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Use of Adhesion-Defective Mutants of *Staphylococcus aureus* To Define the Role of Specific Plasma Proteins in Promoting Bacterial Adhesion to Canine Arteriovenous Shunts

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We used an ex vivo canine arteriovenous shunt model, previously developed to study plasma protein adsorption and thrombogenesis on polymeric biomaterials, to define the role of host proteins in promoting adhesion of *Staphylococcus aureus*. Either polyethylene or polyvinyl chloride tubings were exposed to canine blood for 5, 15, or 60 min at a flow rate of 300 ml/min and then were flushed in phosphate-buffered saline (PBS), cut into 1.5-cm segments, and stored at −70°C. After thawing, each segment was preincubated in 0.5% albumin in PBS to prevent nonspecific staphylococcal attachment to surfaces that were not exposed to blood. Each segment was then incubated with 4×10^6 CFU of [^{3}H]thymidine-labelled *S. aureus* per ml for 60 min at 37°C in an in vitro adhesion assay. Two site-specific mutants of *S. aureus* were tested: one specifically defective in adhesion to fibrinogen (Fg_{Ad}-def). Compared with their respective parental strains, the Fg_{Ad}-def, but not the Fn_{Ad}-def, mutant of *S. aureus* showed a strong (>80%) decrease in attachment to ex vivo tubings. The adhesion of each strain of *S. aureus* onto polyethylene was consistently more than twofold higher than the adhesion onto polyvinyl chloride segments exposed to flowing blood for 5 or 15 min, but adhesion became similar to that on polyvinyl chloride after 60 min of exposure. In conclusion, the specific adhesion-defective mutants of *S. aureus* suggested that fibrinogen was the most active adhesion-promoting protein in a short-term blood-material interaction. The experimental approach described in this study should prove useful for screening materials thought to be resistant to protein-mediated staphylococcal adhesion and colonization.

Staphylococcus aureus is a major pathogenic organism involved in colonization of orthopedic implants or cardiovascular devices and is responsible for life-threatening metastatic infections (20). Blood-contacting materials such as vascular grafts, catheters, kidney dialyzers, and cardiac assist devices trigger multiple and complex events at their interfaces, involving adsorption of numerous plasma proteins and the deposition and activation of platelets. Colonization of cardiovascular biomaterials is thought to occur on plasma proteins and/or platelets (10, 43) that have been adsorbed on the artificial surfaces shortly after their implantation. Several in vitro studies have shown that *S. aureus* attachment to polymeric surfaces is strongly promoted by selectively adsorbed plasma or extracellular matrix proteins such as fibrinogen (2, 10, 12, 21), fibronectin (12, 34, 35, 38–42), collagen (13, 26, 27, 42), vitronectin (3, 18, 28), laminin (12, 19, 42), and thrombospondin (11). The clinical relevance of these in vitro observations was documented by clinical studies (35, 36) showing the in vivo contribution of fibronectin and fibrin or fibrinogen to *S. aureus* attachment to catheters inserted in hospitalized patients.

Progress has been made in elucidating bacterial cell wall-associated surface components that promote specific interactions with individual host proteins. These studies allowed characterization of the genes for the fibrinogen-binding protein (clumping factor [21]), a collagen adhesin (26, 27), and two distinct but related fibronectin-binding proteins (7, 14). Also, site-specific mutants of *S. aureus* specifically defective in adhesion to a single host protein such as fibrinogen (21) or fibronectin (15) have been isolated.

While clinical studies using inserted catheters harvested from patients were instrumental in showing the in vivo contribution of fibronectin (36) and fibrinogen or fibrin (35) to staphylococcal adhesion, they were not designed to characterize the dynamic events of sequential plasma protein adsorption and their specific contribution to *S. aureus* adhesion onto polymer tubing. Previous reports have demonstrated that plasma protein and platelet coating of biomaterials is a dynamic process involving selective adsorption and then release of various components (5, 6, 17, 23, 24). To define the temporal relationship between plasma protein adsorption and bacterial adhesion, we selected an animal model of an arteriovenous shunt previously developed to study thrombogenesis on polymeric biomaterials (4, 5, 17). The ability to make sequential measurements over time in this in vivo model combined with the use of adhesion-defective bacterial mutants allowed identification of fibrinogen or fibrin as the most active plasma component promoting *S. aureus* attachment to the experimental shunt tubing in a brief exposure to whole, non-anticoagulant-treated blood.

