Aggregation of Human Platelets and Adhesion of *Streptococcus sanguis*

MARK C. HERZBERG,^{1,2*} KAREN L. BRINTZENHOFE,¹ AND C. CARLYLE CLAWSON³

Departments of Periodontics¹ and Oral Biology,² School of Dentistry and Department of Pediatrics,³ Medical School, University of Minnesota, Minneapolis, Minnesota 55455

Received 16 July 1982/Accepted 10 December 1982

Platelet vegetations or thrombi are common findings in subacute bacterial endocarditis. We investigated the hypothesis that human platelets selectively bind or adhere strains of *Streptococcus sanguis* and *Streptococcus mutans* and aggregate, as a result, into an in vitro thrombus. Earlier ultrastructural studies suggested that aggregation of platelets over time by *Staphylococcus aureus* was preceded in order by adhesion and platelet activation. We uncoupled the adhesion step from activation and aggregation in our studies by incubating streptococci with platelet ghosts in a simple, quantitative assay. Adhesion was shown to be mediated by protease-sensitive components on the streptococci and platelet ghosts rather than cell surface carbohydrates or dextrans, plasma components, or divalent cations. The same streptococci were also studied by standard aggregometry techniques. Platelet-rich plasma was activated and aggregated by certain isolates of *S. sanguis*. Platelet ghosts bound the same strains selectively under Ca^{2+} - and plasma-depleted conditions. Fresh platelets could activate after washing, but Ca^{2+} had to be restored. Aggregation required fresh platelets in Ca^{2+} -restored plasma and was inducible by washed streptococcal cell walls. These reactions in the binding and aggregometry assays were confirmed by transmission electron microscopy. Surface microfibrils on intact *S. sanguis* were identified. These appendages appeared to bind *S. sanguis* to platelets. The selectivity of adhesion of the various *S. sanguis* strains to platelet ghosts or Ca^{2+} - and plasma-depleted fresh washed platelets was similar for all donors. Thus, the platelet binding site was expressed widely in the population and was unlikely to be an artifact of membrane aging or preparation. Since selective adhesion of *S. sanguis* to platelets was apparently required for aggregation, it is suggested that functionally defined receptors for ligands on certain strains of *S. sanguis* may be present on human platelets. Some differences in the selectivity and rate of the aggregation response were noted among platelet donors, although the meaning of the variability requires further study. Nonetheless, these interactions may contribute to platelet accretion in the initiation and development of vegetative lesions in the subacute bacterial endocarditis.

Interactions between platelets and blood-borne bacteria are likely to be part of the pathophysiological mechanisms of septicemia and disseminated intravascular coagulation (5, 35), as well as bacterial endocarditis (2, 9, 16, 20). In vitro, aggregometry studies have shown that platelets in plasma activate and aggregate in response to incubation with certain fungi (45), gram-positive bacteria, or gram-negative bacteria (10, 11, 13, 26, 44, 50). Ultrastructural studies strongly suggest that an irreversible binding or adhesion with *Staphylococcus aureus*, for example, precedes platelet activation, secretion, and aggregation (8, 10). Although the aggregation response may require plasma cofactors such as fibrinogen (11, 37), immunoglobulin G (26), or

complement system components (44, 45), it is clear that the contact phase, activation, and, in some cases, delayed aggregation can occur under plasma-depleted conditions with certain microorganisms (11).

Viridans group streptococci implant with unusual frequency on the platelet vegetations of subacute bacterial endocarditis. In a recent study, 50% of the cases of endocarditis diagnosed in the years 1970 through 1978 were attributed to these organisms, with *Streptococcus sanguis* identified as the vector three to four times more frequently than *Streptococcus mutans* (42). In contrast, F. J. Roberts (41) reported that the viridans group streptococci accounted for only 2.8% of the 686 episodes of

bacteremia and fungemia documented at Vancouver General Hospital from 1976 through 1977. The ability of viridans group streptococci to adhere to sterile thrombotic vegetations on endocardial tissues is thought to be a major virulence factor. Subsequent colonization and platelet recruitment increases the mass of the vegetation (2, 9, 20). In addition to the resultant hemodynamic disorders, the septic vegetations produce enzymes that may contribute directly to the destruction of the heart valves and walls (46).

The pathogenesis of subacute bacterial endocarditis has been studied in animal models. Viridans group streptococci have been shown to selectively adhere to preformed sterile vegetations in vivo (20) and to platelet-fibrin clots in vitro (43). It has been suggested that those microorganisms that possess surface dextrans adhere better and are more virulent (3, 38, 39, 43).

We sought to learn whether platelet interactions with viridans group streptococci occur preferentially and to provide some explanation for this pattern of infections. To study the adhesion or binding step conveniently and quantitatively in the absence of activation and aggregation responses, platelet ghosts were prepared, incubated with strains of viridans group streptococci, and assayed. To determine whether the viridans group streptococci activate and aggregate platelets, interactions were compared by aggregometry. Our results demonstrate that certain strains of *S. sanguis* among the viridans group streptococci tested both adhered to and aggregated human platelets. Platelet aggregation was strongly suggested to be a consequence of the selective binding of strains of *S. sanguis*. These mechanisms may play roles in the pathogenesis of selected cases of subacute bacterial endocarditis.

MATERIALS AND METHODS

Bacterial strains. *S. sanguis* I 2017-78, I 133-79, I 139-79, II 191-79, and II 2502-78 and *S. mutans* 1014-79, 1106-78, and 658-79 were biotyped after isolation from confirmed cases of subacute bacterial endocarditis and were the generous gift of R. R. Facklam, Center for Disease Control, Atlanta, Ga. *S. sanguis* 10556, derived from an endocarditis isolate and *S. sanguis* M-5 and *S. mutans* GS-5, BHT, and 6715, all originally from human dental plaque, were graciously supplied by C. F. Schachtele, University of Minnesota, Minneapolis. *S. aureus* 502A (protein A positive) was originally the kind gift of Paul G. Quie, University of Minnesota, Minneapolis, and was the same strain employed in previous reports (8, 10, 11).

Growth conditions. Bacteria were harvested by centrifugation from late stationary-phase growth (18 h) in Todd-Hewitt broth ($5,900 \times g$ for 20 min at 4°C) or in the chemically defined synthetic medium, FMC, described by Terleckyj et al. (48). Cells were washed

FIG. 1. PBAA centrifugation conditions. *S. sanguis* I 2017-78 and outdated WP were prepared by standard methods and assayed with partitioning of the interacting cells from free cells by centrifugation ($35 \times g$) for various lengths of time. The resultant A_{620} of the supernatants of the suspension of WP (Δ) or *S. sanguis* ($\blacktriangle$), the mean of a separate suspension of WP + *S. sanguis* (control, ----), and the mean of interacting WP and *S. sanguis* ($\bullet$) were compared.

four times in cold 0.05 M Tris-hydrochloride–0.1 M NaCl–0.02 M EDTA (Tris–NaCl–EDTA buffer)–0.01% sodium azide, pH 7.25 (NaN_3 omitted in all procedures before platelet aggregometry experiments).

Preparation of standardized bacterial suspensions.

