

Adherence of *Staphylococcus epidermidis* to Fibrin-Platelet Clots In Vitro Mediated by Lipoteichoic Acid

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The adherence of two strains of *Staphylococcus epidermidis* to human fibrin-platelet clots in vitro was investigated. Both strains were nonencapsulated, nonhemagglutinating, and nonslime producers. Binding was not related to surface charge, carbohydrate profile, or hydrophobicity of the bacteria. Adherence was reduced four- to sixfold ($P < 0.001$) on pretreatment of bacteria with lipase, while neuraminidase, trypsin, phospholipase C, and sodium periodate did not alter their binding. Pretreatment of bacteria with substances known to bind lipoteichoic acid (LTA), such as human albumin and anti-LTA antibodies, also resulted in a fourfold ($P < 0.001$) reduction in adherence. Prior incubation of clots with free LTA, but not with deacylated LTA, produced a fourfold ($P < 0.001$) decrease in the adherence of homologous and heterologous strains of *S. epidermidis*. A similar reduction was also observed when LTAs derived from *Staphylococcus aureus* and *Streptococcus pyogenes* were used. These data provide evidence that the lipid moiety of LTA has a central role in the adherence of *S. epidermidis* to fibrin-platelet clots in vitro.

Morphological studies with pathological material from human autopsies and experimental models of infective endocarditis (1, 2) strongly suggest that early bacterial colonization of traumatized endocardium occurs on the fibrin-platelet deposits, so called nonbacterial thrombotic endocarditis (NBTE). Results of in vitro studies of bacterial adherence to punch biopsies of human and canine heart valves (11, 19) and to a surface simulating NBTE (20, 22), the fibrin-platelet matrix, correlate closely with observations in vivo and have contributed to our understanding of the complex pathogenesis of endocarditis. These studies have been largely carried out with nonhemolytic and alpha-hemolytic streptococci, the organisms that commonly produce native-valve endocarditis (1, 19; C. H. Ramirez-Ronda and J. Gutierrez, Clin. Res. 28:377a, 1980).

Early prosthetic valve endocarditis, a serious complication of valve surgery, is often caused by a commensal organism, *Staphylococcus epidermidis*. The precise factors that lead to the adherence of *S. epidermidis* to host tissue and to artificial surfaces (prosthetic valves and surgical sutures) are not yet clear. We previously identified bacterial lipoteichoic acid (LTA) as the adhesin which mediates the attachment of *S. epidermidis* to human pharyngeal epithelial cells (8). In the present study, an attempt was made to identify the role of various factors which influence the adherence of *S. epidermidis* to the fibrin-platelet matrix. Here, we describe evidence for LTA being the major adhesin for the interaction of *S. epidermidis* with fibrin-platelet clots. These results may elucidate the early steps of pathogenic lesions and may be relevant to the prophylaxis of prosthetic valve endocarditis.

MATERIALS AND METHODS

Bacterial strains. Two clinically significant strains of *S. epidermidis* isolated from multiple blood cultures of cases of infective endocarditis were used in the present study. These isolates were biotyped (API-Staph; API, Montalieu Vercieu, France) and phage typed. Strain CH2 was of biotype 6300113

and phage type 63/138/245/336, while strain CH5 was of biotype 6706113 and was phage nontypeable. Strains CH2 and CH5 were identified as highly and poorly adherent strains (8), respectively, based on the number of bacteria adherent per pharyngeal epithelial cell in vitro (37.9 ± 4.3 versus 11.1 ± 2.0 , respectively). Neither strain produced slime, was encapsulated, or agglutinated human, rabbit, sheep, or guinea pig erythrocytes (7). The surface charge and hydrophobicity of both strains have been determined previously (8).

Preparation of bacterial suspensions. Cultures were grown in 5 ml of minimal essential medium containing 10 μ Ci of [³H]leucine (Radiochemical Centre, Amersham, England) statically at 37°C for 16 h. The organisms were centrifuged (2,000 rpm, 4°C), washed three times with 0.01 M phosphate-buffered saline (PBS) (pH 7.2), resuspended in the same medium, and passed through an 8- μ m-pore-diameter filter (Millipore Corp., Bedford, Mass.) to remove aggregated forms. The count was adjusted photometrically to give a concentration of 10^7 bacteria per ml.

