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Mechanisms of Platelet Aggregation by Viridans Group Streptococci

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The direct aggregation of platelets is thought to be an important event in the pathogenesis of viridans streptococcal endocarditis, but the mechanisms for platelet activation are unknown. We evaluated the processes by which two endocarditis-producing strains of viridans group streptococci activated human platelets in vitro, as measured by platelet cyclooxygenase activity, secretion, and aggregation. Addition of either streptococcal strain to platelets suspended in whole plasma resulted in a mean lag phase of 15.3 min, followed by platelet secretion and brisk aggregation. The lag phase duration was dependent on the platelet donor and appeared to be a function of direct platelet-bacterial interaction. Aggregation was partially inhibited by 20 M indomethacin and blocked completely by 1 mg of apyrase, an extracellular ADP hydrolase, per ml. Neither strain aggregated washed platelets suspended in Tyrode solution alone. However, both strains produced maximal aggregation when the platelet suspension was supplemented with 10% (final concentration) normal plasma. Studies with factor-deficient plasmas demonstrated that exogenous fibrinogen was required for aggregation. One or more additional plasma components were needed, which eluted with a molecular weight of 67,000 to 130,000 on gel permeation chromatography. These cofactors have not been described for other platelet agonists, which suggests that viridans streptococci may aggregate human platelets by a novel mechanism.

The pathogenesis of bacterial endocarditis is an intricate process, involving complex interactions of bacteria, endocardium, and hematic components. Careful histologic studies indicate that bacterial colonization of the valve surface is followed by the rapid accumulation of fibrin and platelets (15, 19). This local deposition is essential for the development of the macroscopic endocardial vegetation and is thought to render the lesion poorly responsive to antimicrobial therapy (16). Since several species of bacteria have been shown to trigger platelet aggregation in vitro, one proposed mechanism for platelet deposition onto vegetations is their direct aggregation by organisms adherent to the endocardium (8, 11, 23, 30). The pathways by which bacteria trigger aggregation have been delineated for only a few organisms, but it is apparent that the mechanisms employed can differ between species.

Viridans group streptococci are the most frequently implicated pathogen in bacterial endocarditis. Although these organisms have been shown to induce platelet aggregation in vitro, the processes by which they activate platelets have not been characterized fully (24–26). For this reason, we further investigated the mechanisms of platelet aggregation by viridans streptococci by examining the roles of plasma, platelet cyclooxygenase activity, and platelet secretion in mediating aggregation.

MATERIALS AND METHODS

Bacterial strains. A strain of *Streptococcus salivarius* (D1) and one of *Streptococcus sanguis* I (M99) were evaluated. Each strain was recovered from the blood of a patient with endocarditis and was identified to species as described by Facklam (17). Stock cultures of both strains were stored frozen until the time of experimentation.

Preparation of bacteria. Strain D1 was grown in a chemically defined medium (FMC) (48), while strain M99 was cultured in Todd-Hewitt broth. The type of medium employed had no effect on the ability of either strain to aggregate platelets. Eighteen hours after inoculation of the culture medium, each strain was harvested by centrifugation at 2,000 × *g* for 15 min. The bacteria were washed three times in cold 0.05 M Tris hydrochloride–0.1 M NaCl–0.02 M EDTA (pH 7.25) and then suspended in Hanks balanced salt solution (HBSS). To disperse any bacterial clumps, the suspension was sonicated with an ultrasonic processor (model W-225; Heat Systems-Ultrasonics, Farmingdale, N.Y.) for 15 s at an output of 64 W, using a tapered microtip (model 419). The bacterial suspension was examined by phase-contrast microscopy to confirm that all clumps had been dispersed. Bacteria suspended in HBSS were adjusted to a final concentration of approximately 4×10^9 cells per ml, as measured by counting in a Neubauer hemacytometer (American Optical, Buffalo, N.Y.). To confirm that sonication had no effect on bacterial viability, a 0.1-ml sample of the sonicated suspension was serially diluted in phosphate-buffered saline and plated onto blood agar. Colony counts were performed after 24 h of incubation at 37°C. Counts obtained from the same sample before sonication served as controls. Titers increased from $1.68 \times 10^9 \pm 0.57 \times 10^9$ (mean ± standard deviation; $n = 4$) before sonication to $4.15 \times 10^9 \pm 1.36 \times 10^9$ posttreatment, demonstrating that sonication did not reduce viability.

Preparation of fresh platelet suspensions. Blood was collected by venipuncture from normal healthy donors who had not taken any medication for at least 2 weeks before phlebotomy. Nine volumes of blood were drawn into a plastic syringe containing 1 volume of 3.8% buffered citrate solution (0.11 M sodium citrate · $2H_2O$ and 0.02 M citric acid · H_2O, pH 5.5). Platelets were obtained by centrifuging the blood at 100 × *g* for 15 min at 25°C and collecting the upper layer.

