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Staphylococcus aureus Induces Platelet Aggregation via a Fibrinogen-Dependent Mechanism Which Is Independent of Principal Platelet Glycoprotein IIb/IIIa Fibrinogen-Binding Domains

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Platelet aggregation by bacteria is felt to play an important role in the pathogenesis of infective endocarditis. However, the mechanisms involved in bacterium-induced platelet aggregation are not well-defined. In the present study, we examined the mechanisms by which *Staphylococcus aureus* causes rabbit platelet aggregation in vitro. In normal plasma, the kinetics of *S. aureus*-induced platelet aggregation were rapid and biphasic. The onset and magnitude of aggregation phase 1 varied with the bacterium-platelet ratio, with maximal aggregation observed at a ratio of 5:1. The onset of aggregation phase 2 was delayed in the presence of apyrase (an ADP hydrolase), suggesting that this later aggregation phase may be triggered by secreted ADP. The onset of aggregation phase 2 was delayed in the presence of prostaglandin I_2-treated platelets, and this phase was absent when paraformaldehyde-fixed platelets were used, implicating platelet activation in this process. Platelet aggregation phase 2 was dependent on *S. aureus* viability and an intact bacterial cell wall, and it was mitigated by antibody directed against staphylococcal clumping factor (a fibrinogen-binding protein) and by the cyclooxygenase inhibitor indomethacin. Similarly, aggregation phase 2 was either delayed or absent in three distinct transposon-induced *S. aureus* mutants with reduced capacities to bind fibrinogen in vitro. In addition, a synthetic pentadecapeptide, corresponding to the staphylococcal binding domain in the C terminus of the fibrinogen δ-chain, blocked aggregation phase 2. However, phase 2 of aggregation was not inhibited by two synthetic peptides (alone or in combination) analogous to the two principal fibrinogen-binding domains on the platelet glycoprotein (GP) IIb/IIIa integrin receptor: (i) a recognition site on the IIIa molecule for the Arg-Gly-Asp (RGD) sequence of the fibrinogen α-chain and (ii) a recognition site on the IIb molecule for a dodecapeptide sequence of the fibrinogen δ-chain. This differs from ADP-induced platelet aggregation, which relies on an intact platelet GP IIb/IIIa receptor with an accessible RGD sequence and dodecapeptide recognition site for fibrinogen. Furthermore, a monoclonal antibody directed against the RGD recognition site on rabbit platelet GP IIb/IIIa receptors failed to inhibit rabbit platelet aggregation by *S. aureus*. Collectively, these data suggest that *S. aureus*-induced platelet aggregation requires bacterial binding to fibrinogen but is not principally dependent upon the two major fibrinogen-binding domains on the platelet GP IIb/IIIa integrin receptor, the RGD and dodecapeptide recognition sites.

The pathogenesis of infective endocarditis (IE) involves a series of complex interactions between the microorganism and a variety of host components, including cardiac endothelium, platelets, and plasma proteins (e.g., fibrinogen and fibronectin [11, 12, 28]). Platelets are thought to have a central role in the pathogenesis of IE by providing an adhesive surface upon damaged endothelium for microbial binding. In addition, microorganisms attached to the valve surface may induce localized platelet aggregation, resulting in formation of the infected vegetation (12, 13, 28). Studies have demonstrated that the ability of certain bacteria to cause platelet aggregation in vitro correlates with their capacity to induce experimental endocarditis (17, 18).

Staphylococcus aureus is the most common cause of endovascular infections (e.g., intravascular catheter sepsis and hemodialysis access site sepsis), and it is the second leading cause of IE (35). We have previously characterized the following parameters of *S. aureus*-platelet binding interactions in a plasma-free milieu by flow cytometry: binding kinetics, functional numbers of platelet binding sites, and the influence of various potential binding inhibitors (38). However, the precise mechanism(s) by which *S. aureus* binds to the platelet surface in the presence of plasma to trigger subsequent platelet aggregation remains incompletely defined (9). The recent observation that *S. aureus* triggers platelet aggregation in a biphasic manner in rabbit platelets (2) affords an opportunity to dissect the mechanisms of bacterially induced platelet aggregation. For this reason, we examined the staphylococcal and platelet factors that are potentially involved in platelet aggregation in vitro.

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TABLE 1. *S. aureus* strains

Strain[a]	Reference	Description (source)
Lafferty	35	Wild-type clinical isolate that clumps fibrinogen normally (Joseph Wheat, Indianapolis, Ind.)
RN6390	4	Wild-type strain that clumps fibrinogen normally
I	4	Derivative of RN6390 carrying both *sar*::Tn*917* and *agr*::*tetM* mutations; reduced binding to fibrinogen and fibronectin
ISP479	7	Derivative of wild-type strain 8325-4 that contains a thermosensitive plasmid (pI258) as a Tn*551* delivery vehicle; clumps fibrinogen normally
IE3	7	Derivative of ISP479 with *car*::Tn*551* mutation; reduced binding to fibrinogen[b]
Newman	21	Wild-type isolate that clumps fibrinogen normally (Tim Foster, Dublin, Ireland)
DU-5852	21	Derivative of Newman with *clfA*::Tn*917* mutation (clumping factor gene); poor fibrinogen clumping (Tim Foster)
ST021	27	Wild-type microencapsulated type 5 strain; capsule is maximally expressed postexponentially (A. Fattom, Rockville, Md.)

[a] Viridans group streptococcal strain STV287, a clinical isolate from a patient with endocarditis, was kindly provided by Walter Wilson, Rochester, Minn.
[b] *car*, cell-wall-associated regulator (tentative designation).

MATERIALS AND METHODS

Bacterial strains. The sources, contributors, and phenotypic and genotypic descriptions of the *S. aureus* strains utilized in this study are given in Table 1.