Preparation of the platelet-fibrin surface. The platelet-fibrin surface was prepared with blood from healthy individuals who had no coagulation abnormality and had not been on any anticoagulants during the past 4 weeks (22). Blood in 3.8% sodium citrate (4:1) was centrifuged to obtain platelet-rich plasma, platelet-poor plasma, and platelet-free plasma after being filtered twice through 0.45- μ m-pore-diameter filters (Millipore). The platelet counts (Coulter Counter) were 300,000/mm³, 20,000/mm³, and nil in platelet-rich plasma, platelet-poor plasma, and platelet-free plasma, respectively. One milliliter of platelet-rich plasma, platelet-poor plasma, or platelet-free plasma was combined with 0.4 ml of human thrombin (500 U/ml; Sigma Chemical Co., St. Louis, Mo.), and 0.3 ml of the mixture was transferred to each well of a tissue culture plate (24-well plate; well size, 16 by 16 mm; Nunc, Roskilde, Denmark) and incubated at 37°C for 30 min. The plates were stored at 4°C and used within 3 days after preparation.

Preparation of LTA. LTA was prepared from *S. epidermidis* CH2 by cold phenol extraction (13) and purified by

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column chromatography on Sepharose 6B. Nucleic acids were removed with RNase and DNase (overnight at 37°C), and proteins were removed with papain and cysteine (6 h at 37°C). The purified LTA thus obtained had a high phosphorus content (3.2 $\mu\text{mol}/\text{mg}$), a low protein content (0.4%), and a low nucleic acid content (less than 0.8%) (contaminants) and possessed high erythrocyte-sensitizing activity (7.8 $\mu\text{g}/\text{ml}$) and high albumin-binding activity (87.5%) (16, 23). The LTA derived from *S. epidermidis* and the LTAs derived from *Staphylococcus aureus* and *Streptococcus pyogenes* (Sigma) were deacylated by alkaline treatment (17). Deacylation was complete, as judged by the loss of erythrocyte-sensitizing activity ($>500 \mu\text{g}/\text{ml}$) and albumin-binding activity (14.0, 5.5, and 3.6%, respectively).

Anti-LTA antibodies. Anti-LTA antibodies were raised in BALB/c mice by intraperitoneal inoculation of LTA conjugated to methylated bovine albumin and emulsified with Freund complete adjuvant (Sigma). Each mouse received 200 μl of the emulsion containing 50 μg of LTA. After 21 days, mice received the same dose but emulsified in Freund incomplete adjuvant. Boosting was done with 50 μg of free LTA in PBS once per week for 3 weeks. Mice were bled 10 days after the last injection, and the antibody activity was determined by an enzyme-linked immunosorbent assay (4). The optimal concentration of LTA coated onto the wells of microdilution plates was 5 $\mu\text{g}/\text{ml}$. Uncoated antigen was removed by repeated washings with PBS containing 0.05% Tween 20. Test sera were added at various dilutions, unbound antibodies were removed by repeated washings, and rabbit anti-mouse immunoglobulin coupled to horseradish peroxidase (DAKO) was used as the conjugate. The A_{492} values were read with a Titertek Multiscan ELISA reader (Flow Laboratories, Inc., McLean, Va.).

Adherence assay. The bacterial inoculum (0.3 ml) was transferred to each well containing the clot and incubated in a controlled environment in a shaking incubator (Kotterman, Hanigsen, Federal Republic of Germany) at 100 rpm and 37°C for 0 to 60 min. The supernatant was removed, and the clot surface was washed five times with PBS to remove the nonadherent bacteria. This procedure was found to be satisfactory, since no further radioactivity was observed in additional washings. The clot was dissolved by treatment with 0.5 ml of streptokinase (1,600 U/ml; Boehringer GmbH, Mannheim, Federal Republic of Germany) for 2 h at room temperature. The supernatants, the five washings, and the clot were transferred to scintillation vials containing 10 ml of a detergent-based scintillation fluid and counted in a liquid scintillation counter (LKB, Uppsala, Sweden). The counts per minute of known quantities of each bacterial isolate were used to convert data to the number of bacteria. The results represent the number of adherent bacteria after subtraction of values obtained for the control clot incubated without bacteria.

All experiments were carried out in duplicate and repeated three times. The differences between duplicate assays averaged less than 5%. All results represent means \pm standard deviations of three independent experiments. The Student *t* test was used for statistical analysis. The adherence of bacteria to clots was expressed as the adherence ratio (AR), calculated as follows: (average number of bacteria per clot $\times 10^4$)/bacterial count per inoculum.