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Platelet-depleted plasma was prepared by centrifuging the remaining blood specimen at 2,000 × *g* for 10 min. The concentration of the platelets in the upper plasma layer was determined by counting in a hemacytometer, and the final concentration was adjusted to 3×10^8 to 5×10^8 platelets per ml by the addition of autologous platelet-depleted plasma.

Preparation of washed platelets. For some experiments, platelets were separated from plasma by repeated centrifugation (40). Five volumes of human blood were drawn into a plastic syringe containing 1 volume of 0.08 M citric acid–0.17 M sodium citrate (pH 5.5). The platelet-rich supernatant was obtained as described above and then centrifuged at 1,000 × *g* for 10 min. The platelet pellet was recovered and washed three times by suspension in a solution of 0.08 M NaCl–3.8 mM KH_2PO_4–4.0 mM $NaHPO_4$–2.8 mM glucose–16.6 mM citric acid–34.0 mM sodium citrate (pH 6.8), followed by centrifugation at 1,000 × *g* for 10 min. After the third wash, the platelets were suspended in Tyrode solution (pH 7.4). In some studies, the platelet suspensions were supplemented with human plasma. For these experiments, heparin (4 U/ml) was added to the platelet suspensions to prevent clot formation. Addition of heparin did not affect the interaction of platelets with bacteria, as measured by conventional lumi-aggregometry. The platelet concentration was adjusted to 3×10^8 to 5×10^8 cells per ml by the addition of heparinized Tyrode solution.

Preparation of gel-filtered platelets. In some studies, platelets were isolated from plasma by gel permeation chromatography (47). Platelets in plasma (4 ml) were applied to a 20-ml column of Sepharose 4B (Pharmacia, Piscataway, N.J.) and eluted with calcium-free Tyrode solution (pH 7.4) containing 1 mg of bovine serum albumin per ml. Fractions of 1 ml were collected, and the platelet content of each sample was determined by counting. Fractions with greater than 2×10^8 platelets per ml were pooled and supplemented with 1.8 mM $CaCl_2$ and 0.1 mg of fibrinogen per ml.

Preparation of paraformaldehyde-fixed platelets. Platelets were collected as described above and washed three times in Tyrode solution containing 10 mM EDTA and 5 mg of bovine serum albumin per ml (pH 7.2). After the third wash, the platelets were suspended in Tyrode solution plus 1% paraformaldehyde for 5 min (3). The platelets were collected by centrifugation at 1,000 × *g* for 10 min, washed three times in Tyrode solution, and suspended in plasma at a concentration of 4×10^8 platelets per ml.

Platelet aggregometry. Aggregation studies were performed according to the methods of Clawson and White (8). A standard reaction mixture of 0.45 ml of platelets and 0.05 ml of the bacterial suspension was tested at 37°C. Although aggregation can occur at ratios of bacteria to platelets ranging from 1:2 to 10:1, previous work in our laboratory has demonstrated that optimal aggregation occurs at a ratio of 1:1. Therefore, all experiments were performed at this ratio of bacteria to platelets.

Aggregation studies were performed with a single-channel aggregometer (model 330; Chronolog, Haverton, Pa.) connected to a chart recorder. The base line for minimal light transmission was established with platelets in suspension. In experiments with platelets suspended in plasma, the scale was adjusted to indicate maximal light transmission at the optical density of platelet-depleted plasma; for studies with washed or gel-filtered platelets, maximal transmission was set at the optical density of Tyrode solution. Platelet shape change and aggregation were detected as a decrease in the width of the oscillating base line and as an increase in light transmission, respectively. The aggregation lag phase was defined as the time interval between the addition of bacteria to the platelet suspension and the first increase in light transmission. The rate of aggregation was measured as the slope of the line tangential to the midpoint of the aggregation curve. A 50-μl sample of either a 2-mg/ml suspension of calf skin collagen (Sigma Chemical, Co., St. Louis, Mo.) or a 200 μM solution of ADP (Sigma) served as a positive control for the normal aggregation of platelets. In some experiments, platelets were incubated for 1 min with prostacyclin, indomethacin, or apyrase (Sigma) before undergoing aggregation testing. All aggregation studies were performed in duplicate on at least two separate occasions, and representative data are reported.

Platelet lumi-aggregometry. Platelet secretion was assessed by the measurement of ATP release from platelet granules as described by Feinman et al. (18). A lumi-aggregometer (model 500, Chronolog) was used to detect platelet secretion and aggregation simultaneously. Aggregation studies were performed as described, except that 50 μl of a 40-mg/ml luciferin-luciferase solution (0.2 mg of luciferin per ml and 22,000 ^{14}C U of luciferase per ml; Chronolog) was added to the platelet suspension 1 min before the addition of bacteria.