After washing, the bacteria were dispersed by sonication (Sonifier Cell Disruptor, model W185; Heat Systems–Ultrasonics, Inc., Plainview, N.Y.) at 50 W for 8 s and suspended to an absorbance at 620 nm (A_{620}) of 0.5 in Tris–NaCl buffer. Before adhesion experiments, the bacteria were concentrated fourfold, giving a standard suspension with an A_{620} of approximately 2.0 containing 4×10^9 cells per ml as determined by counting in a Petroff–Hausser chamber. For aggregometry experiments, the cells were washed as described and suspended to an A_{620} of approximately 2.0 in complete (with Ca^{2+} and Mg^{2+}) Hanks balanced salt solution (HBSS) (GIBCO Laboratories, Grand Island, N.Y.). Cell walls were prepared by the method of Fischetti et al. (17), examined for minimal breakage by phase contrast or transmission electron microscopy, and prepared to an A_{620} of approximately 2.0 as described above.

Preparation of standardized platelet suspensions.

Outdated human platelet-rich plasma (PRP), anticoagulated with acid citrate–glucose, was supplied by Robert Bowman, American Red Cross Blood Center, St. Paul, Minn. Fresh PRP was also prepared as described previously (10, 11). Briefly, blood was obtained by venipuncture from normal donors who were free of infection and medication for at least 2 weeks before study, with informed consent in accord with the Com-

mittee on the Use of Human Subjects, University of Minnesota. Fresh blood was mixed immediately with 0.1 M trisodium citrate (9:1 [vol/vol]) and centrifuged at $100 \times g$ for 20 min at 20°C. Citrated PRP (supernatant) could be adjusted with autologous citrated platelet poor plasma to provide a platelet count of 4×10^8 per ml in a hemacytometer. PRP to be used in aggregometry experiments was maintained in a water bath at 37°C and tested within 3 to 4 h.

Washed platelets (WP), Ca^{2+} and plasma depleted, were prepared from outdated or fresh PRP as needed. After centrifugation at $2,000 \times g$ for 30 min at 4°C, the platelets were washed three times in Tris-NaCl-EDTA buffer, pH 7.25. The WP were then reconstituted in a known volume of wash buffer to an A_{620} of 0.5, centrifuged at $1,900 \times g$ for 20 min at 4°C, and suspended to one-fourth the volume ($A_{620} \sim 2.0$). The resulting Ca^{2+} - and plasma-depleted outdated WP were unable to activate or aggregate under any of the test conditions described. Functional responses of fresh WP to ADP and collagen could be restored by incubation in complete HBSS at 37°C for 30 min to replenish Ca^{2+} pools (Ca^{2+} -restored fresh WP).

Platelet aggregometry. Streptococci were tested for their ability to induce platelet aggregation with fresh PRP obtained from a single donor. Aggregometry with bacteria was performed as previously described (10, 11). In brief, a standard reaction mixture of 0.45 ml of fresh PRP and 0.05 ml of stock bacterial suspension (final ratio of platelets to bacteria, 1:1) were tested at 37°C, with controlled stirring on a recording aggregometer (model 390; Chronolog Corp., Broomall, Pa.) attached to a linear recorder. After a baseline with PRP was established, the bacterial suspension in complete HBSS was added to the cuvette. Platelet shape change and aggregation were determined as a decrease in the width of the oscillating baseline and an increase in light transmission, respectively. Insoluble particulate collagen (type I, from bovine tendon; Sigma Chemical Co., St. Louis, Mo.), prepared by grinding 250 mg in 5 ml of complete HBSS, or 0.2 μM ADP (type IV, from equine muscle; Sigma) were added to PRP suspensions prepared from each blood collection as positive controls of normal platelet aggregation (6, 7).

PBAA. In the platelet bacterial adhesion assay (PBAA), 105 μl each of standard WP and bacterial suspensions were mixed in the V wells of microtiter plates (Dynatech Laboratories, Inc., Alexandria, Va.) and incubated for 30 min at 4 or 37°C. Control suspensions of 210 μl of WP or bacteria were placed in separate wells and otherwise handled identically to observe and correct for interbacterial or WP adhesion. These interactions were terminated by centrifugation of the microwell plate at $35 \times g$ for 5 min at 4°C. Platelets with bound bacteria were separated from noninteracting cells by this procedure (Fig. 1). Supernatants (200 μl) from each well were diluted 1:5 with buffer and the A_{620} was recorded. For convenience and to facilitate comparisons, adhesion was quantitated by the formula:

$$\% \text{ adhesion} = 100 \times \{1 - [\text{mixture } A_{620} / (\text{bacteria } A_{620} + \text{WP } A_{620}) \div 2]\}$$

which describes the sedimentation of cell mass as the ratio of the A_{620} of the supernatant of the experimental WP-bacteria mixture and the mean of the sum of the

separate WP and bacterial controls. All experiments were performed in duplicate on at least two occasions.

Control experiments showed that pooled WP adhered indistinguishably from the WP of any single donor and that adhesion was unaffected by incubation temperatures between 4 and 37°C.

Modifiers of platelet function and effects on streptococcal adhesion. Platelet ghosts, *S. sanguis* I 2017-78, or both were pretreated or the PBAA was conducted in the presence of cofactors, promoters, or inhibitors of platelet activation and aggregation. In every case it was determined that the agent added did not promote anomalous adhesion scores because of agglutination or lysis of platelet ghosts or *S. sanguis* I 2017-78 cells. Agents that were likely to produce covalent modifications in the platelet ghosts or *S. sanguis* I 2017-78 were also used to treat control substrate macromolecules or cells under similar conditions to verify the efficacy of the procedures.

Pretreatment of streptococci with dextrans. Standard suspensions of bacteria were grown in Todd-Hewitt broth, harvested, and washed (in 0.01 M sodium phosphate buffer, pH 7.0, with 0.9% sodium chloride). These viable (NaN_3 omitted) organisms were suspended to an A_{620} of 0.5 (2×10^8 cocci per ml), centrifuged at $1,900 \times g$ for 20 min at 4°C, and reconstituted to one-eighth the original volume. To study the effects on platelet adhesion of dextrans bound to streptococci, bacteria were pretreated with exogenous high-molecular-weight dextrans in the presence of low-molecular-weight dextran competitors by a modification of the method of Gibbons and Fitzgerald (22). Up to 1 mM (final concentration) dextran 10 (Dextran T_{10} , $M_r = 10^4$; Pharmacia Fine Chemicals, Uppsala, Sweden) in phosphate-buffered saline was added in dose-response manner to an equal volume of concentrated bacteria and incubated at 20 or 37°C for 30 min. To the dextran 10-pretreated suspensions or dextran-free bacteria plus buffer controls, equal volumes of solutions up to 1 M (final concentration) of dextran 2,000 (Dextran T_{2000} , $M_r = 2 \times 10^6$; Pharmacia) or an equal volume of phosphate-buffered saline (control) were added, and preincubation was continued for 120 min at the respective temperatures. A_{620} was recorded after each preincubation step. Agglutination of dextran-pretreated bacteria in each condition was measured directly by spectrophotometry as:

$$\text{agglutination} = (\text{control } A_{620} - \text{dextran } A_{620}) / \text{control } A_{620}$$

Streptococci pretreated with dextran 10, dextran 2,000, or dextran 10 and dextran 2,000 were then used for study in the PBAA.