(The results of this study were presented in part at the 31st Interscience Conference on Antimicrobial Agents and Chemotherapy, 1991 [37].)

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MATERIALS AND METHODS

Canine ex vivo arteriovenous shunt model. The canine ex vivo femoral arteriovenous series shunt model allowing continuous quantitative monitoring of thromboembolization with non-anticoagulant-treated blood under the influence of shear flow (23–25) has been previously described (4, 5, 8, 17). This method also allows the simultaneous measurement of platelet and plasma protein deposition on different polymer surfaces. Either polyvinyl chloride (PVC; 0.125-in. [0.318-cm] internal diameter; Tygon, Norton Plastics, Akron, Ohio) or polyethylene (PE; 0.125-in. [0.318-cm] internal diameter; Intramedic, Parsippany, N.J.) polymer tubing was exposed to canine blood for 5, 15, or 60 min at a flow rate of 300 ml/min. The tubing was then flushed with 0.1 M phosphate-buffered saline (PBS) (pH 7.4), cut into 1.5-cm segments, and stored at −70°C.

Scanning electron microscopy. To determine luminal surface morphology of the polymer tubings after 5, 15, and 60 min of exposure to canine blood, duplicate segments were serially dehydrated in ethanol-water conditions, dried by the critical point method, mounted, and sputter coated with gold for examination in a Jeol-JSM 35C scanning electron microscope with an accelerating voltage of 15 kV (25).

In vitro attachment properties of bacterial strains. The following strains of *S. aureus* were tested. (i) Strain Newman and its Fg_{Ad}-def mutant strain DU5852, which is defective in the production of the fibrinogen-binding protein (clumping factor) were tested. Strain DU5852 is the transposon Tn*917* insertion mutant *clfA1* of strain Newman within the *clfA* gene encoding the fibrinogen adhesin, whose molecular characterization and functional properties have recently been described in detail (21). (ii) Strain 8325-4 and its Fg_{Ad}-def mutant of 8325-4 (DU5880), which carries the same transposon insertion in the *clfA* gene as DU5852 (21), were tested. An additional derivative strain of 8325-4, harboring multicopy plasmid pCF4 bearing the wild-type *clfA* gene enhancing adhesion to surface-bound fibrinogen as previously described (21), was used as a specific indicator of in vivo fibrinogen-promoted adhesion. (iii) Strain 879R4S and a Tn*918* insertion mutant (879R4S/1536), which exhibits defective in vitro adhesion to fibronectin (Fn_{Ad}-def mutant) and reduced in vivo attachment to traumatized rat heart valves (15), were tested. Recent unpublished studies (9) indicate that the transposon insertion in strain 879R4S/1536 occurred between the promoter and coding sequence of the single fibronectin-binding gene carried out by this particular strain. (iv) Strain Cowan I, which adheres strongly to surface-bound fibronectin and surface-bound fibrinogen in a fashion similar to that of the bacteremic isolates (36), was used as an internal control of the bacterial adhesion system.

Canine fibronectin was purified from canine plasma as previously described (16, 33). Canine fibrinogen was purchased from Sigma (St. Louis, Mo.), and contaminating fibronectin was removed by gelatin adsorption (16, 33).

The attachment properties of adhesion-defective mutant and parental strains of *S. aureus* were measured by using a previously described adhesion assay with polymethylmethacrylate (PMMA) coverslips coated with purified proteins (21, 22, 36, 39). Two-sided coverslips, prepared as previously described (39), were coated with increasing amounts of immobilized fibrinogen by incubation for 60 min at 37°C with increasing concentrations (from 0.25 to 2 μg/ml) of purified canine fibrinogen in solution. At the end of the protein coating phase, coverslips were rinsed in PBS as previously described (21, 22). It was ascertained that the PMMA surfaces were coated in a dose-dependent manner with amounts of canine fibrinogen ranging from 32 to 248 ng/cm^2 (41 to 317 ng per coverslip) when fibrinogen, radiolabelled by reductive methylation with sodium boro [^{3}H]hydride (36), was used.