Human IgG. Immunoglobulin G (IgG) was isolated from human serum obtained from a donor known to have platelets that were aggregated by the bacterial strains tested. A crude gamma globulin fraction was prepared by the addition of 25 ml of saturated ammonium sulfate solution (pH 7.8) to 50 ml of serum, followed by centrifugation at 1,400 × *g* for 30 min. The precipitate was dissolved in 50 ml of saline and further purified by two additional ammonium sulfate precipitations, overnight dialysis in phosphate-buffered saline (pH 8.0) at 4°C, and DEAE chromatography (Pharmacia) (31).

IgG was also prepared by affinity chromatography (27). Plasma was filtered through a Sepharose-protein A column (Pharmacia), followed by passage through Sepharose 4B (Pharmacia) that had been conjugated with anti-human IgG antibodies (Atlantic Antibodies, Scarbrough, Maine) by means of cyanogen bromide activation (35). IgG was recovered from both the protein A and the IgG columns by washing with 0.1 M glycine (pH 2.6). The eluents were pooled, dialyzed for 24 h against Tyrode solution, and concentrated with an ultrafiltration unit (Amicon, Lexington, Mass.), using a PM 10 membrane.

As a third source of IgG, commercial pooled human IgG that had been prepared by a low pH process (Gamimune-N) was obtained from Cutter Laboratories (Berkeley, Calif.) (9). The IgG content of all three preparations was confirmed by radial immunodiffusion assay (Behring Diagnostics, La Jolla, Calif.) (34).

C3. C3 was isolated from human plasma as described by Hammer et al. (21). In addition, a commerical preparation of C3 was also evaluated (Behring). The concentration of C3 was determined by radial immunodiffusion assay (Behring).

Fibrinogen. Human fibrinogen (grade L; A. B. Kabi, Stockholm, Sweden) was dissolved in Tyrode solution and purified by gel filtration through Sephadex G-25 M (Pharmacia). The final protein concentration was determined by the Lowry assay (32).

Factor-deficient plasmas. Factor VIII-deficient plasma (less than 1% normal factor VIII activity) was obtained from a donor with Von Willebrand's disease (George L. King Biomedical, Inc., Overland, Kansas). Plasma with less than 0.15 mg of fibrinogen per ml was collected from a donor with congenital afibrinogenemia (George L. King Biomedical, Inc.).

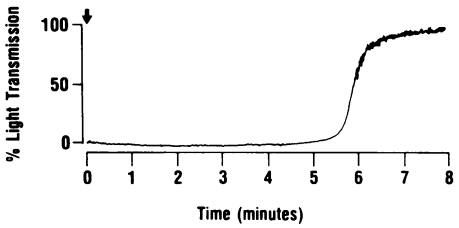

FIG. 1. Aggregation of human platelets by viridans group streptococci. Strain D1 was added to platelets suspended in plasma at 0 min (arrow). After a 5-min lag phase, brisk aggregation was observed, which was complete within 1 min.

Fibrinogen assay. The fibrinogen content of plasma was determined by measurement of the thrombin time (7).

Thromboxane assay. Platelet cyclooxygenase activity was assessed by measurement of thromboxane B_2 (TxB_2) production during aggregation. At the completion of aggregation, the platelet-bacterial suspension was removed from the aggregometer and centrifuged at 10,000 × *g* for 1 min. The supernatant was collected and stored at −70°C until the time of assay. Platelets incubated with indomethacin before aggregation testing were compared with untreated platelets. Unactivated platelets served as controls for background counts. TxB_2 was measured in duplicate by a previously described radioimmunoassay (14, 45), using commercial reagents (New England Nuclear Corp., Boston, Mass.).

Partial purification of plasma cofactors required for aggregation by viridans streptococci. A 30-ml sample of citrated fresh plasma was collected from one donor and fractionated by ultrafiltration (Amicon, PM 30 membrane). The high- and low-molecular-weight fractions were collected and added to separate suspensions of washed platelets. Each suspension was then tested for aggregation by strain D1. The high-molecular-weight fraction was further purified by gel permeation chromatography at 4°C over a Sepharose CL-6B column (Pharmacia; bed volume, 350 ml). The eluent was monitored by UV absorption at 280 nm to identify proteins and collected as 4.4-ml fractions. Dextran (M_r, 2,000,000), human IgG, and bovine serum albumin served as molecular weight standards. Each elution peak was pooled separately, concentrated by ultrafiltration to a volume of 5 ml, and then tested for its ability to support the aggregation of washed platelets by strain D1.

Statistical methods. Differences between donors in lag-phase duration were compared by the Kruskall-Wallis test. The effects of indomethacin on lag phase and rate of aggregation were evaluated by the Wilcoxon paired-sample test.