Electron microscopy. Adhering platelets and bacteria formed in the PBAA or aggregates produced in the aggregometer were initially fixed by making the suspension 0.1% in glutaraldehyde. In each case, individual platelets or bacteria were decanted after differential centrifugation ($35 \times g$ for 5 min at 4°C for the PBAA as described above or $100 \times g$ for 10 min at 24°C) and incubated in 1% osmium tetroxide–1.5% potassium ferrocyanide for 1.5 h at 4°C. Preparations were then embedded in Epon, and thin sections were cut, stained with saturated uranyl acetate–Reynolds lead citrate, and observed in a Philips 301 electron microscope as reported previously (8, 10, 11).

Light microscopy. Light photomicroscopy of plate-

TABLE 1. Platelet aggregometry with viridans group streptococci^a

Organism tested	Aggregation response ^b		Prevalence of unresponsive donors ^c
	Principal donor	All others	
<i>S. sanguis</i>			
I 2017-78	2.0 ± 0.9 (41)	4.7 ± 4.4 (35)	1/17
I 133-79	7.5 ± 0.1 (2)	7.8 ± 4.8 (11)	1/8
I 139-79	4.5 ± 1.3 (3)	— (4)	2/3
II 191-79	— (2)	18.9 ± 6.6 (4)	6/8
II 2502-78	1.3 ± 0.6 (3)	5.9 ± 0.6 (4)	1/4
10556	— (2)	9.1 ± 0.9 (2)	6/7
M-5	+	— (14)	7/8
<i>S. mutans</i>			
1014-79	— (2)	12.3 ± 0.9 (2)	4/5
1106-78	— (3)	12.5 (1)	3/4
658-79	— (2)	— (6)	4/4
GS-5	— (2)	— (6)	4/4
BHT	— (2)	— (6)	4/4
6715	+	— (12)	6/7
<i>S. aureus</i> 502A	10.5 ± 1.3 (4)	1.8 ± 1.1 (10)	0/6

^a PRP from a single donor was equilibrated to 37°C with bacteria at a final cell ratio of 1:1 as described in the text.

^b Response leading to aggregation was recorded as the mean number of minutes or lag time to onset of aggregation ± standard deviation (the number of total separate experimental trials is shown in parentheses); activation without aggregation after 20 min, +; no response, —. In the event of both positively and negatively responding PRPs within the group of "other donors," only the number of positive trials with that organism is shown.

^c PRP from various numbers of different donors was tested with each organism. The frequency of donors whose PRP was nonresponsive with the respective organisms is shown. Platelet activation without aggregation was considered a positive response.

let-bacteria interactions during the PBAA was taken on a Leitz Ortholux microscope equipped with an Orthomat W camera (Ernst Leitz GmbH, Wetzlar, Germany) and Tri-X Pan (Eastman Kodak, Rochester, N.Y.) photographic film.

Chemical reagents. All chemicals or reagents were analytical grade or higher. ADP from equine muscle (grade IX), Tris, disodium EDTA, and sodium chloride (NaCl) were all obtained from Sigma. Magnesium chloride and calcium chloride were obtained from J. T. Baker Chemical Co., Phillipsburg, Pa. Glutaraldehyde (25%, biological grade) was supplied by Polysciences, Warrington, Pa.

RESULTS

Aggregation of platelets by streptococci. Strains of viridans group streptococci were tested for their abilities to promote platelet aggregation in fresh human PRP (Table 1). Replicate experiments were performed on different days. *S. aureus* 502A was used as a nonstreptococcal, positive control for platelet responsiveness to bacteria (8, 10, 11, 26). Of the strains of viridans group streptococci tested and shown in Table 1, *S. sanguis* strains originally isolated from endocarditis patients aggregated PRP most frequently. For example, the PRP of the principal donor aggregated with *S. sanguis* I 2017-78 after a 2.0-min lag in 41 trials, whereas the mean lag time to

aggregation for all other donors was 4.7 min in 35 trials. PRP of only 1 of the 17 donors failed to aggregate in the presence of this organism in an additional two trials. Thus PRP aggregation with *S. sanguis* I 2017-78 was observed in 76 of 78 total trials.

Differences in both the bacterial selectivities and lag times of PRP aggregation response among the principal and other donors were seen. Note that the PRP of the principal donor failed to aggregate in response to *S. sanguis* I 191-79, although PRP of two of the remaining five donors responded in a mean lag time of 19 min in duplicate trials. Similar results were observed with several other organisms. Generally, however, the PRP of all donors aggregated more readily in reaction to *S. sanguis* biotypes I and II (73.2% responsiveness) than with the untyped strains of *S. sanguis* or any of the strains of *S. mutans* (12% responsiveness) tested. After onset, the extent of aggregation (increase in light transmission) was essentially the same for each organism to which a donor PRP responded on a given day.

Experiments were conducted to determine the interdonor variability in PRP response to the test bacteria. The principal donor's PRP reacted as rapidly (±1 min) as those of 7 of the 17 donors

TABLE 2. Platelet adhesion of viridans group streptococci

Organism tested	Mean % adhesion $\pm$ SD (no. of trials) ^a	
	Fresh WP ^b	Outdated WP (ghosts) ^c
<i>S. sanguis</i>		
I 2017-78	70.1 $\pm$ 6.2 (9)	75.3 $\pm$ 6.9 (41)
I 133-79	69.1	83.4 $\pm$ 5.5 (8)
I 139-79	5.9	7.4 $\pm$ 4.9 (6)
II 191-79	12.0	2.5 $\pm$ 2.0 (6)
II 2502-78	39.7	36.4
10556	3.5	2.8
M-5	0	2.1
<i>S. mutans</i>		
1014-79	5.3	0
1106-79	5.3	10.5
658-79	0	1.2
GS-5	0	0
BHT	1.1	0
6715	0	3.2

^a When duplicate trials were performed, only the mean is shown.

^b All platelets were obtained from the principal donor.

^c WP were obtained from multiple donors of all major blood groups as platelet concentrates from the American Red Cross and tested individually.

who were tested with *S. sanguis* I 2017-78 (data not shown). PRP of eight donors showed a protracted lag and one did not respond. Thus, the PRP response of various donors to an organism appeared to cluster, but elucidation of patterns would require further study. In all cases the intradonor variability in response to a single organism was less than that associated with grouped data.

To evaluate the requirement for an intact, viable bacterium or its products, PRP was challenged with a suspension ($A_{620} \sim 2.0$) of washed cell walls of *S. sanguis* I 2017-78. PRP aggregated as rapidly (1.5 ± 0.3 min, seven trials) as with an equivalent suspension of intact organisms, suggesting that cell wall-associated structures on the bacterium were required.

Streptococcal adhesion to WP. The possibility that interactions between the surfaces of WP and *S. sanguis* I 2017-78 may represent an adhesion or binding process necessary for subsequent activation and aggregation of fresh PRP was studied next. WP prepared from fresh or outdated PRP were tested for adhesion with streptococci in the PBAA as described above. Endocarditis strains of *S. sanguis* exhibited relatively high adhesion to these Ca^{2+} - and plasma-depleted WP (Table 2). Essentially the same strains were shown above (Table 1) to aggregate PRP. Controls were performed for every streptococcal-WP experimental mixture. Spontane-

ous association or flocculation of the bacteria or aggregation of platelets during the course of each experiment could be detected and measured. Since control bacteria or platelets remained suspended after differential centrifugation, interactions between the cells in the experimental mixtures were considered to be the basis of their sedimentation. WP prepared for the PBAA from fresh PRP adhered to the same spectrum of organisms as preparations from outdated American Red Cross PRP. These observations were confirmed by Gram- or Wright-stained smears of the experimental streptococcal-WP mixtures in comparison with the individual WP and streptococcal controls. Single or short chains of gram-positive cocci were associated with platelets, often appearing to connect or bridge neighboring platelets into platelet-bacteria clusters.