To optimize adsorption of purified canine fibronectin from concentrations below 1 μg/ml, PMMA coverslips were precoated with gelatin (1 mg/ml) as previously described (36, 39). After a rinsing in PBS, gelatin-coated PMMA coverslips were incubated for 60 min at 37°C with low concentrations (ranging from 0.125 to 1 μg/ml) of fibronectin, followed by rinsing in PBS (36, 39). These conditions allowed a linear coating of PMMA surfaces with ^{3}H-labelled fibronectin ranging from 15 to 132 ng/cm^2 (22 to 190 ng per coverslip).

The adhesion characteristics of the different parental and mutant strains of *S. aureus* were evaluated by incubating the fibrinogen- or fibronectin-gelatin-coated PMMA coverslips with 4×10^6 CFU of washed log-phase cultures, metabolically radiolabelled with [^{3}H]thymidine in Mueller-Hinton broth as previously described (39). Each protein-coated PMMA coverslip was incubated with the suspension of radiolabelled bacteria for 60 min at 37°C as previously described (22, 39). The adhesion medium was PBS with 1 mM Ca^{2+} and 0.5 mM Mg^{2+}, supplemented with 5 mg of human serum albumin per ml, which prevented nonspecific adhesion of *S. aureus* (21, 22, 36, 39). Coverslips were then rinsed, and the number of attached bacteria was estimated from radioactive counts (39). Albumin-coated and gelatin-coated PMMA coverslips were used as controls of adhesion to fibrinogen- and fibronectin-coated surfaces, respectively (21, 22, 39).

Attachment of *S. aureus* to protein-coated PMMA coverslips was scored as the number of adherent CFU (10^3/cm^2).

Bacterial adhesion to ex vivo polymer tubing. The conditions for assaying adhesion of *S. aureus* onto ex vivo polymer tubing segments were essentially similar to those described above for protein-coated PMMA coverslips. Frozen segments of blood-exposed PVC or PE tubing were thawed just before the assay and preincubated for 60 min at 20°C with 0.5% human albumin in PBS to prevent nonspecific attachment to the external, blood-unexposed surface.

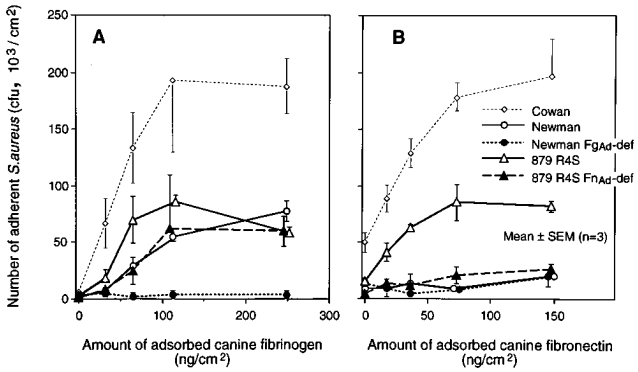

FIG. 1. Attachment properties of adhesion-defective mutant and parental strains of *S. aureus* tested on PMMA coverslips coated in vitro with either purified canine fibrinogen (A) or fibronectin (B). SEM, standard error of the mean.

Duplicate segments for each time point of blood exposure were then tested for promotion of bacterial adhesion by incubation with suspensions of each strain of *S. aureus* for 60 min at 37°C. The concentration (4×10^6 CFU/ml) of radiolabelled bacteria and the conditions for the adhesion assay were identical to those of the assay performed with PMMA coverslips. Attachment of *S. aureus* to each tubing segment was scored as the number of adherent CFU (10^3/cm^2), to facilitate the comparison with attachment to protein-coated PMMA coverslips.

Bacterial adhesion to in vitro fibrinogen-coated polymer tubing. When the specific contribution of fibrinogen to *S. aureus* attachment to polymer tubing was studied, 1.5-cm-long segments of native PVC or PE, coated in vitro for 60 min at 37°C with concentrations of canine fibrinogen ranging from 1 to 16 μg/ml, were incubated in the bacterial adhesion assay as described above. These concentrations of canine fibrinogen were selected from dose-response curves of canine fibrinogen immobilized on either type of polymer tubing, whose amounts were determined with ^{3}H-labelled canine fibrinogen as described above.