RESULTS

Platelet activation by bacteria: aggregation of platelets suspended in plasma. Strains D1 and M99 aggregated platelets suspended in plasma in 12 of 14 donors tested. A mean lag phase of 15.3 min (range, 5.0 to 33.5 min) was observed initially, followed by platelet shape change and brisk aggregation (Fig. 1). Aggregation was complete within 1 min of onset. No further aggregation was observed after the addition of 20 μM ADP or 200 μg of collagen per ml, indicating that both strains had induced complete aggregation. Platelet secretion occurred in parallel with aggregation, as measured by ATP release on lumi-aggregometry (Fig. 2).

The average lag phase varied significantly between donors ($P \leq 0.005$) and ranged from 6.3 ± 1.0 to 23.1 ± 6.6 min (mean ± standard deviation). However, it showed little intradonor variation when platelets were tested on different days or with either streptococcal strain. To determine whether the lag phase was predominantly due to plasma binding by bacteria or to the activation of plasma by the organisms, an overnight culture of strain D1 was washed as described, suspended in HBSS containing 50% (vol/vol) plasma, and incubated at 37°C for 30 min. Strain D1 preincubated in HBSS alone served as a control. Both bacterial suspensions were then tested for their capacity to aggregate platelets. Preincubation in plasma resulted in a mean reduction in lag phase of 2.0 min (range, 0 to 3.7 min; five donors tested), which represented a mean relative shortening of 25.0%. In no donor was the lag phase abolished by preincubation, indicating that it was not exclusively a product of plasma-bacterial interaction.

These studies suggested that the lag phase results predominantly from direct bacteria-platelet interaction. To examine this further, washed platelets from one individual were suspended in plasmas obtained from donors with different lag phases. When tested with strain D1, no change in lag phase was observed, suggesting that the lag phase is an intrinsic property of the platelets themselves.

To confirm that physiologic aggregation rather than passive agglutination was being observed, strain D1 was added to platelets that had been preincubated for 1 min with 130 μM prostacyclin, a potent inhibitor of aggregaton (49, 51). No aggregation was detected after 20 min of observation. In contrast, strain D1 aggregated untreated platelets from the same donor within 6 min. Strain D1 was then tested with paraformaldehyde-fixed platelets, which can undergo agglutination but not aggregation (3). Addition of D1 to fixed platelets failed to induce agglutination in two separate trials. In control experiments, fixed platelets were agglutinated by ristocetin (1.5 mg/ml), but were not affected by ADP (20 μM) or collagen (200 μg/ml).

To determine whether aggregation was mediated by the direct interaction of intact streptococci with platelets, as opposed to a soluble bacterial product, a suspension of strain D1 in HBSS or plasma (4×10^9 bacteria per ml) was incubated at 37°C for 30 min and then pelleted by centrifugation at 2,000 × *g* for 10 min. A 50-μl sample of each supernatant was added separately to platelets in plasma. After 30 min of observation, no aggregation was detected. In control studies, platelets from the same donors were aggregated within 11 min by D1. Thus, aggregation by strepto-

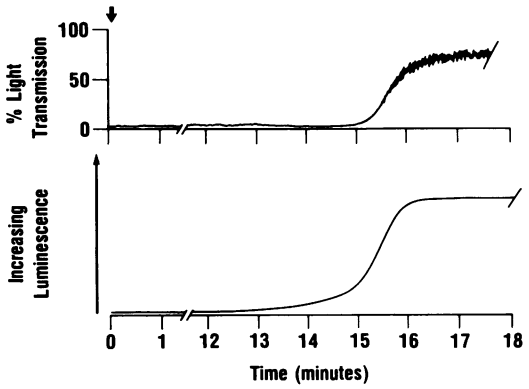

FIG. 2. Simultaneous measurement of platelet secretion and aggregation (lumi-aggregometry). Addition of strain D1 (arrow) to platelets in plasma resulted in a 14-min lag phase, followed by platelet secretion (bottom tracing) and aggregation (top). Platelets were obtained from a different donor than for Fig. 1.

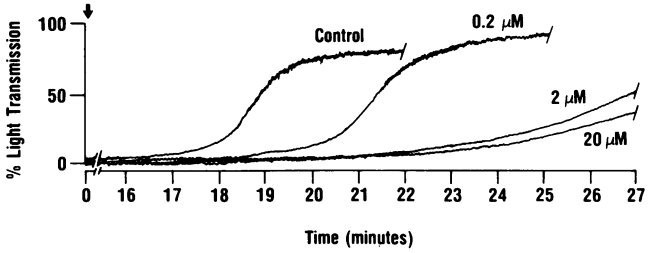

FIG. 3. Role of platelet cyclooxygenase in aggregation: inhibition by indomethacin. Platelets were incubated for 1 min with 0, 0.2, 2.0, or 20 μM indomethacin and then tested for their aggregation response by the addition of strain D1 (arrow). Note the prolonged lag phase and diminished rate of aggregation produced by indomethacin. Although delayed, maximal (100%) aggregation was observed at all concentrations of indomethacin evaluated.