Pretreatment of bacteria and clots. Bacteria were treated with the modifying agents lipase VI, trypsin IX, phospholipase C, neuraminidase VI, and sodium metaperiodate (8). Bacteria ($10^7/\text{ml}$) were also treated in equal volumes with (i) PBS containing human albumin (0.0, 0.1, 1, 10, 100, and

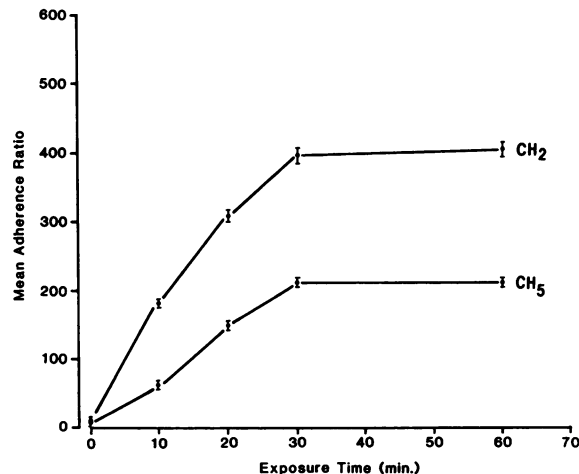


FIG. 1. Effect of time of exposure of *S. epidermidis* CH2 and CH5 on adherence to platelet-rich fibrin clots.

1,000 $\mu\text{g}/\text{ml}$) for 60 min at 37°C and (ii) normal or immune mouse serum for 18 h at 4°C and were washed three times with PBS. No bacterial multiplication was observed during the incubation period, as measured by viable counts. Clots were pretreated with 0.1 ml of PBS containing LTA or deacylated LTA in various concentrations (3, 6, 12, 25, 100, and 200 $\mu\text{g}/\text{ml}$) at 37°C for 60 min and washed three times with PBS. Hemoglobin and DL-glycerolphosphate (100 $\mu\text{g}/\text{ml}$) were used as controls. Control clots and organisms were treated with buffer alone. The adherence test was performed as described above.

RESULTS

Conditions influencing bacterial adherence to fibrin-platelet clots. Adherence was facilitated by slow agitation of microdilution plates containing clots and bacterial suspensions. There was a minimal loss of radioactivity in bacteria incubated with streptokinase over a 2-h incubation period (3.3% \pm 0.7%; $P < 0.1$). The AR increased for both strains as a function of time, being maximum at 30 min (Fig. 1), and further incubation up to 60 min did not lead to any significant increase ($P < 0.1$). The AR increased with bacterial count in the inoculum until it attained a plateau when a constant number of bacteria in the initial inoculum were adherent irrespective of the surface and bacterial strain (Fig. 2). The AR was determined for equal bacterial titers but various volumes (data not shown). The AR was relatively constant at 50 to 100 μl for all surfaces studied. A standard volume of 100 μl of 10^7 bacteria per ml and an incubation period of 30 min were therefore used for all subsequent experiments. Organisms were neither serum sensitive, as their counts remained unaltered, nor able to agglutinate when incubated with normal or immune mouse serum at 37°C for 30 min.

Both strains of *S. epidermidis* were capable of adhering to clots, although strain variation was observed, strain CH2 being more adherent than strain CH5. This property correlated with their degree of adherence to pharyngeal epithelial cells. However, there was no correlation of the degree of bacterial adherence to clots with adherence to ion exchangers, with surface hydrophobicity, or with adherence to lectin-Sepharose.

Effect of platelets. The AR for the two strains of *S. epidermidis* increased in the presence of platelets. The mean ratios of adherence of strains CH2 and CH5 to human fibrin

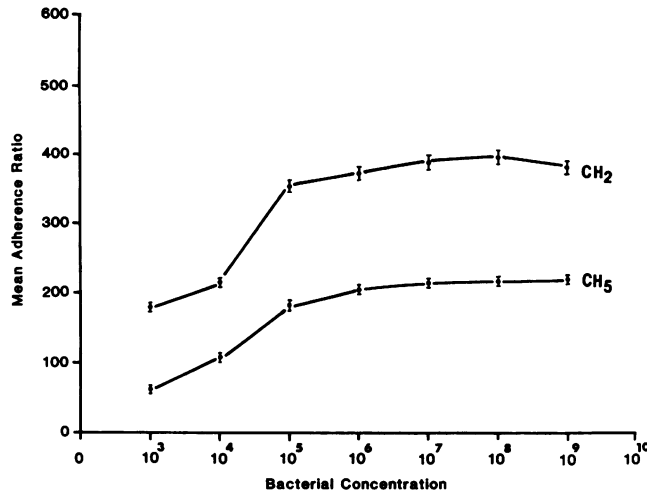


FIG. 2. Effect of concentration of *S. epidermidis* CH2 and CH5 on adherence to platelet-rich fibrin clots.

clots free of platelets were 184 ± 7 and 87 ± 6, respectively. The corresponding values for their adherence to platelet-rich fibrin clots were 398 ± 13 and 217 ± 10 (*P* < 0.001). However, this effect was not dependent on platelet concentration over the range studied (20,000 to 300,000 platelets per mm³). The corresponding values for their adherence to platelet-poor fibrin clots were 384 ± 12 and 204 ± 9.