RESULTS

Selective attachment of parent and mutant strains of *S. aureus*. Figure 1A shows that promotion of bacterial attachment by immobilized canine fibrinogen is optimal at a concentration of 100 ng/cm^2, both for the reference strain Cowan and for the parental strains Newman and 879R4S. The Fg_{Ad}-def (clumping factor defective) strain DU5852 (21) of Newman, but not the Fn_{Ad}-def mutant strain (879R4S/1536) of 879R4S (15), showed a selective defect in attachment to canine fibrinogen. Figure 1B shows that immobilized canine fibronectin promoted attachment of strains 879R4S and Cowan to the same extent as fibrinogen, but adhesion of strain Newman and its Fg_{Ad}-def mutant was not promoted. The Fn_{Ad}-def mutant strain of *S. aureus* 879R4S showed a selective decrease in attachment to surface-bound fibronectin.

Control experiments confirmed that bacterial attachment of parental and mutant strains of *S. aureus* promoted by canine fibrinogen or fibronectin was equivalent to that of the same proteins of human origin (data not shown).

Bacterial attachment to ex vivo polymer tubings. The promotion of *S. aureus* adhesion by segments of ex vivo polymer tubing exposed to canine blood for either 5, 15, or 60 min is shown in Fig. 2. After 5 min of blood exposure, adhesions of all parental and mutant strains to either PVC or PE tubing segments had already reached their maximum values. Significant differences were found between PVC and PE tubing segments exposed for either 5 or 15 min. Whereas adhesion of Newman, 879R4S, and Cowan strains to PVC tubing segments remained constant throughout the 60-min period of blood exposure (Fig. 2A), each of these strains showed an approximately twofold increase in attachment to PE compared with PVC exposed for 5 or 15 min (Fig. 2B). However, at 60 min, adhesion of strains

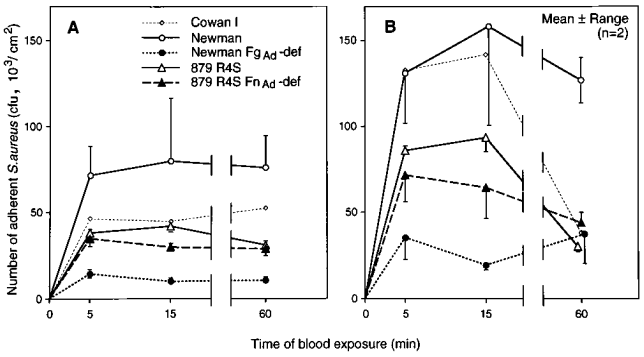

FIG. 2. Attachment of adhesion-defective mutant and parental strains of *S. aureus* to segments of ex vivo PVC (A) and PE (B) tubing exposed to canine blood for 5, 15, or 60 min.

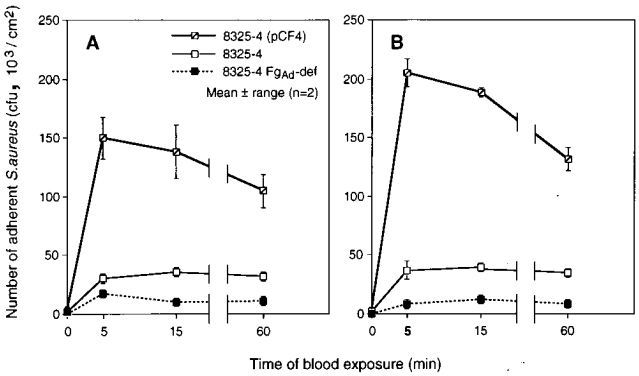

FIG. 3. Attachment of parental and mutant strains of *S. aureus* 8325-4 expressing different levels of affinity for fibrinogen onto ex vivo PVC (A) and PE (B) tubing exposed for 5, 15, or 60 min to canine blood.

Cowan, 879R4S, and, to a lesser extent, Newman onto ex vivo PE tubing segments decreased to levels almost equivalent to those found on PVC (Fig. 2B). On both types of polymer tubing, the Fg_{Ad}-def mutant of strain Newman, but not the Fn_{Ad}-def mutant of strain 879R4S, showed a strong (>80%) reduction in attachment compared with the parental strain. The selective reduction in the attachment of an Fg_{Ad}-def mutant of *S. aureus* to both types of ex vivo polymer tubing identified fibrinogen or its derivative fibrin as the most active plasma component promoting *S. aureus* attachment to the canine shunt tubing.

