells coated with 0.05 mg of human fibronectin per ml were incubated with 25 μl of the indicated concentrations of peptides D1 (▲), D2 (■), and D3 (●) and the fusion protein ZZ-FR (○) or with PBS alone (control) at 37°C for 1 h. ^{125}I-labeled bacteria (2×10^7 cells in 25 μl of PBS–0.1% BSA) were then added to each well, mixed well, and incubated further at 37°C for 2 h. Bacterial attachment was quantitated after the wells were washed as described in Materials and Methods. Bars represent standard deviations from the means of quadruplicate determinations. (A) *S. aureus* Newman; (B) *S. aureus* 8325-4.

bacteria were seeded on a substrate composed of thrombin-induced clots of intact and fibronectin-depleted human plasma. The number of cells (*S. aureus* Newman) adhering to plasma clots was unaffected by the depletion of plasma fibronectin when the attachment assay was carried out at low bacterial densities (Fig. 5). However, bacterial attachment to plasma clots depleted of plasma fibronectin was significantly less (up to 50%) than was attachment to clots made of intact plasma when the number of cells seeded on the substrate was increased, indicating that fibronectin present in the plasma clots can serve as a substrate for bacterial adhesion. This observation is consistent with earlier studies (17) which showed decreased bacterial adherence to plasma clots depleted of fibronectin. When low numbers of bacteria are seeded on the plasma clot, it appears that components other than fibronectin (e.g., fibrin) can efficiently mediate adherence of bacterial cells. However, when these sites are saturated, the incorporated fibronectin becomes an impor-

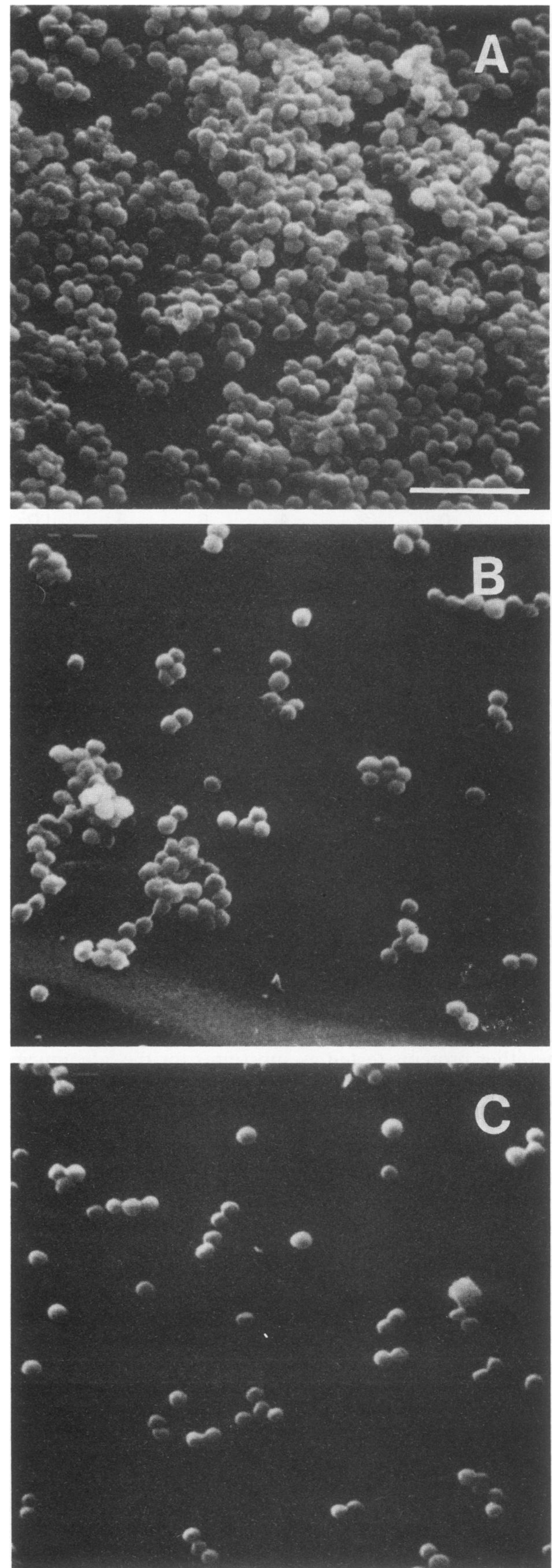

FIG. 4. Scanning electron microscopy of cells of *S. aureus* Newman adhering to fibronectin-coated cover slips in the absence (A) or presence (B) of the fusion protein ZZ-FR (500 μg/ml). (C) Electron micrograph of *S. aureus* Newman cells attached to BSA-coated cover slips. Magnification, × ~4,000 in all panels. Bar, 5 μm.