Preparation of bacteria for platelet aggregometry. *S. aureus* strains were grown for 2 h (logarithmic phase) at 37°C in brain heart infusion (BHI) broth (Difco Inc., Detroit, Mich.). For the isogenic mutants listed above, our previous studies revealed virtually identical growth kinetics and stable phenotypic traits in the presence or absence of the specific antibiotics to which they were resistant (4, 8). For the present studies, all such strains were grown in antibiotic-free BHI broth to circumvent any potential antibiotic-mediated influences on bacterium-platelet interactions. The viridans group streptococcal strain was grown to logarithmic phase (~3 h) in Todd-Hewitt broth (Difco Inc.). All bacterial cultures were pelleted, washed three times, suspended in Tyrode's buffer (Sigma Chemicals, St. Louis, Mo.) to the desired final concentration, and tested for their ability to aggregate platelets as described below.

In certain studies, the ability of logarithmic-phase staphylococcal cells to induce platelet aggregation was compared with that of stationary-phase cells (grown for 18 h at 37°C). In parallel experiments, bacteria were heat killed (100°C for 30 min), fixed in 1% paraformaldehyde for 5 min (followed by washing), or trypsinized (trypsin at 1 mg/ml for 30 min at 37°C, followed by soybean trypsin inhibitor [2 mg/ml for 30 min at 37°C; Sigma Chemicals]) prior to aggregometry (16).

To determine whether platelet aggregation induced by staphylococci could be simulated by inert particles of the same size, aggregometry was performed following addition of polystyrene latex beads (0.8-μm diameter; Sigma Chemicals) to platelet-rich plasma (PRP) at the same final inoculum as *S. aureus*, to achieve a final particle-platelet ratio of 5:1.

Production of cell wall-free bacterial protoplasts. A late-logarithmic-phase culture of *S. aureus* Lafferty (~2×10^9 CFU/ml) was pelleted by centrifugation at 2,000 × *g* for 25 min and then resuspended in 600 μl of Tyrode's buffer containing 30% raffinose (osmotic stabilizing agent for protoplast integrity), 0.05 M Tris-HCl, and 0.145 M NaCl (pH 7.6). To this suspension were added lysostaphin (Sigma Chemicals; final concentration = 250 μg/ml) and DNase (Sigma Chemicals; final concentration = 500 μg/ml) (14); the resulting reaction mixture was incubated at 37°C for 1 h on a rotating drum. The tube was then centrifuged at 4,500 × *g* for 15 min to pellet the protoplasts, which were then reconstituted to the desired final concentration in 30% raffinose-containing Tyrode's buffer prior to platelet aggregometry. Lysostaphin-induced cell wall solubilization was confirmed by lack of crystal violet uptake by staphylococcal cells on Gram's stain (29). The protoplast inoculum was routinely confirmed by quantitative culture on BHI agar plates containing 30% raffinose as an osmotic stabilizing agent.

Preparation of platelets. Rabbit blood was collected in polypropylene tubes (3.8% sodium citrate anticoagulant-to-blood ratio, 1:9). Centrifugation (100 × *g*, 15 min, 25°C) produced an upper PRP suspension. The upper two-thirds of this PRP fraction yielded a platelet-rich suspension having <1% leukocyte contamination. For certain studies, washed platelet suspensions were prepared from PRP by centrifugation and washing in Tyrode's buffer and resuspended in either normal or afibrinogenemic plasma prior to aggregation assays (see below).

Platelet aggregometry. A dual-channel platelet aggregometer (Peyton Associates, Buffalo, N.Y.) attached to a chart recorder was used to assess platelet aggregation by turbidometry (33). Platelet concentrations in PRP were determined with a Coulter Counter (Coulter Industries, Hialeah, Fla.) and adjusted to ~2.5×10^8 platelets per ml. PRP (250 μl) was warmed to 37°C in a siliconized glass cuvette stirred at 900 rpm with a magnetic microrod. Platelet-poor plasma (PPP) obtained by centrifugation of PRP (1,000 × *g*, 10 min) was used to establish the 100% light transmission baseline, while PRP served as the 0% aggregation baseline. A range of bacterium-platelet ratios (1:1 to 10:1) was examined for maximal aggregation in pilot studies; in most studies, a bacterium-platelet ratio of 5:1 was used, as this ratio provided optimal platelet aggregation for the test strains. Bacterial counts approximated by optical density at 620 nm were confirmed by quantitative plate cultures onto BHI and Todd-Hewitt agars for staphylococci and viridans group streptococci, respectively (Difco Inc.). Aggregometry was performed by recording light transmission for 25 to 30 min or until platelet aggregation was complete, as indicated by a minimum of 10 min of recorder chart equilibrium (no change in optical density). ADP (20 μM; Sigma Chemicals)-induced aggregation was performed prior to each experimental run to ensure the responsiveness of each day's PRP preparation.

Platelet activation. (i) PGI_2. To define whether platelet activation influences staphylococcus-induced aggregation of rabbit platelets, 10 μM prostaglandin I_2 (PGI_2; Sigma Chemicals) was added to PRP for 15 min prior to addition of the *S. aureus* inoculum. This concentration of PGI_2 completely blocked ADP- and thrombin-induced aggregation of rabbit platelets in pilot studies.

(ii) Platelet fixation. As an additional assessment of the role of platelet activation in staphylococcus-induced aggregation, platelets were paraformaldehyde fixed as described by Sullam et al. (33). After multiple washings in Tyrode's buffer, fixed platelets were suspended in rabbit PPP, at the appropriate final concentration, prior to aggregometry. Fixed platelets can undergo agglutination but not aggregation (33). The adequacy of the fixation procedure was thus confirmed by a lack of aggregation following addition of ADP (20 μM) to the fixed-platelet–PPP preparation.

(iii) Platelet ATP secretion. The ability of bacteria (1:1 bacterium-platelet ratio) to induce platelet ATP secretion was assessed by platelet luminoaggregometry as previously described, using the luciferin-luciferase reaction, with a model 500 Chronolog luminoaggregometer (33). Exogenous ATP (1 to 4 μM; Sigma Chemicals) served as a standard for quantifying ATP secretion. Final platelet concentrations were ~2×10^8/ml.

(iv) ADP inhibition. To determine whether secreted ADP mediates *S. aureus*-induced platelet aggregation, an ADP hydrolase, apyrase (0.5- to 5-mg/ml final concentrations; Sigma Chemicals), was added to PRP 10 min prior to aggregation assays.