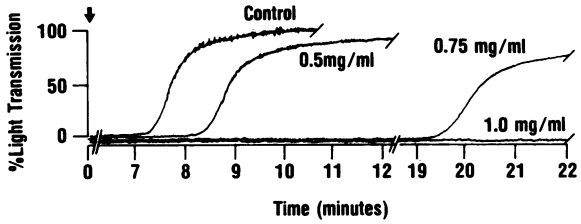

FIG. 4. Role of extracellular ADP in platelet aggregation: inhibition by apyrase. Platelets were incubated for 1 min with 0 to 1.0 mg of apyrase per ml before the addition of strain D1 (arrow). Note prolongation of the lag phase at the lower concentrations of apyrase. Complete inhibition of aggregation was seen at 1.0 mg/ml.

cocci required direct platelet-bacterial interaction and was not mediated exclusively by a soluble bacterial product or by the activation of a plasma component (e.g., thrombin).

Mechanisms of platelet activation. (i) Role of cyclooxygenase. To assess whether aggregation was dependent on platelet cyclooxygenase activity, both strains D1 and M99 were tested with platelets preincubated with various concentrations of indomethacin. Cyclooxygenase activity was evaluated by radioimmunoassay of TxB_2 production and by measuring the response of platelets to 1.6 mM arachidonic acid, an agonist that produces aggregation by the cyclooxygenase pathway exclusively (6, 22, 37). In all donors tested, indomethacin increased the duration of the lag phase and diminished the rate of aggregation, as indicated by a decrease in the slope of the aggregation tracing (Fig. 3). These effects varied directly with the concentration of indomethacin. Since the lag phases differed significantly between donors, the effect of indomethacin on the average lag-phase duration could not be calculated. However, it was possible to assess the mean change in lag phase and rate of aggregation with various concentrations of the drug. At a concentration of 1 μM indomethacin increased the lag-phase duration by 2.8 ± 3.7 min (mean ± standard deviation; $N = 4$) as compared with controls (Table 1). The rate of aggregation decreased from a mean slope of 2.42 ± 0.81 in controls to 1.13 ± 2.34. At 20 μM indomethacin, the aggregation lag phase was increased by 3.7 ± 2.3 min over controls, and the slope decreased to 0.72 ± 0.73, a significant change in both parameters ($n = 6$; $P \leq 0.05$, compared with untreated controls). Although it was delayed and prolonged, complete platelet aggregation was observed at 20 μM indomethacin in all donors tested.

At 20 μM indomethacin, cyclooxygenase activity was inhibited maximally (2, 12, 28). Platelets pretreated with this concentration of indomethacin failed to aggregate in response to 1.6 mM arachidonic acid. Moreover, TxB_2 production was reduced markedly. Aggregation of control platelets by strain D1 produced 30,000 and 140,000 pg of TxB_2 per ml (two donors evaluated). However, platelets preincubated with 20 μM indomethacin generated only 600 and 1,250 pg/ml, respectively, when aggregated.

TABLE 1. Effect of indomethacin on aggregation of human platelets by viridans streptococci (strain D1)

Indomethacin concn (μM)	n^a	Lag phase increase (min) (% change)	Slope
0	6		2.42 ± 0.81
1	4	2.8 ± 3.7 (12)	1.29 ± 2.34
2	3	4.1 ± 3.9 (26)	0.83 ± 1.05
10	4	6.8 ± 4.9 (49)	1.11 ± 0.53
20	6	3.7 ± 2.3* (39)	0.72 ± 0.73[b]

[a] n, Number of donors tested.
[b] $P < 0.05$, compared with controls.

(ii) Role of extracellular ADP. For some platelet agonists, aggregation is mediated by the release of ADP from platelet granules and its subsequent binding to specific receptors on the platelet surface (1, 5, 36). To determine whether aggregation by viridans streptococci was dependent on the release of platelet ADP, strain D1 was tested with platelets in plasma containing apyrase, an extracellular ADP hydrolase (44). At concentrations of 0.30 to 0.75 mg/ml, apyrase produced a prolongation of the lag phase and a slight diminution in the rate of aggregation (Fig. 4). Aggregation was blocked totally by 1 mg of apyrase per ml in five of five donors evaluated. Similarly, 0.3 mg of apyrase per ml delayed the onset and rate of platelet aggregation by strain M99; complete inhibition occurred at 1 mg/ml.