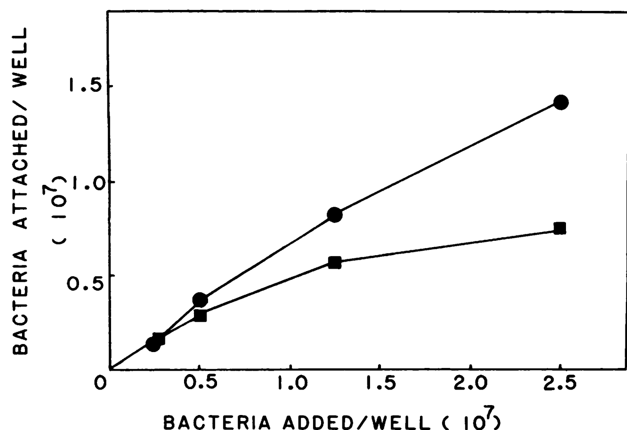

FIG. 5. Effect of fibronectin depletion on attachment of ^{125}I-labeled cells of *S. aureus* Newman to human plasma clots. ^{125}I-labeled bacteria at the indicated cell densities were incubated at 37°C with plasma clots prepared from regular (●) or fibronectin-depleted (■) plasma. After 25 min the clots were washed, and bacterial attachment was quantitated as described in Materials and Methods.

tant alternate substrate for adherence of staphylococcal cells.

The effects of various concentrations of the peptides D1, D2, and D3 and the fusion protein ZZ-FR on the adherence of cells of strain Newman to plasma clots were studied. The results (Fig. 6) suggest that the fusion protein ZZ-FR could at the most reduce the number of bacteria adhering to the clots made from intact plasma by 50%. These data are consistent with the observed 50% maximal reduction in the number of bacteria adhering to fibronectin-depleted plasma clots. The presence of the ZZ-FR fusion protein did not affect the number of bacterial cells adhering to clots made from fibronectin-depleted plasma. Hence, the fusion protein ZZ-FR appears to efficiently inhibit fibronectin-dependent adhesion also when this protein is incorporated in a plasma clot. None of the three synthetic peptides tested reduced the number of bacterial cells adhering to regular clots by more than 20% (data not shown).

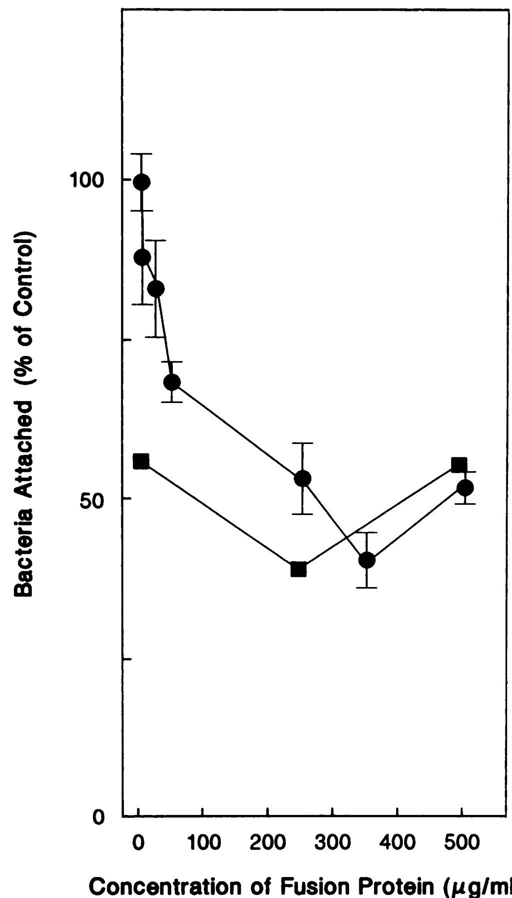

FIG. 6. Effect of increasing concentrations of fusion protein on attachment of ^{125}I-labeled *S. aureus* Newman to in vitro plasma clots. Plasma clots prepared from either regular (●) or fibronectin-depleted (■) plasma were incubated with 25 µl of the indicated concentrations of the fusion protein at 37°C for 1 h. Bacteria (2×10^7 cells in 25 µl of PBS–0.1% BSA) were added to each well, and incubations were continued at 37°C for 25 min. The clots were then washed, and bacterial attachment was quantitated as described in Materials and Methods.