(v) Platelet cyclooxygenase pathway. To determine if cyclooxygenase pathways are involved in the aggregation of platelets by *S. aureus*, indomethacin (0.1 to 20 μM; Sigma Chemicals) was added to PRP 10 min prior to aggregation assays.

Bacterial binding to platelet surface. To determine if differences in efficiency of bacterial binding to platelets influenced subsequent platelet aggregation, *S. aureus* binding to rabbit platelets was assessed by flow cytometry (fluorescence-activated cell sorting) as described previously (38). In brief, bacteria were cultured to logarithmic phase, washed in Tris buffer, and labelled with the DNA-intercalating fluorochrome Hoechst 33342 (25 μg/ml; Polysciences, Warrington, Pa.) for 2 h at room temperature; this was followed by washing and resuspension in Tyrode's buffer. Platelets were labelled internally with 5-chloromethyl fluorescein diacetate (30 μg/ml; Molecular Probes, Eugene, Oreg.) for 1 h and then washed in citrate buffer. Binding studies were performed at room temperature by mixing bacteria and platelets (10:1 ratio) in Tyrode's buffer. Immediately after mixing, suspensions were analyzed by using a FACStar^{Plus} (Becton Dickinson, San Jose, Calif.) with two argon lasers (Coherent, Mountain View, Calif.) operating at 150 mW and with the appropriate combinations of excitatory wavelengths and filters (35). The percentage of bacteria bound to platelets was determined by dividing the number of dually labelled particles (representing bacteria bound to platelets) by the number of particles labelled only with Hoechst 33342 (i.e., total number of bacteria in suspension) and multiplying by 100 (38).

Afibrinogenemic plasma. Afibrinogenemic rabbit plasma was prepared by the method of Cheung et al. (6). In brief, affinity-purified goat anti-rabbit fibrinogen antibody (5 mg) was covalently attached to 1 g of glutardialdehyde-activated beads (Boehringer Mannheim, Irvine, Calif.). Citrate-containing PPP was exposed to the beads in a column (1.5 by 20 cm) at 4°C overnight, and then the fibrinogen-deficient plasma was collected in the fall-through. This process was repeated four times after bound fibrinogen was removed from the affinity column by glycine (pH 2). Untreated PPP contained 2.31 mg of fibrinogen per ml prior to column adsorption, as quantified by activated thrombin time (courtesy of Nichols Institute, Los Angeles, Calif.); after four such treatments, fibrinogen was undetectable in treated PPP (assay sensitivity = 0.05 mg/ml). *S. aureus* Lafferty-induced aggregation was then carried out in either normal or afibrinogenemic plasma. In a parallel study, rabbit fibrinogen (2-mg/ml final concentration; Sigma Chemicals) was added back to afibrinogenemic PPP to reconstitute a normal fibrinogen level prior to the assessment of *S. aureus*-induced platelet aggregation.

Fibrinogen-binding capacity. Three distinct strategies were employed to assess the requirement for bacterial fibrinogen-binding capacity to execute rabbit platelet aggregation, as follows.

(i) Anti-fibrinogen-binding-protein antibody studies. We evaluated whether antibody directed against the *S. aureus* fibrinogen-binding protein would mitigate platelet aggregation. Rabbit polyclonal antibody (immunoglobulin G [IgG]) against purified fibrinogen-binding protein (14) and preimmune rabbit IgG from subsequently immunized rabbits were kindly provided by F. Esperson (Copenhagen, Denmark). In parallel studies, preimmune or immune IgG (final concentrations = 50 to 1,000 μg/ml) was incubated with Lafferty strain cells for 1 h at room temperature on a rotating drum. Bacterial cells were then washed twice in phosphate-buffered saline, resuspended in Tyrode's buffer to the desired final bacterial inoculum, and added to PRP for the platelet aggregation assay as before.

(ii) Mutant *S. aureus* strains defective in fibrinogen binding. *S. aureus* strains (I, IE3, and DU5852) with reduced fibrinogen-binding capacity in vitro were compared with their respective parental strains (RN6390, ISP-479, and Newman) for the ability to induce aggregation.

(iii) Competitive inhibition of fibrinogen binding. Attachment of fibrinogen to *S. aureus* involves the interaction of the staphylococcal fibrinogen-binding protein(s) with a 15-amino-acid peptide domain representing the extreme C terminus of the δ chain of fibrinogen (GQQHHLGGAKQAGDV [30]). An analogous, synthetic pentadecapeptide (Sigma) was utilized to additionally determine whether competitive inhibition of *S. aureus*' fibrinogen binding influences *S. aureus*-induced platelet aggregation. The Lafferty strain was preincubated with the pentadecapeptide (0.8 to 3.3 mM) for 30 min at 37°C, pelleted by centrifugation, washed, and resuspended in Tyrode's buffer prior to aggregometry. In parallel experiments, the pentadecapeptide was added to PRP either immediately after addition of the bacterial inoculum (within 30 s) or just after phase 1 of aggregation was completed (~3 min after addition of the bacterial inoculum). In pilot studies, concentrations of the pentadecapeptide exceeding 2 mM prevented rabbit fibrinogen agglutination by the Lafferty strain in a previously described slide agglutination assay (30).

Role of platelet GP IIb/IIIa receptor in aggregation. Several strategies were used to define whether the glycoprotein (GP) IIb/IIIa receptor is involved in *S. aureus*-induced platelet aggregation, as follows.

(i) Inhibition with antibody to GP IIb/IIIa complex. A monoclonal antibody to the RGD-dependent fibrinogen-binding site on the human GP IIb/IIIa receptor (OPG2) was used to determine the influence of this binding domain on *S. aureus*-induced platelet aggregation (kindly provided by T. Kunicki, Scripps Institute, La Jolla, Calif.). This antibody contains the Arg-Tyr-Asp (RYD) motif in its heavy chain, which confers its blocking specificity. The binding of this antibody to GP IIb/IIIa is itself inhibited by exogenous RGD-containing peptides (20, 34). Antibody OPG2 has been previously shown to completely block fibrinogen binding to rabbit platelets in vitro (19a). Washed platelets were incubated with the antibody (10 to 100 μg/ml) or Tyrode's buffer at room temperature for 30 min prior to reconstitution in PPP and addition of *S. aureus* for the aggregation assay. The adequacy of antibody-mediated inhibition of the GP IIb/IIIa receptor was confirmed by complete block of ADP-induced aggregation. Bacteria were then added to the same reaction mixture, and subsequent aggregation was measured.