To document further the contribution of fibrinogen to *S. aureus* attachment in the canine shunt model, we incubated ex vivo polymer tubing segments with a derivative of *S. aureus* 8325-4, which contains a multicopy plasmid leading to overexpression of clumping factor, whose molecular and functional properties have been previously described in detail (21). Figure 3 demonstrates that attachment of the plasmid-containing strain 8325-4 (pCF4) to both PVC and PE polymer tubing segments exposed to canine blood was markedly higher than that of the plasmid-free parental strain. These differences in the adhesion of the plasmid-enriched and nonenriched strains reflected the higher affinity of the former strain for fibrinogen immobilized on polymer surfaces as previously described in detail (21). As also expected from previous studies (21), the Fg_{Ad}-def mutant strain DU5880 of 8325-4, which contains the same *clfA1* mutation as strain Newman, showed a strong defect in attachment to each type of polymer tubing exposed to canine blood (Fig. 3).

Luminal surface morphology. Figure 4 shows scanning electron micrographs of either PVC or PE tubing after a 15-min exposure to canine blood. Significant differences were found between the two types of polymer tubing. Whereas a large number of platelets were adherent to the surface of PVC, they did not show significant spreading on this surface (Fig. 4A). In contrast, extensive spreading of the adherent platelets occurred on the surface of PE tubing (Fig. 4B). The differences in platelet morphology on PE versus PVC tubing were also observed after 5 and 60 min of blood exposure (data not shown).

Contribution of fibrinogen to the material-dependent differences in *S. aureus* attachment. To study the specific contribution of fibrinogen to the transient increase in bacterial adhesion promoted by PE compared with PVC on blood-exposed shunt tubing, further in vitro experiments were performed. The most relevant concentrations of immobilized fibrinogen used for studying the promotion of bacterial adhesion to either type of polymer tubing were selected from adsorption isotherms of ^{3}H-labelled canine fibrinogen to each type of polymer tubing shown in Fig. 5A. Protein adsorption was dose dependent from 1 to 16 μg of soluble canine fibrinogen per ml to both types of polymer tubing, followed by a plateau at higher protein concentrations. The plateau phases of fibrinogen adsorption on both PVC and PE tubings (Fig. 5A) likely reflected monolayer amounts of fibrinogen on these surfaces. Of note, the saturating amounts of radiolabelled fibrinogen on PVC were somewhat higher on PVC than on PE tubing, as shown in Fig. 5A. In contrast to this, *S. aureus* adhesion promoted by canine fibrinogen immobilized as a monolayer on PE (Fig. 5C) was higher than that on PVC tubing (Fig. 5B). The increases in adhesion were 65 and 45% for the parental strains Newman and 879R4S, respectively. As expected, the Fg_{Ad}-def mutant of Newman showed a completely defective attachment to either type of in vitro fibrinogen-coated polymer tubing. Because we could exclude the possibility that the increased adhesion of parental strains Newman and 879R4S of *S. aureus* to fibrinogen-coated PE compared with PVC tubing reflected a higher amount of protein on the former surface, the most likely explanation was that the monolayer amounts of canine fibrinogen expressed a higher specific activity for promoting *S. aureus* attachment when the protein molecules were adsorbed onto PE than onto PVC. Such a difference in the specific activity of the protein immobilized on either type of polymer surface might contribute, at least in part, to the higher bacterial attachment to blood-exposed PE compared with PVC tubing in the ex vivo shunt model.

DISCUSSION

Thrombogenesis is the most frequent complication of blood-contacting polymeric implants and devices. A relationship between catheter-related thrombogenesis and infection has been proposed at both the subclinical microscopic level (29, 35, 36) and the clinical level, as recently demonstrated with central venous catheters used in cancer patients (32). Despite their importance in the clinical situation, central venous catheters are not suitable for time course studies of thrombogenic events. Therefore, most experimental studies designed to evaluate the thrombogenic potential of various materials used for blood-contacting applications were performed in short-term ex vivo systems, including various types of arteriovenous shunt models (see review in reference 6). The major advantage of such ex vivo systems is they allow evaluation in a dynamic way of how the composition and structure of the polymer surface