DISCUSSION

Previous studies have shown that fibronectin-containing matrices formed in vitro (11, 13, 16, 18) or in vivo (19, 20) may serve as substrates for the attachment of staphylococcal cells. On the basis of these observations, it has been proposed that the recognition of fibronectin plays a role in the initial phase of staphylococcal wound and foreign-body infections. If this hypothesis is correct, then the development of reagents that interfere with bacterial attachment to fibronectin may be a useful strategy for prevention and treatment of such infections. Soluble fibronectin is effective in blocking attachment of certain types of bacteria, e.g., *E. coli*, to cultured cells (5). However, in other cases, e.g., adhesion of staphylococci to fibronectin substrates (11), soluble fibronectin seems less effective in inhibiting bacterial attachment. It is possible that these bacteria bind fibronectin in a manner that allows the bound fibronectin to associate with other fibronectin molecules in the substratum, thus serving as a bridging ligand. Alternatively, conformational differences between soluble fibronectin and fibronectin deposited in a matrix may account for the apparent preferential binding of bacteria to the adsorbed protein. In this study, we have examined the usefulness of analogs of the staphylococcal FNBP as inhibitors of bacterial attachment to fibronectin-containing substrata.

When analogs of the active site of the staphylococcal FNBP were tested as inhibitors of bacterial attachment, the peptides D1, D2, and D3 as well as the fusion protein ZZ-FR inhibited staphylococcal attachment to plastic substrates coated with intact fibronectin or the 29-kDa amino-terminal fragment of fibronectin. Peptide D3 and the fusion protein ZZ-FR were more active than peptides D1 and D2 in inhibiting bacterial attachment to intact fibronectin. All three peptides were more active than intact fibronectin in inhibiting adhesion to the 29-kDa domain, suggesting that there are conformational differences in the bacterial binding site in the two forms of fibronectin.

A comparison of the inhibitory effects of the FNBP analogs on staphylococcal attachment with the earlier study (15) on the effect of FNBP analogs on binding of soluble fibronectin reveals some interesting differences. In binding assays using soluble fibronectin, the fusion protein ZZ-FR was at least 1,000-fold more active on a molar basis in

inhibiting fibronectin binding than were any of the isolated peptides. In the adhesion assays, however, we found only marginal differences in the abilities of ZZ-FR and peptide D3 to inhibit staphylococcal attachment to plastic surfaces coated with intact fibronectin or the 29-kDa fragment.

The D peptides were essentially ineffective in inhibiting the adherence of staphylococci to plasma clots formed in vitro, although the fusion protein ZZ-FR was able to decrease bacterial adherence to plasma clots to the same level as adherence of bacteria to clots prepared from fibronectin-depleted plasma. These results suggest that fibronectin in solution or incorporated into a plasma clot (7) is conformationally different from fibronectin adsorbed on a plastic surface, which may affect how the protein is presented to staphylococcal fibronectin receptors.

Since depletion of fibronectin from plasma causes at most a 50% decrease in the number of bacteria adhering to plasma clots, it appears that additional mechanisms may be involved. Preliminary studies in this laboratory (R. Raja, L. Switalski, and M. Hook, unpublished data) have shown that an antibody directed to the staphylococcal fibrinogen receptor in combination with the fusion protein ZZ-FR further decreases the number of bacteria adhering to a plasma clot, suggesting that fibrinogen-fibrin binding also is an adhesion mechanism.

The results presented above show that the D peptides and the fusion protein ZZ-FR significantly reduced bacterial adherence to fibronectin-containing substrata and may be useful in reducing bacterial attachment in fibronectin-rich environments such as surface wounds and implanted prosthetics. Although it is attractive to speculate that peptide analogs to the *S. aureus* FNBP may be of clinical use in the treatment and prevention of staphylococcal infections, the studies reported here have been performed in vitro and it appears likely that in vivo bacteria use multiple mechanisms for attachment; consequently, several adhesins might have to be blocked.

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