In addition, a monoclonal antibody directed against the GP IIb/IIIa receptor of rabbit platelets (AZ-1) was used (kindly provided by M. Azrin [Farmington, Conn.] and M. Ezekowitz [New Haven, Conn.]). As previously described (1), AZ-1 was added to PRP to a final concentration of ~50 μg/ml at 37°C 3 min prior to addition of ADP (10 μM). After confirmation of antibody-mediated inhibition of ADP-induced aggregation, *S. aureus* (Lafferty) was added to this reaction mixture, and aggregation was measured as before.

(ii) Integrin binding inhibition. The GP IIa component of the GP IIb/IIIa receptor complex recognizes and binds to an RGDX sequence on the α-chain of fibrinogen (26). To determine whether *S. aureus*-induced platelet aggregation utilizes the fibrinogen RGDX motif to execute platelet aggregation, synthetic RGDS peptide (Arg-Gly-Asp-Ser; 150 to 1,500 μg/ml; Sigma Chemicals) was added to PRP 30 min prior to the addition of the bacterial inoculum. Pilot studies showed that ADP-induced platelet aggregation was blocked by 500 μg of RGDS per ml. In a parallel study, bacteria were added to RGDS-containing PRP 10 min after addition of ADP (10 μM).

(iii) Dodecapeptide binding inhibition. A dodecapeptide domain (HHLGG AKQAGDV) on the δ-chain of fibrinogen is thought to participate in platelet aggregation by binding to a recognition site on the IIb component of the platelet GP IIb/IIIa receptor complex (15). To delineate whether *S. aureus*-induced platelet aggregation is mediated through a dodecapeptide-dependent mechanism, synthetic dodecapeptide (0.1 to 1 mM; Sigma Chemicals) was added to PRP 30 min prior to addition of the bacterial inoculum. Pilot studies showed that a 0.2 mM concentration of the dodecapeptide blocked ADP-induced platelet aggregation. In a separate experiment, bacteria were added to dodecapeptide-containing PRP at 10 min after addition of ADP (10 μM). In a parallel strategy, both RGDS peptide and the dodecapeptide were added to PRP 30 min prior to addition of the bacterial inoculum.

RESULTS

Influence of bacterium-platelet ratios on platelet aggregation. When tested at a bacterium-platelet ratio of 5:1, addition of the Lafferty strain to PRP resulted in biphasic aggregation. Intervals between the first and second aggregation phases were short (~1 to 3 min), and aggregation was completed within 16 ± 2 min (Fig. 1a). At bacterium-platelet ratios of 2.5:1 and 1:1, the onset of phase 2 of aggregation was delayed (Fig. 1b and c). Similar results were seen with *S. aureus* RN6390; intervals between phases 1 and 2 (lag time) were ~8 ± 1 min and 24 ± 1.5 min at bacterium-platelet ratios of 5:1 and 2.5:1, respectively (data not shown). In contrast, viridans group streptococcal strain STV287 caused less extensive, uniphasic aggregation at bacterium-platelet ratios of 5:1 (Fig. 1d) and 10:1 (data not shown).

Influence of bacterial growth phase and viability on platelet aggregation. Both logarithmic- and stationary-phase staphylococcal cells caused biphasic platelet aggregation. However, logarithmic-phase cells triggered phase 2 of aggregation earlier than stationary-phase cells. For example, at a 5:1 bacterium-platelet ratio, logarithmic-phase Lafferty cells initiated phase 2 of aggregation within 8 ± 0.6 min of adding the bacterial inoculum to PRP, compared with 16 ± 1 min for stationary-phase cells. Similarly, strain ST021 triggered phase 2 of aggregation substantially faster when in logarithmic growth phase than when grown to stationary phase (10 ± 1 min versus 20 ± 0.5 min). Of note, this strain is known to maximally express its polysaccharide capsule postexponentially (27).

In contrast, after the Lafferty strain was killed with heat or paraformaldehyde, only phase 1 of aggregation was seen. Latex beads of the same size as staphylococcal cells (0.8-μm diameter) yielded a uniphasic aggregation curve virtually identical to that seen with killed staphylococci.

Influence of bacterial cell wall on platelet aggregation. When osmotically stabilized *S. aureus* protoplasts were added to PRP (5:1 or 10:1 protoplast-platelet ratio), only phase 1 of aggregation was observed. This could not be attributed to reagents used in making or stabilizing the protoplasts, since neither lysostaphin, DNase, nor 30% raffinose altered platelet aggregation by intact bacteria. After trypsinization, the onset of phase 2 of Lafferty-induced aggregation was markedly delayed (36 min versus 8 min for the untrypsinized control strain).

Influence of bacterial fibrinogen-binding capacity on platelet aggregation. Exposure of *S. aureus* cells to preimmune rabbit IgG did not alter typical biphasic platelet aggregation. In contrast, exposure of Lafferty cells to fibrinogen-binding-protein antibody at concentrations from 200 to 1,000 μg/ml substantially delayed the onset of phase 2 of aggregation compared with onset in bacterial cells exposed to preimmune IgG (22 ± 2 min versus 8 ± 1 min, respectively [Fig. 2]).