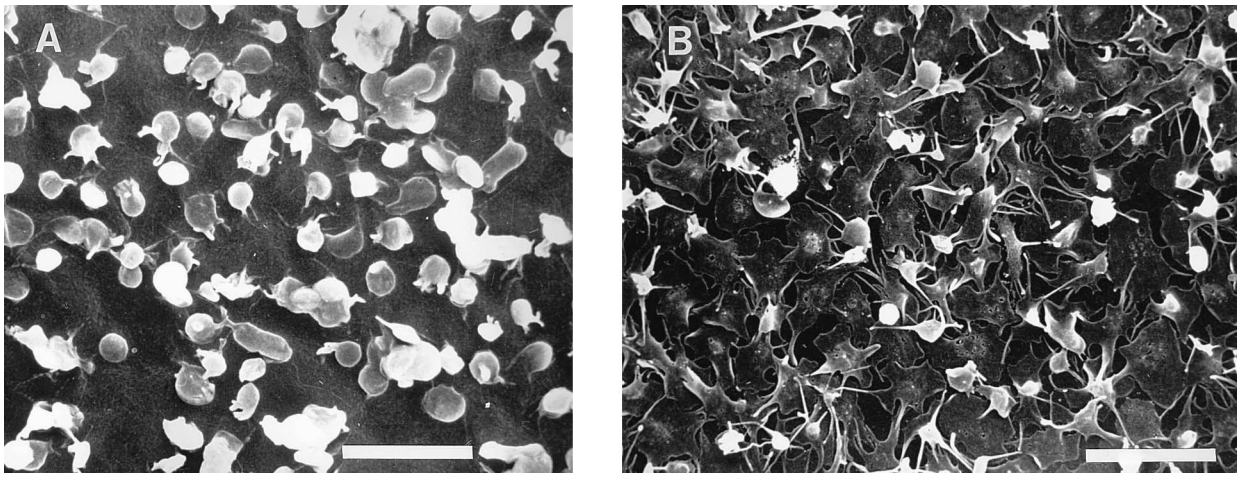

FIG. 4. Scanning electron micrographs showing platelets adherent to the luminal surface of polymer tubing exposed to canine blood for 15 min. (A) Nonspread platelets on PVC. (B) Extensively spread platelets on PE.

can influence thrombus and embolus formation within the first hours of blood-material interaction (6). In the canine acute ex vivo femoral arteriovenous shunt model, an early peak of platelet deposition, activation, and aggregation was shown to occur on a wide range of thrombogenic surfaces after 15 to 20 min of blood contact (17, 31). More detailed studies demonstrated that the thrombogenicity of a surface was determined by the composition of the initial protein layer rather than the total concentration of protein on the polymer surfaces (25). The most important protein component involved in thrombogenesis was shown to be fibrinogen (6, 17, 23–25), which can directly interact with the platelet receptors GPIIb/IIIa and contribute to platelet activation (30). Thus, the initial 60-min period of blood contact with the two different polymer surfaces tested in this model was considered appropriate for evaluating the short-term impact of plasma protein adsorption on *S. aureus* attachment.

In blood, which contains hundreds of proteins, the competitive and sequential adsorption of proteins is very complicated (31). In a preliminary analysis of ex vivo polymer tubing-associated proteins performed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), we found a large number of silver-stained protein components extracted from PVC or PE tubing segments at either 5, 15, or 60 min (unpublished data). Identification of major tubing-associated protein components will require the use of immunoblots with specific antibodies to a large panel of plasma and platelet components. In view of the large number of proteins bound to the tubing segments and because several of them might potentially interact with *S. aureus* and promote bacterial adhesion to artificial surfaces (2, 3, 10–13, 18, 19, 21, 26, 27, 34, 35, 38–42), identification of fibrinogen as the most active adhesion-promoting component would have been very difficult without the use of specific adhesion-defective mutants of *S. aureus* which lack attachment to either fibrinogen, fibrin, or fibronectin. Further studies and a greater range of experimental conditions and materials are required to precisely evaluate the contribution of fibronectin and additional plasma or platelet components, such as vitronectin (1, 18), thrombospondin (11), or collagen (26, 27), to *S. aureus* adhesion in the ex vivo system. This will require the development of further bacterial mutants exhibiting complete specific defects in adhesion to each of these proteins.