Characteristics of WP-*S. sanguis* adhesion. The duration and temperature of the incubation of platelet ghosts with *S. sanguis* I 2017-78 were varied in the PBAA. Adhesion was invariant after 15 and up to 60 min of incubation. The rates of adhesion at 4 and 37°C were indistinguishable. In addition, cold (4°C) storage of platelet ghosts in assay buffer for up to 4 days after preparation did not appear to affect adhesion with *S. sanguis* I 2017-78. Thus, 30 min of incubation at 4°C was routinely used to observe maximal temperature-independent interactions. Buffers with final pH in the ranges of 5 to 7.4 and 7.15 to 9.0 were prepared from 0.05 M sodium phosphate and 0.05 M Tris-hydrochloride (4°C), respectively,

FIG. 2. Optimal ratios. *S. sanguis* I 2017-78 and platelet ghosts were counted and mixed in different ratios in the standard 210- μ l reaction volume. Controls consisted of separate 210- μ l suspensions of the respective concentrations of ghosts or *S. sanguis* tested.

each with 0.1 M NaCl and 0.027 M disodium EDTA. After washing as described above, platelet ghosts and *S. sanguis* I 2017-78 were suspended and incubated in one of the buffers of various pH. Adhesion with *S. sanguis* I 2017-78 showed only mild dependence on medium pH, with a maximum at pH 7.25.

Since platelet ghosts are bridged or cross-linked by *S. sanguis*, we next studied the stoichiometry of these adhesion clusters. Various ratios of *S. sanguis* I 2017-78 and platelet ghosts, as determined spectrophotometrically and by Petroff-Hausser or hemacytometer counts, were incubated in a standard 210- μ l reaction volume for 30 min at 4°C in Tris-hydrochloride, pH 7.25, assay buffer. Only one cell concentration was varied at a time. The data shown in Fig. 2 indicate that cell ratios of about 1:1 were optimal.

To examine the effect of increased ionic strength of the medium, *S. sanguis* I 2017-78 and platelet ghosts were washed conventionally but suspended and incubated in assay buffer with or without various concentrations of added NaCl. At low concentrations of added NaCl, adhesion was essentially unaffected. After physiological concentrations of NaCl were exceeded (>200 mM), adhesion decreased. The standard assay buffer incorporated 100 mM NaCl. Thus, adhesion between *S. sanguis* and WP would appear to be insensitive to physiological concentrations of saline.

In contrast, cell-to-cell adhesion might be augmented or dependent upon addition of divalent cations such as Ca^{2+} . Although selective adhesion between platelet ghosts (Ca^{2+} and plasma depleted) and certain strains of *S. sanguis* occurs in the absence of divalent cations, Ca^{2+} is required for platelet aggregation responses to these organisms. To determine whether divalent cations modified adhesion, platelet ghosts were suspended and incubated with various concentrations of Ca^{2+} or Mg^{2+} without disodium EDTA. Incubation with *S. sanguis* in the presence of Ca^{2+} concentrations up to the 2.5 mM range (physiological) was without apparent effect. At high Ca^{2+} concentrations, adhesion was inhibited. Like Ca^{2+} , MgCl_2 addition up to 10 mM had little effect on the percent adhesion, and in higher, unphysiological concentrations it was inhibitory.

Expression of sites for adhesion to platelets during growth of *S. sanguis*. We evaluated the ability of *S. sanguis* to adhere to platelet ghosts as a function of stage of growth and the pH of either Todd-Hewitt broth or chemically defined FMC (48) medium (Fig. 3A and B, respectively). The rate and extent of growth in each medium were similar, although the acidification of the chemically defined synthetic medium was great-

FIG. 3. *S. sanguis* expression of adhesion to platelet ghosts during growth. *S. sanguis* I 2017-78 was grown, harvested, washed, and tested for adhesion to platelet ghosts at the various times indicated. Growth as determined by the A_{620} (log) and pH of the media were measured at the time of harvest. (A) Growth in Todd-Hewitt broth. (B) Growth in chemically defined synthetic medium FMC. Symbols: $\circ$, A_{620} (log); $\bullet$, pH; $\blacktriangle$, % adhesion.

er. *S. sanguis* harvested from either medium after various growth times between 1 and 24 h adhered to platelet ghosts with consistency at the 80% level.

Effects of dextrans on streptococcal adhesion to platelet ghosts. To examine the contribution of low- and high-molecular-weight dextrans to the adhesion of *S. sanguis* I 2017-78 to platelet ghosts, we established suitable conditions by experimentation with *S. mutans* 6715. We used measurements of streptococcal agglutination (no platelet ghosts in the incubate) as evidence of dextran binding. *S. mutans* 6715 showed 55% agglutination in the presence of 0.01 M dextran 2,000 (M_r , 2×10^6) at 37°C (Table 3). This streptococcal agglutination was blocked by prior treatment with 1 mM dextran 10 (M_r , 10^4), which did not promote agglutination itself. Under identical conditions and throughout a matrix of dose-response dextran concentrations, no comparable agglutination of *S. sanguis* I 2017-78 was observed. No agglutination was noted for either

TABLE 3. Effect of dextrans on streptococcal adhesion to platelet ghosts

Incubation conditions ^a		Adhesion of platelet ghosts with: ^b	
Temp	Addition	<i>S. sanguis</i> I 2017-78	<i>S. mutans</i> 6715
37°C	Control	59.5 (0)	13.2 (0)
	1 mM dextran 10 (30 min)	53.1 (2.3)	8.9 (-22.1)
	0.01 μ M dextran 2,000 (120 min)	43.6 (-10.5)	9.4 (55.6)
	Dextran 10 (30 min), dextran 2,000 (120 min)	57.4 (18.0)	2.2 (-12.9)
20°C	Control	69.1	0
	1 mM dextran 10 (30 min)	56.7	5.1
	0.01 μ M dextran 2,000 (120 min)	56.8	8.5

^a *S. sanguis* and *S. mutans* were preincubated under the conditions listed with dextran 10 (M_r , 10^4) or dextran 2,000 (M_r , 2×10^6) as described in the text. Controls were pretreated identically but without dextrans.