Addition of *S. aureus* cells (Lafferty strain) to PRP after 30 min of incubation with the pentadecapeptide (3.33 mM) eliminated phase 2 of aggregation. However, addition of the pentadecapeptide either immediately after addition of the bacterial inoculum or at the end of phase 1 of aggregation did not alter the usual biphasic platelet aggregation curve of PRP. The role of fibrinogen in platelet aggregation was further examined by using isogenic mutants that exhibited reduced fibrinogen-binding capacity compared with their respective parental strains. In these studies, mutant strains I and IE3 induced phase 1 of platelet aggregation normally. However, these strains exhibited either no phase 2 of aggregation or a prolonged lag period prior to the onset of phase 2 of aggregation compared with their respective parental strains. For example,

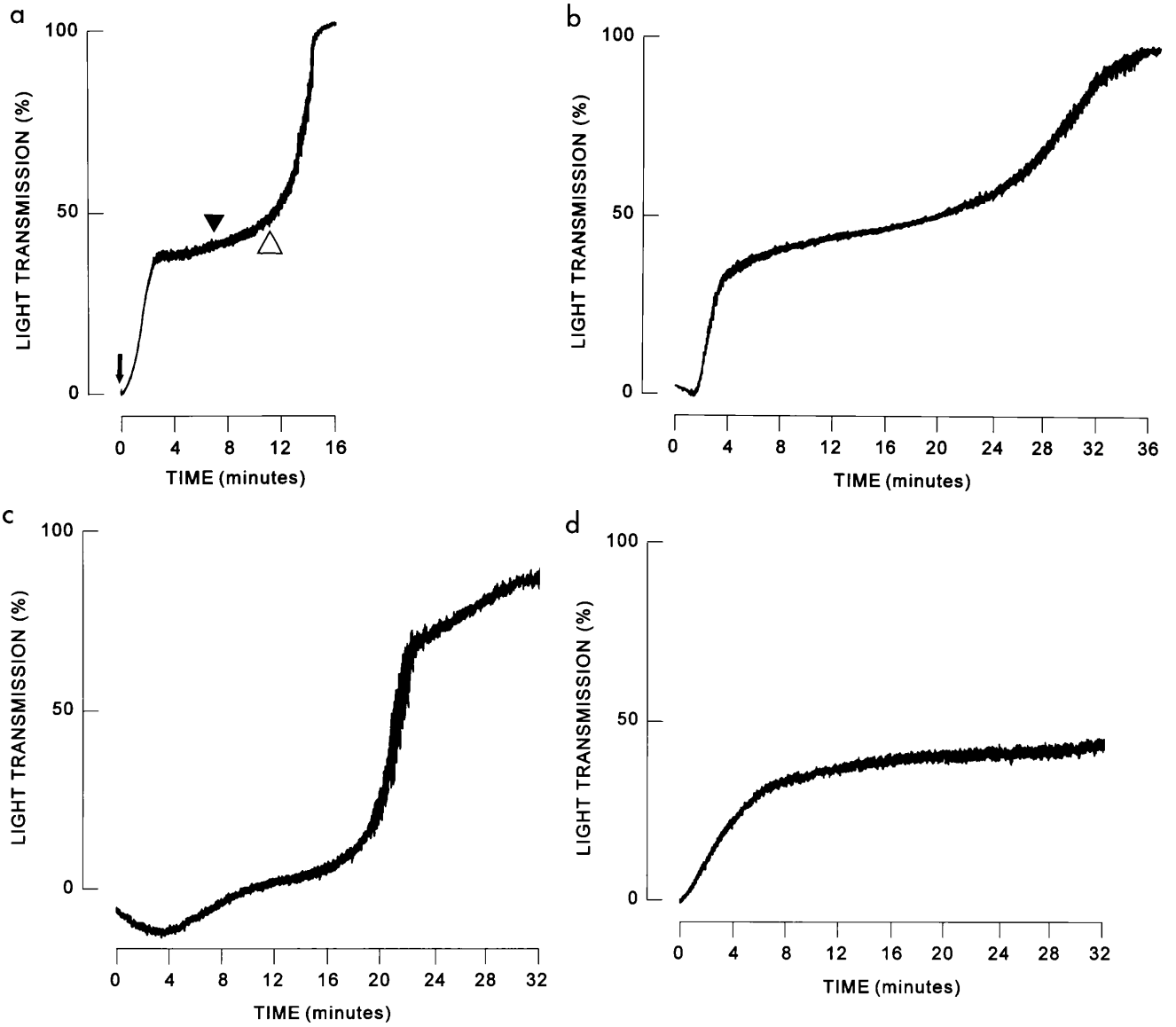

FIG. 1. Platelet aggregation induced by *S. aureus* Lafferty at bacterium-platelet ratios of 5:1 (a), 2.5:1 (b), and 1:1 (c) and by viridans group streptococcal strain STV287 (d). The arrow indicates addition of bacterial inoculum to PRP followed by phase 1 of aggregation; the closed arrowhead indicates the lag period between phases 1 and 2 of aggregation; the open arrowhead indicates onset of phase 2 of aggregation.

parental strain RN6390 induced phase 2 of platelet aggregation at 13 ± 1 min after addition of the bacterial inoculum to PRP, while phase 2 of aggregation induced by its isogenic variant (I) did not begin until ~28 ± 3 min after mixing. Similarly, the onset of phase 2 of aggregation induced by mutant strain IE3 was not observed over a 32-min period after addition of bacteria to PRP, compared with 14 ± 2 min for its isogenic parent strain ISP-479. Mutant strain DU-5852 exhibited similar deficiencies in triggering phase 2 of aggregation, compared with its parental strain (Newman), which aggregated platelets with typical biphasic kinetics.

Role of plasma fibrinogen in bacterially induced platelet aggregation. In afibrinogenemic PRP, *S. aureus* (Lafferty) caused normal phase 1 aggregation but a prolonged lag period prior to the onset of phase 2 of aggregation (Fig. 3). The reconstitution of normal fibrinogen concentrations in PRP by addition of exogenous rabbit fibrinogen (2 mg/ml) to PRP resulted in typical *S. aureus*-induced biphasic aggregation.

ATP secretion during bacterially induced platelet aggregation. Maximal ATP secretion (~3 μM) was seen 2 min after addition of bacteria to PRP. This peak of ATP secretion coincided with the maximal point of phase 1 of aggregation (Fig. 4). Comparable ATP secretion and platelet aggregation were seen with paraformaldehyde-killed *S. aureus* cells. Strains RN6390 and ISP479, as well as their respective isogenic mutants (I and IE3), caused ATP secretion and platelet aggregation curves similar to those observed with Lafferty cells. For all strains, no ATP secretion was seen during phase 2 of aggregation.