Plasma cofactors for aggregation. (i) Requirement for plasma components. To evaluate whether aggregation required one or more plasma cofactors, strains D1 and M99 were tested with platelets that had been separated from plasma by washing. Neither strain aggregated washed platelets suspended in Tyrode solution alone. However, washed platelets in Tyrode solution supplemented with 10% (vol/vol) heparinized plasma (4 U/ml) were aggregated normally by both strains.

Similarly, strain D1 did not activate platelet cyclooxygenase in the absence of plasma. Strain D1, added to washed platelets, produced levels of TxB_2 (300 to 850 pg/ml) that did not differ from those found in platelets alone or in platelets suspended in plasma. However, addition of strain D1 to washed platelets in 10% plasma generated 340,000 to 475,000 pg of TxB_2 per ml ($n = 2$).

Plasma also was required for platelet secretion. Addition of strain D1 to washed platelets in 10% plasma resulted in a normal lag phase, followed by platelet secretion of ATP and aggregation. However, strain D1 failed to induce either the secretion or aggregation of washed platelets suspended in Tyrode solution alone. After 20 min of observation, 50 μl of heparinized plasma was added to the platelet-bacterial suspension, resulting in a lag phase of less than 1 min, followed by rapid secretion and aggregation (Fig. 5). In control studies, addition of heparinized plasma to washed platelets failed to produce either ATP release or aggregation.

(ii) Characterization of plasma cofactors. To define better the plasma cofactors required for aggregation, strain D1 was tested with washed platelets in Tyrode solution containing

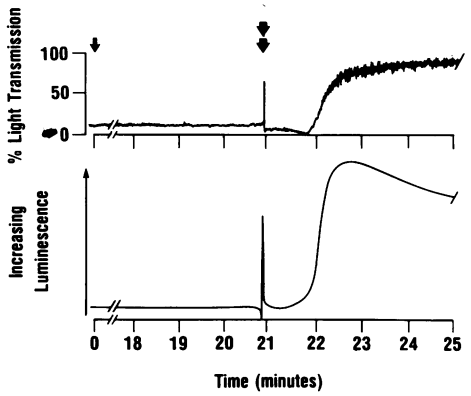

FIG. 5. Requirement of plasma for platelet activation. Strain D1 was added to washed platelets, suspended in Tyrode solution, which were obtained from the same donor as for Fig. 2. After 20 min of observation, no secretion or aggregation was detected. Addition of 50 μl of heparinized autologous plasma to the bacteria-platelet mixture (arrows) produced prompt platelet activation.

one or more plasma components (Table 2). Platelets suspended in 10% factor VIII-deficient plasma were aggregated normally. However, strain D1 failed to aggregate washed platelets in fibrinogen-deficient plasma. Normal aggregation was restored by the addition of fibrinogen (final concentration; 0.1 to 0.6 mg/ml) to the suspension. These results demonstrated that aggregation by strain D1 required exogenous fibrinogen. However, D1 failed to aggregate platelets in Tyrode solution supplemented with fibrinogen only (0.1 to 2 mg/ml), indicating that additional plasma components were needed (seven donors tested). Similarly, strain M99 failed to aggregate either washed platelets alone or washed platelets supplemented with fibrinogen (0.6 mg/ml). However, platelets in 10% plasma (final fibrinogen concentration, 0.1 to 0.2 mg/ml) were aggregated normally by strain M99.

To determine whether IgG or C3 was the essential cofactor, strain D1 was tested with washed platelets suspended in Tyrode solution with fibrinogen (0.1 to 0.6 mg/ml) and various concentrations of IgG or C3. Despite IgG concentrations (0.1 to 5 mg/ml) that exceeded those in Tyrode solution plus 10% normal plasma (0.5 to 1.0 mg/ml), no aggregation was observed with any of the three IgG preparations tested. Similarly, strain D1 failed to aggregate washed platelets supplemented with C3 (0.25 mg/ml) or with both C3 and IgG.

TABLE 2. Assessment of plasma cofactors for aggregation of human platelets by viridans streptococci[a]

Platelet medium	Aggregation[b]
Tyrode solution (T)	−
T + 10% normal plasma	+
T + 10% factor VIII-deficient plasma	+
T + 10% fibrinogen-deficient plasma	−
10% Fibrinogen-deficient plasma + fibrinogen (0.1–2 mg/ml)	+
T + fibrinogen	−
T + IgG + fibrinogen	−
T + C3 + fibrinogen	−
T + C3 + IgG + fibrinogen	−

[a] Washed platelets were suspended either in Tyrode solution alone or in Tyrode solution supplemented with one or more plasma components. Strains of viridans streptococci were prepared as described in the text and tested for their ability to aggregate washed human platelets in various media.