^b The PBAA was performed conventionally at 4°C and is expressed as the mean of replicate trials in duplicate experiments. After preincubation at 37°C, in the presence of dextrans, streptococcal agglutination (no platelet ghosts) was quantitated (see equation 2 in the text), and the values are listed in parentheses. Neither organism agglutinated as a consequence of dextran preincubation at 4 or 20°C.

microorganism at 4 or 20°C. After dextran pretreatment, the bacteria were recovered and tested for adhesion to platelet ghosts. Dextran-pretreated organisms adhered to platelet ghosts at levels comparable to the control organisms. Adhesion levels for *S. sanguis* I 2017-78 and *S. mutans* 6715 clearly differed from each other under all conditions of study, even after 20°C pretreatment. Therefore adhesion to platelet ghosts depended on factors other than the binding of exogenous dextrans by *S. mutans* 6715 or identical pretreatment of *S. sanguis* I 2017-78. Additional adhesion experiments were performed with *S. sanguis* I 2017-78 grown in Todd-Hewitt broth supplemented with 5% sucrose. These streptococci were washed (with NaN_3) and assayed in the PBAA according to our standard methods. Sucrose-grown *S. sanguis* I 2017-78 adhered at a 69.5% level in comparison to the 75.3% level for the sucrose-free control, suggesting that growth in conditions suitable for dextran synthesis did not promote adhesion to platelet ghosts.

Ultrastructure of streptococcal interactions with platelets. The interactions between *S. sanguis* I 2017-78 and human platelets were examined by transmission electron microscopy. Aggregation of PRP in the aggregometer is shown in Fig. 4A; masses of tightly compacted platelets, platelet membranes, and granules have incorporated two cocci, though commonly more, into a thrombotic aggregate. The microbes appear in close association with platelet membranes, a consistent finding. Note that non-interacting platelets and bacteria were effectively separated from aggregates by the preparative procedures used.

A normal unaffected platelet (Ca^{2+} -restored, plasma-depleted fresh WP) is discoid in shape, with other granules and subcellular structures distributed throughout the cytoplasm (Fig. 4B).

When observed with bound cocci, platelets typically changed shape to spheroid, formed pseudopods, and centralized their granules and organelles. Fewer dense granules were observed in any plane of section in these activated but not aggregated platelets. When incubated with poorly adhering strains, platelets showed no signs of activation or aggregation.

Platelets (Ca^{2+} -restored, plasma-depleted fresh WP) appeared to bind streptococci by close approximation of microfibrillar structures on the respective contacting surfaces (Fig. 4C), not unlike many related systems in the literature. These interactions were typical also of the binding of streptococci to outdated WP (Ca^{2+} - and plasma-depleted) observed among cells recovered from the PBAA (Fig. 4D). Although their plasma membranes remained often intact, these platelets generally appeared to be ghosts. Cocci bound at complementary plasma membrane concavities on the ghost. Morphological evidences of platelet activation (Fig. 4B) or aggregation (Fig. 4A) were not noted.

Streptococcal adhesion and platelet function. Platelet functional responses were not observed by aggregometry or morphologically upon selective adhesion of *S. sanguis* strains to platelet ghosts. Adhesion might, however, be promoted or modified by biochemical events in the platelet plasma membrane associated with activation and aggregation. To evaluate this possibility, *S. sanguis* and platelet ghosts were incubated with cofactors, activators or inhibitors of platelet function.

Normal plasma calcium concentrations of about 2.5 to 5.0 mM are required in the medium as a cofactor for platelet aggregation (10). As shown above, restoration of these divalent cation concentrations from depletion was without effect.

ADP in millimolar concentrations will cause

FIG. 4. Ultrastructure of streptococcal interactions with platelets. Transmission electron microscopy was performed as described in the text. (A) PRP-*S. sanguis* I 2017-78 aggregate from the aggregometer. (B) Ca^{2+} -restored, plasma-depleted fresh WP and *S. sanguis* from the aggregometer. (C) higher magnification of cell surface of *S. sanguis* in contact with plasma membrane of activated platelet. (D) Platelet ghosts with adhering *S. sanguis* from PBAA.

platelet activation-aggregation of fresh PRP within seconds (6, 7). The PBAA was performed in the presence of final ADP concentrations of from 12.5 to 50 mM. No evidence of aggregation

of platelet ghosts was noted. Adhesion with *S. sanguis* I 2017-78 at each concentration of ADP was the same as that with the ADP-free control.

Fresh PRP will aggregate in response to the

TABLE 4. Effect of proteases on adhesion

Protease	Adhesion of treated cells (mean % $\pm$ SD) ^a		
	Platelet ghosts + <i>S. sanguis</i> ^b	Platelet ghosts only ^c	<i>S. sanguis</i> only ^c
Control	71.2 $\pm$ 2.4	82.0 $\pm$ 4.2	82.0 $\pm$ 4.2
Trypsin (2 mg/ml)	4.6 $\pm$ 6.9	35.1 $\pm$ 2.4	0
Soybean trypsin inhibitor (2 mg/ml)	72.7 $\pm$ 2.4	77.1 $\pm$ 0.6	80.5 $\pm$ 1.7
Trypsin + trypsin inhibitor	70.6 $\pm$ 1.8	76.2 $\pm$ 1.9	72.6 $\pm$ 3.0
Control	68.5 $\pm$ 1.8 ^d	75.0 $\pm$ 2.2 ^e	75.0 $\pm$ 2.2 ^e
Pronase (0.2 mg/ml)	0	23.6 $\pm$ 2.4	0

^a Platelet ghosts were prepared from two American Red Cross platelet concentrates and tested separately.

^b This assay was performed in 1 $\times$ HBSS (pH 8.0) at 37°C for 30 min when platelet ghosts and *S. sanguis* I 2017-78 were tested together in the presence of trypsin (or related reagents), and the reaction was stopped by the addition of an equal concentration of trypsin inhibitor by modification of the method of Nachman and Ferris (34).

^c Platelet ghosts or *S. sanguis* were treated with trypsin by preincubation as described in footnote *b* and centrifuged; the supernatants were aspirated, reconstituted in Tris-hydrochloride-NaCl without enzyme (inhibitor), and assayed by standard procedures.

^d Assayed in 0.05 M Tris-hydrochloride buffer (pH 7.25) at 37°C in the presence of 0.2 mg of pronase per ml. After 10 min, disodium EDTA was added to a final concentration of 15 mM to inhibit Ca²⁺ and Mg²⁺-dependent proteases. Incubation of WP and *S. sanguis* was continued for an additional 30 min at 37°C before reading.

^e Platelet ghosts or *S. sanguis* were treated with pronase by preincubation (37°C, 10 min) and centrifuged; the supernatants were aspirated, reconstituted in Tris-hydrochloride-NaCl-EDTA and assayed by standard procedures.

presence of trypsin, presumably because of its thrombin-like substrate attack (32). Treatment of platelet ghosts with 2 mg of trypsin per ml in our system did not cause aggregation in the platelet aggregometer; this was also evidenced by their sedimentation in the PBAA. However, incubation of platelet ghosts and *S. sanguis* I 2017-78 in the presence of trypsin reduced adhesion to 4.6% (Table 4). This effect was blocked by soybean trypsin inhibitor, which had no effect on adhesion itself. When incubated with untreated bacteria, platelet ghosts pretreated with trypsin had an adhesion score of 35.1%. Thus, about half of the adhesion or binding capacity of the platelet ghosts was lost. In contrast trypsinization of only *S. sanguis* I 2017-78 resulted in complete loss of adhesion. Pronase treatment of platelet ghosts and *S. sanguis* I 2017-78 similarly abrogated interactions. Evaluation of experimental controls suggested that cell lysis was avoided.