Platelet surface bacterial binding. The adherence of bacteria to washed platelets in a plasma-free milieu was independent of the organism's fibrinogen-binding capacity. For example, at bacterium-platelet ratios of 10:1, there were no significant differences between the percentage of the bacterial inoculum bound to the platelet surface, as determined by flow cytometry, for parent strain ISP-479 and the percentage for its

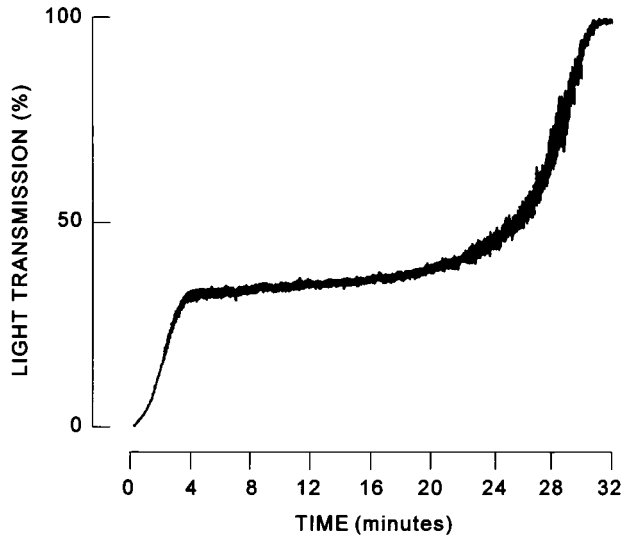

FIG. 2. Platelet aggregation induced by *S. aureus* Lafferty after exposure of bacteria to anti-fibrinogen-binding-protein antibody.

isogenic derivative, IE3 (91% ± 1.8% versus 96.3% ± 0.8%, respectively).

Antibody to GP IIb/IIIa platelet receptor. Both monoclonal antibodies OP-G2 and AZ-1 inhibited ADP-induced aggregation. However, addition of *S. aureus* to such blocked PRP preparations resulted in typical aggregation of PRP.

Synthetic RGDS peptide and synthetic dodecapeptide inhibition of bacterially induced platelet aggregation. RGDS (1,500 μg/ml) inhibited ADP-induced platelet aggregation of PRP. Addition of *S. aureus* to this RGDS-blocked PRP preparation resulted in typical biphasic aggregation. Similarly, addition of RGDS to PRP 30 min prior to addition of the bacterial inoculum (in the absence of prior ADP) resulted in a normal *S. aureus*-induced platelet aggregation profile.

The dodecapeptide (0.2 mM) also blocked ADP-induced aggregation of PRP. However, addition of *S. aureus* (Lafferty) to this dodecapeptide-blocked PRP preparation resulted in typical biphasic platelet aggregation. Similarly, *S. aureus*-induced platelet aggregation proceeded normally despite addition of both the synthetic RGDS and dodecapeptide to PRP 30 min prior to addition of the bacterial inoculum (Fig. 5).

ADP inhibition. Preincubation of platelets with apyrase at concentrations ranging from 3 to 5 mg/ml delayed the onset of *S. aureus*-induced aggregation phase 2. In contrast, a 1-mg/ml concentration of apyrase completely prevented viridans group streptococcus-induced aggregation.

Platelet activation. Inhibition of platelet activation by PGI_2 resulted in a substantial reduction in the amount of secreted ATP following addition of *S. aureus* Lafferty (<20% of normal). Addition of *S. aureus* to PGI_2-treated PRP yielded an intact phase 1 of aggregation, with a prolongation of the lag phase before the onset of an intact phase 2 of aggregation compared with normal PRP (14 versus 8 min). Addition of *S. aureus* to paraformaldehyde-fixed platelets resulted in only phase 1 of aggregation.

Cyclooxygenase pathway. When 20 μM indomethacin was added to PRP, ADP-induced aggregation was completely inhibited. This concentration of indomethacin had little effect on phase 1 of aggregation or on ATP secretion (Lafferty or ISP 479), while completely blocking phase 2 of aggregation.

DISCUSSION

The ability of an organism to induce IE and promote the evolution of valvular vegetations appears to be linked to its capacity to bind to the platelet surface and initiate irreversible aggregation (12, 17, 18, 23). In experimental endocarditis, compounds which interfere with platelet activation or responsiveness, or antiplatelet antibodies that result in immune thrombocytopenia, cause substantial reductions in vegetation weight, confirming that platelets account for the majority of the vegetation mass (23, 32). However, neither the specific bacterial adhesin(s) nor the platelet receptor(s) responsible for bacterially induced platelet aggregation has been clearly identified to date.

The initial binding of *S. aureus* to the platelet surface is a rapid, saturable, and reversible process that is dependent upon the relative numbers of platelet surface receptors available to the bacterium (38). In the present study, no significant difference in platelet surface binding by isogenic *S. aureus* strains

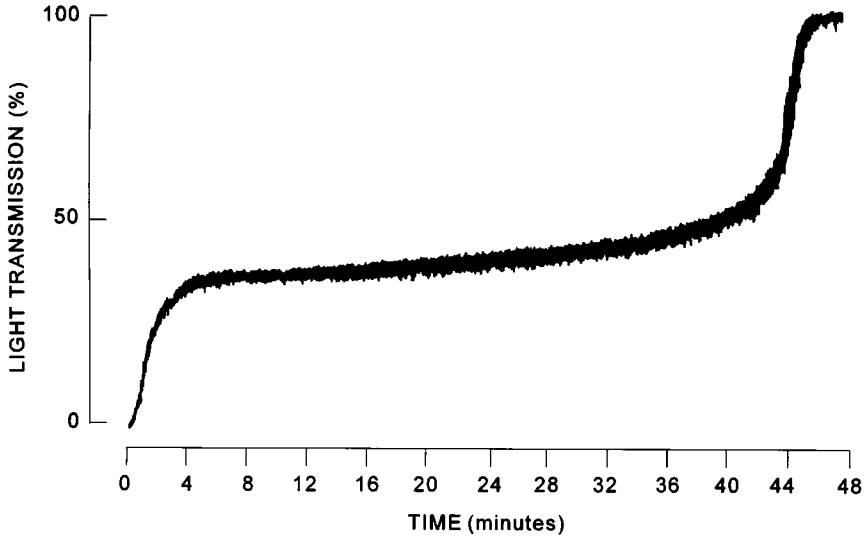

FIG. 3. Platelet aggregation induced by *S. aureus* Lafferty in the presence of afibrinogenemic plasma.