Pretreatment of bacteria. Lipase treatment caused a marked decrease in the adherence of both bacterial strains to clots without a loss of viability or altered morphology of bacteria (Table 1) (the percentages of inhibition of adherence were 85.7 ± 0.9 and 78.5 ± 1.2 for CH2 and CH5, respectively; *P* < 0.001). Pretreatment with trypsin, neuraminidase, phospholipase C, or sodium metaperiodate did not alter their adherence.

The adherence of both homologous CH2 and heterologous CH5 strains was significantly (*P* < 0.005) decreased by pretreatment with polyclonal mouse anti-LTA serum (raised against LTA derived from strain CH2), whereas the adherence of cells incubated in pooled normal mouse serum did not change (*P* < 0.1). The degree of inhibition correlated with the titer of anti-LTA antibodies. Prior treatment of

TABLE 1. Effect of pretreatment of *S. epidermidis* on adherence to platelet-rich clots

Pretreatment with:	% Inhibition of adherence of <i>S. epidermidis</i>	
	CH2	CH5
Lipase (10 µg/ml)	85.7 ± 0.9	78.5 ± 1.2
Human albumin (µg/ml)		
0.1	6.9 ± 0.3	9.1 ± 0.7
1	10.7 ± 0.7	15.9 ± 1.0
10	28.6 ± 1.0	20.6 ± 1.2
100	68.7 ± 1.2	59.7 ± 1.3
1,000	78.8 ± 1.0	75.5 ± 1.0
Mouse anti-LTA serum		
1:5	73.6 ± 1.1	80.6 ± 1.3
1:10	50.1 ± 0.8	42.6 ± 0.9
1:40	30.6 ± 1.0	31.3 ± 1.4
Normal mouse serum, 1:5	7.6 ± 0.4	10.9 ± 0.7

TABLE 2. Adherence of *S. epidermidis* to platelet-rich clots pretreated with LTA and deacylated LTA

Pretreatment with (µg/ml):	% Inhibition of adherence of <i>S. epidermidis</i>	
	CH2	CH5
LTA		
3	6.4 ± 1.3	4.0 ± 0.3
6	11.1 ± 1.6	7.1 ± 1.2
12	20.3 ± 1.9	11.3 ± 1.2
25	34.2 ± 3.7	29.2 ± 1.9
50	60.4 ± 3.2	51.2 ± 2.1
100	73.4 ± 2.9	66.7 ± 3.2
200	76.3 ± 3.9	70.2 ± 3.6
Deacylated LTA, 100	10.8 ± 1.0	6.6 ± 1.1

bacteria with albumin blocked their adherence to clots. This effect was dose dependent, and the maximum inhibition was observed at a concentration of 1,000 µg/ml (the percentages of inhibition of adherence were 78.8 ± 1.0 and 75.5 ± 1.0 for CH2 and CH5, respectively; *P* < 0.001).

Pretreatment of clots. Prior incubation of clots with LTA produced a marked inhibition of bacterial adherence (Table 2) (*P* < 0.001). This inhibition was dose dependent and saturable at a concentration of 100 µg/ml. The blocking effect was similar on both homologous and heterologous strains of *S. epidermidis* (the percentages of inhibition of adherence were 73.4 ± 2.9 and 66.7 ± 3.2, respectively). The corresponding treatment with deacylated LTA did not cause a significant inhibition (the percentages of inhibition of adherence were 10.8 ± 1.0 and 6.6 ± 1.1, respectively; *P* < 0.1). The inhibition of adherence of *S. epidermidis* to fibrin-platelet clots after treatment with LTA derived from *S. epidermidis*, *S. aureus*, or *S. pyogenes* was significantly different, since 50% inhibition was observed with 22.4, 38.4, and 51.7 µg of LTA per ml, respectively (*P* < 0.05; paired *t* test).