Treatment with protease inhibitors (3, 29, 49) or disodium EDTA (51) has been shown previously to block platelet aggregation responses without altering platelet shape change (activation). Soybean trypsin inhibitor has been shown to inhibit platelet aggregation, but as shown in Table 4, it did not affect *S. sanguis* adhesion to platelet ghosts. Similarly, the protease inhibitors toluenesulfonyl chloride (0.4 mM), phenylmethylsulfonyl fluoride (0.4 mM), and *N*-ethylmaleimide (1 mM) were tested for their effects on adhesion. Incubation of platelet ghosts, *S. sanguis*, or both in the presence of these agents or disodium EDTA (0.027 M) resulted in little, if

any, differences in adhesion from the levels observed in control buffer. Fresh WP were tested also since they might be expected to be more sensitive to inhibition of protease-dependent effects. Adhesion of *S. sanguis* to fresh WP, protease inhibitor treatment notwithstanding, was indistinguishable from the buffer-treated control.

Vinblastine sulfate (12, 28) and cytochalasin B (18, 52) promote microtubule depolymerization and inhibit microfilament polymerization, respectively, and were tested under standard PBAA conditions as inhibitors of platelet granule release or shape change-associated reactions (Table 5). When added to the assay buffer, both agents appeared to be associated with small reductions (16 to 18%) in the adhesion of *S. sanguis* to platelet ghosts. To clarify whether these agents affected the *S. sanguis* or ghosts, each was preincubated in the presence of either of the two agents. After washing, each was tested with untreated cells or ghosts. Treated platelet ghosts bound *S. sanguis* to the same extent as untreated control ghosts. However, when treated *S. sanguis* were incubated with untreated platelet ghosts, adhesion was reduced relative to untreated controls. The magnitude of this reduction was similar to the levels of adhesion observed when the inhibitors were present in the PBAA medium. This inhibition was, therefore, due to an effect on *S. sanguis*. Platelet ghosts were not affected by these agents.

Participation of other substituent chemical groups. In addition to the sensitivity to proteases and the insensitivity to *N*-ethylmaleimide (free

TABLE 5. Effects of microfilament, microtubule disaggregation

Agent in medium	Adhesion of treated cells (mean % $\pm$ SD) ^a		
	Platelet ghost + <i>S. sanguis</i> ^b	Platelet ghost only ^c	<i>S. sanguis</i> only ^c
Control	64.5 $\pm$ 0.9		
Vinblastine (0.2 mM)	54.1 $\pm$ 0.9	64.1 $\pm$ 6.3	47.1 $\pm$ 6.7
Control	71.4 $\pm$ 3.1		
Cytochalasin B (0.2 mM)	58.6 $\pm$ 0.6	69.2 $\pm$ 2.3	54.4 $\pm$ 2.8

^a Platelet ghosts were prepared from two American Red Cross platelet concentrates and tested separately.

^b Platelet ghosts and *S. sanguis* I 2017-78 were incubated together under standard assay conditions in the presence of agent.

^c Preincubation (10 min, 4°C) of platelet ghosts or *S. sanguis* in the presence of agent was followed by reconstitution in Tris-NaCl assay buffer. Conditions were sufficient to inhibit physiological aggregation of platelets.

sulfhydryl residues) that was shown above for the adhesion reaction(s), the participation of epsilon amino groups and vicinal hydroxyl groups of platelet ghosts (sialic acid was not detected; data not shown) or *S. sanguis* I 2017-78 were studied. Pretreatment or PBAA incubation in the presence of 1% (vol/vol) glutaraldehyde (10) or 1 mM sodium metaperiodate (1) did not affect adhesion. Since platelets may carry major blood group determinants (14), the possibility that these might define structural and functional cell membrane subsets that affect adhesion was investigated. Platelet ghosts prepared from ABO typed donors (American Red Cross Blood Center, St. Paul) were tested in the PBAA. Adhesion of platelet ghosts of A, B, or O donor blood groups, or A/AB, A/B, or A/B/AB mixtures to *S. sanguis* I 2017-78 were similar, suggesting that adhesion was independent of the major blood group type of the donor.

Sugar inhibition studies. To determine whether the addition of simple sugars would inhibit the adhesion between platelet ghosts and *S. sanguis*, the following sugars were incorporated into the assay buffer: D-glucose; D-galactose; D-mannose; D-ribose; L-fucose; D-galactosamine; N-acetyl-D-mannosamine; N-acetylmuramic acid (each 200 mM, highest final concentrations); N-acetylneuraminic acid (100 mM); β -D-lactose; and 1-O-methyl- α - and 1-O-methyl- β -D-galactopyranoside (125 mM). The PBAA was then performed as usual. In additional experiments, platelet ghosts or *S. sanguis* were separately preincubated at 4°C for 15 min with each sugar, followed by assay for adhesion with untreated ghosts or *S. sanguis*. In no case was any inhibition observed.

DISCUSSION

Our observations showed that certain strains of viridans group streptococci adhered to human platelet ghosts. When bound, the same strains

activated and aggregated fresh human platelets. Selective microbial adhesion thus may precede activation and aggregation of platelets. The in vitro aggregates consisted largely of compacted platelets. *S. sanguis* were incorporated, apparently bound to platelet plasma membranes. Morphologically these aggregates resembled vegetations from human valve lesions (2) and probably reflect a hemostatic function of platelets. In contrast, the adhesion of *S. sanguis* to platelets occurred independently of clotting mechanisms.

Strains of viridans group streptococci were compared for their reactions with: (i) Ca²⁺- and plasma-depleted outdated WP ghosts; (ii) identically treated fresh WP; (iii) Ca²⁺-restored fresh WP; and (iv) fresh PRP. Strains of *S. sanguis* originally isolated from endocarditis patients reacted preferentially with each of the platelet preparations in comparison with endocarditis- and dental plaque-derived strains of *S. mutans*. Adhesion did not appear to require Ca²⁺, plasma components, viable bacteria, or platelets. Although not rigorously excluded, a role for soluble bacterial- or platelet-derived components in the interactions seemed unlikely, since all bacteria and platelets were washed carefully (except PRP). In addition, sodium azide treatment of WP ghost preparations or bacteria did not affect the selectivity of adhesion relative to aggregation. Furthermore, washed cell walls of at least one strain, *S. sanguis* I 2017-78, were sufficient to promote aggregation of PRP.

The preparation of WP ghosts (Fig. 4D) permitted the study of adhesion of *S. sanguis* without the concurrent complications of the normal metabolic processes of platelets. The functional responses of the platelet (shape change, intracellular reorganization, and aggregation) were clearly uncoupled from the adhesion event. The presence of agents associated with the activation and aggregation of fresh intact platelets did not affect adhesion. In addi-

tion, cytochalasin B or vinblastine sulfate, inhibitors of platelet shape change and secretion, were without effect on the WP ghosts. For reasons that are not clear, these agents did attenuate the binding of *S. sanguis* by untreated WP ghosts. During adhesion, WP ghosts and *S. sanguis* cross-link into clusters that are measurable spectrophotometrically in the PBAA. Divalent cations or the ionic strength of the medium do not appear to contribute to the selectivity of the adhesion interactions. The ability of *S. sanguis* to be bound by platelet ghosts is expressed from early log-phase growth through late stationary-phase growth, irrespective of growth media. Adhesion also occurs when the *S. sanguis* is grown or preincubated in citrated human plasma (unpublished observations). It is clear that aspects of the functional aggregation response characteristic of fresh platelets are not the basis for adhesion. Since bound *S. sanguis* are associated with aggregation of platelets, a cause-effect relationship is strongly suggested. In addition, we observed that the platelet selectivity for adhesion of viridans group streptococci is the same as for in vitro aggregation. Therefore, platelet bacterial adhesion may play a role in the colonization of sterile endocarditis vegetations, whereas in situ platelet aggregation could contribute to the growth of intracardiac vegetations, as long as virulent, though not necessarily viable, bacteria persist in the circulation.