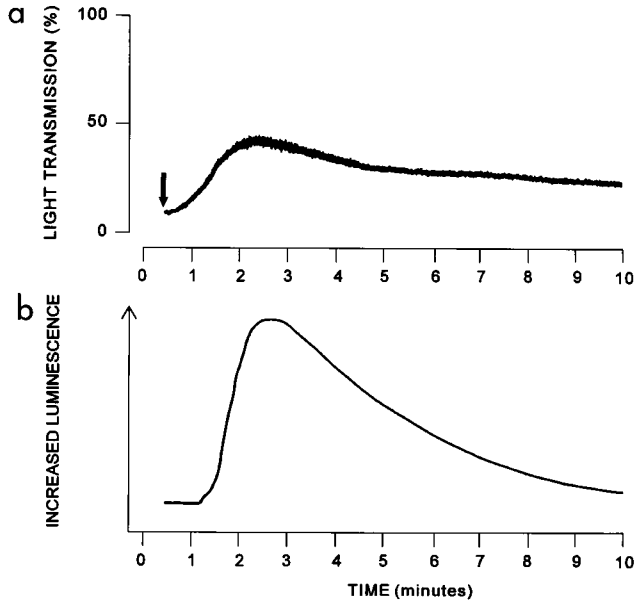

FIG. 4. Release of ATP from platelets following addition of *S. aureus* to platelets (arrow). The bacterium-platelet ratio was 1:1. (a) Aggregation curve; (b) ATP release measured by chemiluminescence.

which differed in fibrinogen binding was found. However, subsequent platelet aggregation by these same strains differed substantially. Thus, the earliest bacterium-platelet interactions (i.e., platelet surface binding) do not determine the course of later aggregation events.

Aggregation of rabbit platelets by *S. aureus*, at bacterium-platelet ratios of $\geq$5:1, proceeds with biphasic kinetics, similar to that described for human platelets exposed to serotonin or high concentrations of ADP (24, 37). At ratios of $<$5:1, a reduction of aggregation phase 1 and a prolongation of the lag period prior to normal phase 2 aggregation were observed. These observations suggest that a critical number of staphylococcal cells must bind to the platelet surface to initiate and fully execute platelet aggregation.

The necessity for an intact staphylococcal cell wall in biphasic platelet aggregation was confirmed in the present study. *S. aureus* protoplasts lacking cell walls induced uniphasic platelet aggregation but did not trigger phase 2 of aggregation. Similarly, trypsinization of *S. aureus* surface proteins resulted in an intact phase 1 of aggregation, with substantial prolongation in the lag period preceding phase 2. Despite equivalent phase 1 aggregation profiles, logarithmic-phase *S. aureus* cells initiated phase 2 of aggregation more rapidly than stationary-phase cells. This finding may be explained by the observation that maximal production of cell wall-associated staphylococcal proteins, such as fibrinogen-binding proteins, occurs during exponential growth but is repressed postexponentially (5, 19). A corollary to this concept is that the polysaccharide capsule of *S. aureus*, maximally expressed postexponentially (27), was shown to inhibit platelet aggregation in the present study, paralleling previous data for viridans group streptococcus-induced platelet aggregation (31).

Paraformaldehyde-killed and heat-killed staphylococcal cells, staphylococcal protoplasts, and latex beads of the same size as staphylococcal cells induced uniphasic aggregation, with no phase 2. Similarly, viable staphylococcal cells induced aggregation of PGI_2-treated and indomethacin-treated platelets with either delayed onset or absence of phase 2 of aggregation. Moreover, viable staphylococci induced only uniphasic aggregation in the presence of paraformadehyde-fixed platelets. Collectively, these data suggest that phase 1 of aggregation may well represent agglutination between staphylococcal cells and platelets (i.e., it is a process not requiring platelet activation or staphylococcal viability), while phase 2 of aggregation requires viable staphylococcal cells with intact cell wall surface proteins and is accompanied by platelet activation and ATP secretion.

The GP IIb/IIIa receptor is the most abundant of the platelet integrin receptors, and when activated by various agonists, it becomes a receptor for several soluble plasma proteins, including von Willebrand factor, fibronectin, vitronectin, and fibrinogen (24, 25). In turn, fibrinogen has been shown to be critical in irreversible platelet aggregation (24). There is controversy regarding which specific motifs on the fibrinogen molecule are principally responsible for aggregation of activated platelets via the GP IIb/IIIa receptor. It appears that at least two loci on the GP IIb/IIIa receptor are important in fibrinogen binding to activated platelets: RGD and dodecapeptide recognition sites. The recognition site for the N-terminal RGD sequence of the α-chain of fibrinogen lies within the N-terminal region of the GP IIIa molecule, at residues 109 to 171. The dodecapeptide sequence found at the extreme C terminus of the fibrinogen δ-chain binds to a recognition site on the GP IIb molecule at residues 294 to 314 (3). There may be coordinate control of fibrinogen binding to these distinct recognition sites on the GP IIb/IIIa heterodimer, such that fibrinogen binding to one recognition sequence makes the other functionally unavailable for binding. This concept is supported by site-directed mutagenesis studies, which show that the fibrinogen δ-chain is essential for optimal ADP-induced platelet aggregation while the α-chain RGD sequences alone are not sufficient to induce complete aggregation (15). In a recent study (38), we showed that initial *S. aureus* binding to the platelet surface, in the absence of plasma factors, probably does not involve the GP IIb/IIIa receptor. In that study, we used a fluorescein-labelled monoclonal antibody to the GP IIb/IIIa receptor to label platelets (38). This antibody labelling did not interfere with staphylococcal binding to the platelet surface, which oc-