DISCUSSION

Fibrin-platelet clots were previously used to study the adherence of *Streptococcus sanguis* (14, 22), *Streptococcus pneumoniae* (1), and *Candida* spp. (15), and their in vitro adherence correlated closely with in vivo observations. However, Crawford and Russel (9), using several species of streptococci (*S. sanguis*, *S. mutans*, *S. mitior*, *S. milleri*, *S. faecalis*, and *S. salivarius*), did not find any apparent relationship between the incidence of endocarditis and the in vitro ability of bacteria to adhere to fibrin clots. The present study is the first to show that *S. epidermidis* is capable of binding to an in vitro surface simulating NBTE. Optimal binding occurred above a certain threshold concentration of bacteria in the inoculum and was time dependent. These results agree with earlier observations in rabbits (3); the effective contact was obtained in vivo with circulating bacterial microcolonies trapped over the diseased valves and fresh fibrin. The presence of platelets in a fibrin matrix significantly increased bacterial adherence, although the critical number of platelets required was not high. These results agree with previous observations made with streptococci in vitro and in rabbits pretreated with antiplatelet drugs; in these rabbits NBTE developed, platelets were seen in the fibrin, and the drugs were unable to increase the infectious dose required to produce the disease (20). Beachey et al. (5) observed that the LTA of group A

streptococci bound spontaneously to human platelets and that the binding was dependent on the concentration of platelets and the time of incubation. They further observed that deacylated LTA was not able to do so, confirming the requirement of ester-linked fatty acids for binding. This result is similar to our observations with LTA from *S. epidermidis*. The physicochemical features and the biological consequences of the interaction of LTA with platelets are not fully understood. LTA is reported to interfere with collagen-induced aggregation of platelets, although collagen is still able to attach to binding sites to trigger the release reaction (5). A study of the biological events associated with LTA-mediated binding of *S. epidermidis* to human platelet-rich clots may shed light on the potential role of such interactions in the pathogenesis of infective endocarditis.

The results of studies on the mechanism of adherence of *S. epidermidis* to artificial surfaces have been contradictory. Hydrophobicity (12), slime production (8), a protein adhesin (12), polysaccharide (25), and LTA (G. D. Christensen and H. S. Courtney, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, B43, p. 25) have been reported to mediate bacterial adherence. The nature of the binding of *S. epidermidis* to eucaryotic cells is under investigation in our laboratory. We previously demonstrated the presence of a lipase-sensitive material on the surface of *S. epidermidis* which binds these organisms to human pharyngeal epithelial cells (8), and we further identified this adhesin as LTA (unpublished data).

A majority of gram-positive bacteria have LTA complexed to proteins on their surfaces in such a way that the fatty acid ends of the LTA molecules are exposed at the outer ends to interact with specific receptors on host tissues (18). LTA has been reported to be the principal adhesin of group A and B streptococci, alpha-hemolytic streptococci, *S. aureus*, and *Staphylococcus saprophyticus* (6, 24; Ramirez-Ronda and Gutierrez, Clin. Res. 28:377a, 1980). Our present results clearly showed that treatment of *S. epidermidis* cells with agents known to bind LTA, polyclonal mouse anti-LTA antibodies and human albumin, led to decreased bacterial adherence to fibrin-platelet clots. Also, treatment of clots with free LTA blocked the adherence of the homologous and heterologous strains. These observations are similar to those reported earlier (10, 21, 22): decreased adherence of *Candida albicans* (0 to 7.8% of control values; $P < 0.001$) and *S. sanguis* (40% of control values; $P < 0.001$) to the constituents of fibrin-platelet clots was reported when these organisms were preincubated with homologous rabbit immune sera. However, Adler et al. (1) reported that rabbit anti-pneumococcal sera increased the in vitro adherence of pneumococci to fibrin-platelet surfaces ($P < 0.001$) and rabbit aortic valve cusps ($P < 0.05$). Our results showed that antibody to LTA was capable of blocking the early attachment or colonization of fibrin-platelet clots and functioned as a protective mechanism.

Although immunization against infective endocarditis is impractical because of a wide variety of causative organisms, common adherence factors may exist between them. Our observations that LTA is capable of blocking the adherence of homologous as well as heterologous strains of *S. epidermidis* and that LTAs derived from *S. aureus* and *S. pyogenes* are capable of causing significant inhibition of the adherence of *S. epidermidis* suggest that LTA may be the common adhesin between staphylococci and streptococci which frequently cause endocarditis. Further studies are needed to explore these possibilities.

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