[b] +, Aggregated normally by streptococci; −, no aggregation.

The roles of fibrinogen, IgG, and C3 in mediating platelet secretion were also examined. Addition of strain D1 to washed platelets suspended in fibrinogen-deficient plasma failed to produce secretion. When the test suspension was supplemented with purified fibrinogen (0.2 mg/ml), this strain induced normal platelet activation. Addition of strain D1 to washed platelets suspended in Tyrode solution containing IgG (0.8 mg/ml), C3 (0.25 mg/ml), and fibrinogen (0.2 mg/ml) failed to trigger secretion. As was observed for aggregation, therefore, induction of platelet secretion by strain D1 required exogenous fibrinogen as well as additional plasma components, which were not IgG or C3 alone.

(iii) Partial purification of plasma cofactors. In an attempt to characterize better the plasma cofactors for platelet aggregation by viridans group streptococci, whole plasma was divided by ultrafiltration into low-molecular-weight (≤30,000) and high-molecular-weight fractions. These fractions were then tested for cofactor activity, i.e., the ability to support aggregation. Strain D1 failed to aggregate washed platelets suspended in heparinized Tyrode solution supplemented with the low-molecular-weight plasma components (10%, vol/vol) and 0.2 mg of fibrinogen per ml. However, platelets suspended in Tyrode solution with 10% high-molecular-weight components were aggregated normally by D1. The high-molecular-weight plasma components were further fractionated by gel permeation chromatography (Fig. 6). The eluent fractions were combined into six pools, concentrated by ultrafiltration to a 3-ml volume each, and tested for their ability to support aggregation. Fibrinogen (0.2 mg/ml) was added to each pool before testing. Washed platelets in Tyrode solution containing either 10% fraction 4 or 10% fraction 5 were aggregated normally by strain D1. No cofactor activity was present in the remaining pools (fractions 1, 2, 3, and 6). Fractions 4 and 5 represented two major adjacent protein peaks, corresponding to elution volumes 255 to 339 ml (73 to 93% of the bed volume). Based on their elution position between IgG and bovine serum albumin, the

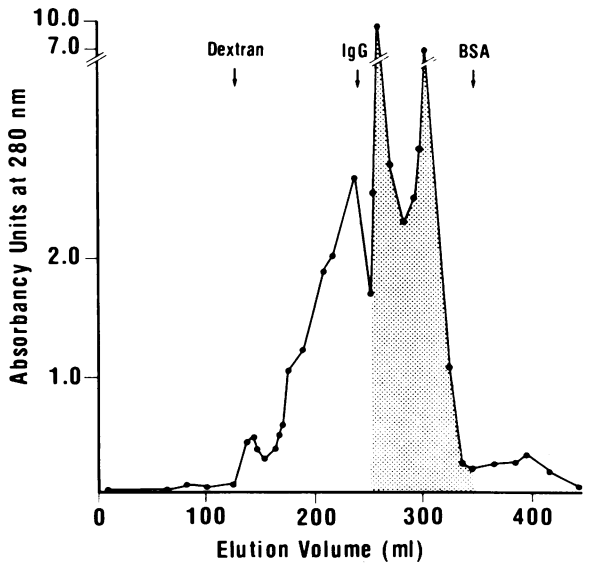

FIG. 6. Gel permeation chromatography (Sepharose CL-6B) of plasma cofactors for platelet aggregation. The high-molecular-weight plasma fraction (>30,000) produced by ultrafiltration was further purified by chromatography on Sepharose CL-6B. Only materials recovered in the M_r 67,000 to 130,000 fraction (shaded region) supported platelet aggregation by strain D1.

plasma components in both peaks were estimated to have molecular weights of 67,000 to 130,000.

(iv) Cofactor specificity for aggregation by streptococci. These experiments demonstrated that platelet aggregation by viridans streptococci required exogenous fibrinogen and one or more additional plasma cofactors in the above molecular-weight range. As discussed previously, however, aggregation was also dependent on extracellular ADP. In addition to fibrinogen (36), the aggregation of washed platelets by ADP often requires additional cofactors that are not well defined and that vary with the technique employed for washing (4, 10, 13, 41, 46, 50). Thus, it was not clear whether the M_r 67,000 to 130,000 cofactors were unique to aggregation by viridans streptococci or merely represented cofactors for ADP-mediated aggregation.

To resolve this issue, platelets were separated from plasma by gel permeation chromatography and then tested for their aggregation response to ADP or viridans streptococci. In control studies, gel-filtered platelets suspended in Tyrode solution (supplemented with 0.1 mg of fibrinogen per ml) were aggregated normally by 20 μM ADP. In contrast, strain D1 failed to aggregate gel-filtered platelets suspended in this medium. However, platelets in Tyrode solution supplemented with 10% plasma were aggregated normally by strain D1. As was found with washed platelets, the cofactors for aggregation were located in the M_r 67,000 to 130,000 region of plasma.