The fresh WP and WP ghosts of different donors showed similar patterns of selectivity for binding *S. sanguis* (Table 2). More variation in both the selectivity and the rate of functional responses was observed among the PRPs of different donors (Table 1). Nevertheless, the mean functional responses of PRP from all donors showed a pattern similar to that seen for adhesion. We speculate that platelets of different donors may differ somewhat in their capacity for functional responses. Whether this is due to intrinsic differences in platelets or in plasma cofactors of platelet aggregation remains to be clarified.

Although *S. sanguis* is frequently recovered as an infectious agent in subacute bacterial endocarditis (42), dental plaque is held to be its natural ecological niche in humans (24). Among the numerous organisms in the complex microflora of the oral cavity, *S. sanguis* is believed to be an initiator of plaque formation (24, 30). It has been shown to adhere selectively to the pellicles of salivary proteins and glycoproteins, which absorb to the enamel surface (30, 31, 47). Lilje-mark and Bloomquist (31) recently reported that this attachment of salivary pellicle may be mediated, at least partially, by a surface protein(s) on *S. sanguis*. Indeed, our studies show that adhesion between WP ghosts and trypsin- or pro-

nase-treated *S. sanguis* is abolished. However, the WP ghosts-*S. sanguis* adhesion mechanism appears to be different.

Earlier studies suggest that *S. sanguis* can bind the *N*-acetylneuramic acid residues of complex oligosaccharides present on mammalian glycoproteins (30, 47). An *N*-acetylneuraminic acid-binding lectin on *S. sanguis* may mediate this attachment (33). Our sugar inhibition studies suggest no role for *N*-acetylneuraminic acid, or any of numerous other sugars, in platelet-*S. sanguis* adhesion. In addition, platelet ghosts contain virtually no *N*-acetylneuraminic acid detectable with the thiobarbituric acid assay (M. C. Herzberg, and K. L. Brintzenhofe, unpublished observation), and destruction of periodate-sensitive sugars on *S. sanguis* or platelet ghosts did not affect adhesion. Furthermore, *S. sanguis* adhesion to platelet ghosts was unaffected by the ABO blood type. This was not altogether surprising since the inhibition studies with high concentrations of the determinant sugars showed no effect. Other studies by Gibbons and Qureshi (23) showed that many strains of *S. sanguis* agglutinate human erythrocytes, but not on the basis of major blood group type.

Although added or newly synthesized dextrans (22, 36, 38, 43) have been shown to enhance adhesion of *S. sanguis* to preformed platelet-fibrin clots in in vitro models of endocarditis vegetations, our studies present several lines of evidence to show that either the dextran interaction occurs between *S. sanguis* and fibrin (or other clotting proteins) or that the basis of selectivity of adhesion resides with other mechanisms. The streptococci in our experiments were grown in the absence of the sucrose required for dextran synthesis (24). However, neither the pretreatment with low- ($M_r, 10^4$) or high-molecular-weight ($M_r, 2 \times 10^6$) dextrans nor the growth of *S. sanguis* I 2017-78 in media supplemented with 5% sucrose had any effect on adhesion to WP ghosts. Furthermore, control experiments with *S. mutans* 6715, a strain that shows weak adhesion to platelet ghosts, demonstrated that although the organisms were agglutinated by high-molecular-weight dextran, adhesion was unchanged. Therefore, protease-sensitive components of *S. sanguis*, rather than exogenous or endogenous dextrans, mediate adhesion to WP ghosts and may constitute virulence factors.

In subacute bacterial endocarditis, sterile vegetations appear to form on human (2, 16) and experimental rabbit (16, 20, 27) endocardium in association with cardiac disease or trauma. Once platelets have accumulated at the endocardial site, other studies (15, 19, 27, 43) suggest that infection is a consequence of direct adherence of viridans group streptococci. These microbes may be carried in the circulation as free

chains or in association with the platelet microaggregates often found in sepsis (5, 35). With *S. sanguis*, our aggregometry experiments suggested that the latter may occur. Thus our work may provide explanations for the observation (2, 15, 20, 40) that, subsequent to adhesion of streptococci, the vegetation enlarges by apparent recruitment and aggregation of platelets. *S. sanguis* may bind directly to platelets on the damaged heart tissues and subsequently to platelets from the circulation to promote aggregation. Preformed septic microaggregates would reasonably adhere to damaged endocardium or to its platelet covering, contributing somewhat more passively to the growth of the vegetation. Alternatively, even if viridans group streptococci bound to damaged endocardium directly, subsequent interactions with circulating platelets could initiate vegetations.

We were intrigued that none of the strains of *S. mutans* that we tested interacted appreciably with platelets. Nevertheless, selective adhesion of strains of *S. sanguis* to platelets and their subsequent aggregation may represent an efficient, although specialized, pathogenic mechanism in subacute bacterial endocarditis. Since selective binding of viridans group streptococci by platelets was shown to be accompanied by functional activation and aggregation responses, our observations suggest that platelets may have binding sites and receptors for certain *S. sanguis* strains. After further study, this system may be a useful model for the study of other streptococcal interaction mechanisms with mammalian cells (4, 21, 25).

ACKNOWLEDGMENTS

We thank Greg R. Germaine for his expert advice, Patricia Mottaz for outstanding assistance with the electron microscopy, and Louise Ruppert for fine secretarial support.

Supported in part by U.S. Public Health Service grants DE-05501 (M.C.H.) and HS-11880 (C.C.C.), funds from the Graduate School of the University of Minnesota (M.C.H.), and a special allocation for dental research from the State of Minnesota (M.C.H.).