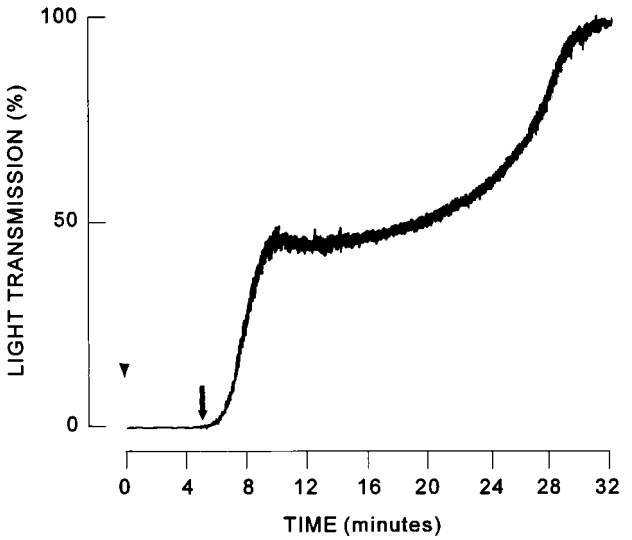

FIG. 5. Block of ADP-induced aggregation of PRP by combination of synthetic RGDS peptide and synthetic dodecapeptide (arrowhead indicates addition of ADP). *S. aureus* Lafferty added to this reaction mixture (arrow) induces normal biphasic aggregation.

TABLE 2. Effects of plasma fibrinogen and fibrinogen binding on *S. aureus*-induced platelet aggregation

Variable	Effect on phase 2 aggregation[a]
Presence of antibody to fibrinogen-binding protein	Prolonged lag phase
Use of fibrinogen-binding-defective mutant:	
I	Prolonged lag phase
IE3	No phase 2
DU-5852	No phase 2
Presence of afibrinogenemic plasma	Prolonged lag phase
Inhibition of fibrinogen binding site on GP IIb/IIIa receptor:	
RGDS	None
Dodecapeptide	None
Both	None
Presence of antibody to GP IIb/IIIa receptor:	
Rabbit	None
Human	None
Inhibition of fibrinogen-*S. aureus* binding by pentadecapeptide	No phase 2

[a] Phase 1 aggregation was normal in every case. "None" indicates normal phase 2 aggregation.

curred at rates similar to those observed in the present study with internally labelled platelets.

To determine if *S. aureus* binding to the platelet GP IIb/IIIa integrin receptor via fibrinogen bridging was involved in staphylococcus-induced platelet aggregation, known GP IIb/IIIa recognition motifs for fibrinogen were blocked prior to addition of the bacteria to PRP. Monoclonal antibodies directed against rabbit and human GP IIb/IIIa, exogenous RGDS peptide, and exogenous dodecapeptide failed to prevent typical biphasic *S. aureus*-induced platelet aggregation. In contrast, each of these blocking maneuvers prevented ADP-mediated platelet aggregation. These findings suggest that *S. aureus*-induced aggregation is independent of bacterial binding to and cross-linking of fibrinogen previously bound to known GP IIb/IIIa recognition sites. It is still possible, however, that *S. aureus* may bind to non-RGD and/or nondodecapeptide recognition sites within the GP IIb/IIIa complex during platelet aggregation. Of interest, a recent study has confirmed that *Borrelia burgdorferi* utilizes the GP IIb/IIIa receptor to bind to human platelets (10). Studies are in progress to characterize the influence of monoclonal antibodies against other domains on the GP IIb/IIIa molecule upon *S. aureus*-induced platelet aggregation.

Several findings in the present study suggest that the fibrinogen-binding capacity of *S. aureus* is of pivotal importance in phase 2 of rabbit platelet aggregation. *S. aureus* mutants with reduced ability to bind to fibrinogen in vitro caused platelet aggregation with either marked diminution or absence of phase 2 compared with their respective parental strains. In addition, polyclonal antibody directed against a fibrinogen-binding protein (clumping factor) substantially prolonged the lag time between the two platelet aggregation phases. Moreover, an exogenous synthetic pentadecapeptide representing the 15-residue C terminus of the δ-chain of fibrinogen that attaches to the fibrinogen-binding protein of *S. aureus* blocked phase 2 of aggregation. Lastly, we documented a substantial delay prior to the onset of phase 2 of aggregation in afibrinogenemic PRP, with full reconstitution of typical *S. aureus*-induced platelet aggregation following addition of exogenous fibrinogen. Table 2 summarizes the influence of various modulators upon phase 2 of *S. aureus*-induced platelet aggregation.

The collective data from this study contribute to an evolving paradigm which indicates that *S. aureus* adherence to and aggregation of platelets are mechanistically distinct. *S. aureus* adherence to the platelet surface is rapid (complete within 15 to 30 s), saturable, and reversible and proceeds in the presence or absence of plasma proteins (38). In contrast, *S. aureus*-induced aggregation of platelets requires the presence of plasma, is initiated rapidly after bacterial addition (~1 min), and exhibits biphasic kinetics. Aggregation phase 1 is independent of staphylococcal viability, bacterial growth phase, intact bacterial cell wall, bacterial fibrinogen-binding protein, platelet cyclooxygenase activity, and fibrinogen-binding domains on the platelet GP IIb/IIIa receptor, suggesting that passive bacterium-platelet agglutination may be occurring. After a variable lag period, platelet aggregation phase 2 is triggered. As with phase 1 of aggregation, this second aggregation phase is independent of the two major fibrinogen-binding domains on platelet GP IIb/IIIa. However, in contrast to phase 1 of aggregation, phase 2 requires both viable staphylococci with intact fibrinogen-binding capacity and adequate plasma fibrinogen, and it appears to be facilitated in the presence of platelet activation. Therefore, it seems likely that maximal, irreversible platelet aggregation is dependent upon fibrinogen cross-linking between bacterial cells bound to the surface of adjacent, activated platelets. Of note, a critical role for fibrinogen binding in the pathogenesis of *S. aureus* endocarditis was recently supported by Moreillon et al. (22). These investigators showed that *S. aureus* mutants with insertional inactivation of structural genes mediating fibrinogen binding had a markedly reduced ability to induce experimental endocarditis in catheterized rats. Thus, the fibrinogen-binding protein(s) of *S. aureus* may well represent a novel bacterial target amenable to prophylactic or therapeutic interventions in IE. These issues are under investigation in our laboratories.

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