The significant transient increase in bacterial adhesion promoted by PE compared with PVC blood-exposed tubing may reflect the higher intrinsic acute thrombogenicity of PE over PVC tubing, which has been documented in previous studies (see review in reference 6). Of note, the peak of *S. aureus* adhesion to blood-exposed PE tubing occurs at the same time points (5 and 15 min) as that of platelet deposition and activation (6, 17). Previous studies of fibrinogen and platelet deposition with radiolabeling techniques (6, 17) showed marginally different profiles on PE compared with PVC blood-exposed tubings. In contrast, ex vivo PE but not PVC tubing promoted major changes in platelet morphology which consisted of extensive spreading and activation of the PE-deposited platelets after 5 and 15 min of blood exposure. To evaluate the specific contribution of fibrinogen to the transient increase in bacterial attachment to ex vivo PE compared with PVC, comparative *S. aureus* adhesion to each material coated in vitro with purified fibrinogen was recorded. The results of these experiments showed that despite the lower in vitro affinity of PE versus PVC for fibrinogen leading to slightly lower protein surface coatings, fibrinogen immobilized as a monolayer on PE was significantly more active in promoting attachment of *S. aureus* than when on PVC. Therefore, these data seem to indicate that PE tubing not only activates deposited platelets but also activates fibrinogen for promoting bacterial attachment. It is possible that immobilized fibrinogen molecules undergo confor-

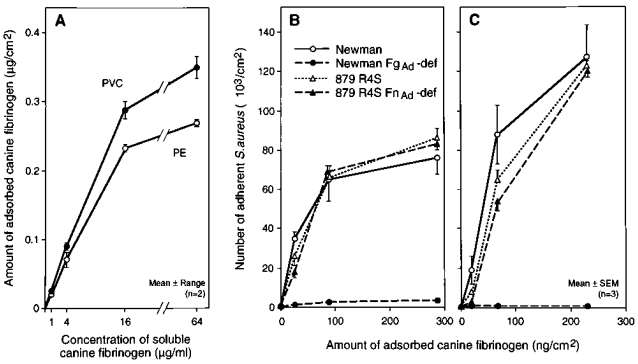

FIG. 5. In vitro adsorption (A) of purified canine fibrinogen and promotion of the adhesion of parental and mutant strains of *S. aureus* to PVC (B) or PE (C) protein-coated tubing segments. SEM, standard error of the mean.

mational changes which promote both platelet activation and *S. aureus* adhesion after adsorption on PE tubing. Further support for a link between the activation of deposited platelets and fibrinogen-mediated adhesion of *S. aureus* is suggested by a recent in vitro study using purified human platelets adsorbed onto PMMA coverslips in the presence of plasma (10). In this simplified in vitro system, platelets which spread on the surface bound more *S. aureus* than nonspread platelets and the interaction of fibrin or fibrinogen with thrombin-activated surface-bound platelets greatly increased *S. aureus* adhesion to the polymer surfaces.

The findings of this study with a short-term model of ex vivo blood-material interaction differ in several respects from those of a previous clinical study (36) which analyzed promotion of *S. aureus* adhesion by plasma proteins adsorbed onto central venous catheters used in patients for several days or weeks. In the latter study, chronically inserted catheters were found to be coated with a restricted number of plasma protein components, with fibrinogen and fibrin, plasminogen, and fibronectin being the predominant components identified by SDS-PAGE and immunoblots (41). In addition, central venous catheters showed a selective proteolytic inactivation of fibrin or fibrinogen over fibronectin which occurred during long-term insertion and led to a significant decrease in *S. aureus* adhesion (36, 41). Under the latter conditions, fibronectin and fibronectin proteolytic fragments seemed to be the most active protein component or components promoting *S. aureus* attachment to chronically implanted catheters (41). The very different results recorded with human intravenous catheters and the canine ex vivo system might be explained by significant differences in the time frame, material composition, clinical use, and hemodynamic conditions of both types of blood-contacting devices.

In conclusion, the canine arteriovenous shunt combined with the use of adhesion-defective mutants of *S. aureus* is a useful model for identification of the most active components promoting bacterial adhesion and colonization among plasma proteins adsorbed on various types of blood-contacting polymers. This study illustrates the role of fibrinogen or fibrin as a very active component promoting in vivo *S. aureus* adhesion in a short-term blood-material interaction, as opposed to fibronectin and fibronectin proteolytic fragments, which play a more important role in chronically implanted catheters (36). Finally, the experimental approach described in this study should prove useful for screening materials that might reduce protein-mediated staphylococcal adhesion and colonization.

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