DISCUSSION

These studies demonstrate that viridans group streptococci aggregate human platelets by a series of complex mechanisms, of which some are typical of many platelet agonists while others may be unique to these organisms. Aggregation by streptococci is characterized by an initial lag phase of over 5 min, followed by platelet secretion and the rapid formation of aggregates. In contrast, conventional platelet agonists, such as ADP or thrombin, normally produce secretion and aggregation within 1 min of exposure to platelets (6). Although a brief lag phase is observed with aggregation by collagen, it seldom exceeds 2 min (33). The extensive lag phase seen with aggregation by viridans streptococci has been reported for other organisms, including group A streptococci, *Staphylococcus aureus*, and *Escherichia coli* (8, 30). Therefore, it appears to be a characteristic feature of platelet aggregation by bacteria.

The etiology of the lag phase remains obscure. Since evidence of platelet activation could be detected during this period, it is likely that the lag phase represents the time required for one or more events on the platelet surface. Studies by Clawson and White (8) and by Herzberg et al. (24–26) indicate that direct bacteria-platelet binding occurs during the lag phase. However, the time required for binding has not been described. Thus, it is possible that the lag phase represents predominantly the time needed for bacteria-platelet binding. Alternatively, binding may occur rapidly, but subsequent events on the platelet surface, which in turn produce activation, may take several minutes to occur. Both models of platelet activation have been reported for other agonists. For example, ADP is thought to trigger aggregation by binding two groups of protein receptors on the platelet surface (1, 5). In contrast, aggregation by thrombin is produced by its binding to one class of membrane ligands (glycoprotein Ib), followed by hydrolysis of a separate surface receptor, glycoprotein V, which may then result in platelet activation (20, 29, 42).

The interaction of plasma with bacteria or platelets contributes minimally to the lag phase, as indicated by three studies. First, preincubation of bacteria with plasma produced only a 25% reduction in its duration, suggesting that plasma-bacterial binding, if present, was not the rate-limiting step. Second, the suspension of platelets in plasmas obtained from different donors produced no change in the lag phase. Third, the addition of plasma to suspensions of bacteria and washed or gel-filtered platelets resulted in rapid aggregation with little delay. These data suggest that the lag phase is derived predominantly from bacterium-platelet interaction.

As mentioned, the lag phase terminates in rapid platelet secretion and aggregation. Our studies demonstrate that viridans streptococci aggregate platelets in part by the cyclooxygenase pathway, as shown by a delay in the onset of aggregation and a decrease in the rate of aggregation when platelets were pretreated with indomethacin. However, aggregation was still observed at concentrations of indomethacin (20 μM) that produce maximal inhibition of cyclooxygenase activity (2, 28). Thus, aggregation by these organisms can be mediated by mechanisms that are independent of cyclooxygenase function, such as the calcium-dependent phospholipase C pathway (38).

For some agonists, the terminal events in platelet aggregation are mediated by the extracellular release of ADP from platelet dense granules, which in turn induces fibrinogen binding to specific receptors (glycoprotein IIb/IIIa) on the platelet surface (39, 41, 43). Our studies indicate that aggregation by viridans streptococci is dependent on this process. Preincubation of platelets with 0.30 to 0.75 mg of apyrase per ml, which hydrolyzes ADP (44), delayed the onset of aggregation. Moreover, complete inhibition of aggregation was observed at 1 mg of apyrase per ml.

In addition to extracellular ADP, platelet aggregation by viridans streptococci requires plasma. Exogenous fibrinogen is one plasma cofactor necessary for aggregation. Additional plasma components are needed which do not appear to be IgG, C3, or factor VIII. By means of ultrafiltration and gel permeation chromatography, we have isolated the cofactors to the M_r 67,000 to 130,000 fraction of plasma. The plasma components contained within this fraction are not required for the aggregation of platelets by conventional agonists such as thrombin or collagen (40, 47). Moreover, they are not cofactors for ADP-mediated aggregation, as demonstrated by our studies with gel-filtered platelets. Although IgG and complement have been implicated as cofactors for the aggregation of platelets by *Staphylococcus aureus* and *Listeria monocytogenes*, respectively, the requirement for plasma components in the M_r 67,000 to 130,000 range has not been described previously (11, 23). It is possible, therefore, that these cofactors are unique to aggregation by viridans streptococci.

The precise role of these plasma components in mediating aggregation by these organisms is not known. The failure of the strains tested to trigger either platelet secretion or aggregation in the absence of the cofactors suggests that the cofactors are critical for an early step in the activation process. Studies to characterize further these cofactors, and to delineate their function in aggregation, are in progress.

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