LITERATURE CITED

- Andersson, L. C., and G. Gahmberg. 1978. Surface glycoproteins of human white blood cells. Analysis by surface labeling. *Blood* 52:59-67.
- Angrist, A., M. Oka, and K. Nakao. 1967. Vegetative endocarditis. *Pathol. Annu.* 2:155-212.
- Aoki, N., K. Naito, and N. Yoshida. 1978. Inhibition of platelet aggregation by protease inhibitors. Possible involvement of proteases in platelet aggregation. *Blood* 52:1-12.
- Beachey, E. H. 1981. Bacterial adherence: adhesin-receptor interactions mediating the attachment of bacteria to mucosal surfaces. *J. Infect. Dis.* 143:325-345.
- Bick, R. L. 1978. Disseminated intravascular coagulation and related syndromes: etiology, pathophysiology, diagnosis, and management. *Am. J. Hematol.* 5:265-282.
- Born, G. V. R. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nature (London)* 194:927-929.
- Born, G. V. R., R. Dearnley, J. R. Foulks, and D. E. Sharp. 1978. Quantification of the morphological reaction of platelets to aggregating agents and of its reversal by aggregation inhibitors. *J. Physiol.* 280:193-212.
- Clawson, C. C. 1973. Platelet interaction with bacteria. III. Ultrastructure. *Am. J. Pathol.* 70:449-464.
- Clawson, C. C. 1979. The role of platelets in the pathogenesis of endocarditis. *Am. Heart Assoc. Monogr.* 52:24-27.
- Clawson, C. C., and J. G. White. 1971. Platelet interaction with bacteria. I. Reaction phases and effects of inhibitors. *Am. J. Pathol.* 65:367-380.
- Clawson, C. C., J. G. White, and M. C. Herzberg. 1980. Platelet interaction with bacteria. VI. Contrasting the role of fibrinogen and fibronectin. *Am. J. Hematol.* 9:43-53.
- Crawford, N., L. A. Amos, and A. G. Castle. 1980. Platelet microtubule subunit proteins: assembly and disassembly factors, p. 171-188. In A. Rotman, F. A. Meyer, C. Gitler, and A. Silberberg (ed.), *Platelets: cellular response mechanisms and their biological significance*. John Wiley & Sons, Inc., New York.
- Czuprynski, C. J., and E. Balish. 1981. Interactions of rat platelets with *Listeria monocytogenes*. *Infect. Immun.* 33:103-108.
- Duquesnoy, R. J. 1978. Donor selection in platelet transfusion therapy of alloimmunized thrombocytopenic patients, p. 229-243. In T. J. Greenwalt and G. A. Jamieson (ed.), *The blood platelet in transfusion therapy*. Alan R. Liss, New York.
- Durack, D. T. 1975. Experimental bacterial endocarditis. IV. Structure and evolution of very early lesions. *J. Pathol.* 115:81-89.
- Durack, D. T., and P. B. Beeson. 1978. The pathogenesis of infective endocarditis, p. 1-53. In S. H. Rahimatoola (ed.), *Infective endocarditis*. Grune & Stratton, New York.
- Fischetti, V. A., E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie. 1976. Streptococcal M-protein extracted by non-ionic detergent. I. Properties of the antiphagocytic and type-specific molecules. *J. Exp. Med.* 144:32-53.
- Fox, J. E. B., and D. R. Phillips. 1981. Inhibition of actin polymerization in blood platelets by cytochalasins. *Nature (London)* 292:650-652.
- Francioli, P. B., and L. R. Freedman. 1979. Streptococcal infection of endocardial and intravascular vegetations in rabbits: natural history and effect of dexamethasone. *Infect. Immun.* 24:483-491.
- Freedman, L. R., and J. V. Valone, Jr. 1979. Experimental infective endocarditis. *Prog. Cardiovasc. Dis.* 22:169-180.
- Gibbons, R. J. 1977. Adherence of bacteria to host tissue, p. 395-406. In D. Schlessinger (ed.), *Microbiology—1977*. American Society for Microbiology, Washington, D.C.
- Gibbons, R. J., and R. J. Fitzgerald. 1969. Dextran-induced agglutination of *Streptococcus mutans*, and its potential role in the formation of microbial dental plaques. *J. Bacteriol.* 98:341-346.
- Gibbons, R. J., and J. V. Qureshi. 1976. Interactions of *Streptococcus mutans* and other oral bacteria with blood group reactive substances, p. 163-181. In H. M. Stiles, W. J. Loesche, and T. C. O'Brien (ed.), *Proceedings: Microbial Aspects of Dental Caries (a special supplement to Microbiology Abstracts)*, vol. 1. Information Retrieval, Inc., Washington, D.C.
- Gibbons, R. J., and J. VanHoute. 1980. Bacterial adherence and the formation of dental plaques, p. 61-104. In E. H. Beachey, (ed.), *Bacterial adherence*. Chapman and Hall, London.
- Hamada, S., and H. D. Slade. 1980. Biology, immunology, and cariogenicity of *Streptococcus mutans*. *Microbiol. Rev.* 44:332-384.
- Hawinger, J., S. Steckley, D. Hammond, C. Cheng, S. Timmons, A. D. Glick, and R. M. DesPrez. 1979. Staphylococci-induced human platelet injury mediated by protein A and immunoglobulin G Fc fragment receptor. *J. Clin. Invest.* 64:931-937.
- Hook III, E. W., and M. A. Sande. 1974. Role of the

- vegetation in experimental *Streptococcus viridans* endocarditis. Infect. Immun. **10**:1433-1438.
28. Kenney, D. M., and F. C. Chao. 1978. Microtubule inhibitors alter the secretion of β -glucuronidase by human blood platelets: involvement of microtubules in release reaction. II. J. Cell. Physiol. **96**:43-52.
 29. Kosaki, G., T. Nomura, and J. Kambayashi. 1980. The mechanism of the inhibitory effect of proteinase inhibitors on platelet aggregation and cellular synthesis of prostaglandins. I. Effect of the release of arachidonic acid from phospholipids. Thromb. Res. **20**:587-598.
 30. Levine, M. J., M. C. Herzberg, M. S. Levine, S. A. Ellison, M. W. Stinson, H. C. Li, and T. Van Dyke. 1978. Specificity of salivary-bacterial interactions: role of terminal sialic acid residues in the interaction of salivary glycoproteins with *Streptococcus sanguis* and *Streptococcus mutans*. Infect. Immun. **19**:107-115.
 31. Liljemark, W. F., and C. G. Bloomquist. 1981. Isolation of a protein-containing cell surface component from *Streptococcus sanguis* which affects its adherence to saliva-coated hydroxyapatite. Infect. Immun. **34**:428-434.
 32. Martin, B. M., R. D. Feinman, and T. C. Detwiler. 1975. Platelet stimulation of thrombin and other proteases. Biochemistry **14**:1308-1314.
 33. Murray, P. A., M. J. Levine, L. A. Tabak, and M. S. Reddy. 1982. Specificity of salivary-bacterial interactions. II. Evidence for a lectin on *Streptococcus sanguis* with specificity for a NeuAc α 2.3GalNAc sequence. Biochem. Biophys. Res. Commun. **106**:390-396.
 34. Nachman, R. L., and B. Ferris. 1972. Studies in the proteins of human platelet membranes. J. Biol. Chem. **247**:4468-4475.
 35. Neame, P. B., J. G. Kelton, I. R. Walker, I. O. Stewart, H. L. Nossel, and J. Hirsch. 1980. Thrombocytopenia in septicemia: the role of disseminated intravascular coagulation. Blood **56**:88-92.
 36. Pelletier, L. L., Jr., M. Coyle, and R. Petersdorf. 1978. Dextran as a possible virulence factor in streptococcal endocarditis. Proc. Soc. Exp. Biol. Med. **158**:415-420.
 37. Pfueller, S. L., and L. J. Cosgrove. 1980. Staphylococcal-induced platelet injury. Thromb. Res. **19**:733-735.
 38. Ramirez-Ronda, C. H. 1978. Adherence of glucan-positive and glucan-negative streptococcal strains to normal and damaged heart valves. J. Clin. Invest. **62**:805-814.
 39. Ramirez-Ronda, C. H. 1980. Effects of molecular weight of dextran on the adherence of *Streptococcus sanguis* to damaged heart valves. Infect. Immun. **29**:1-7.
 40. Riba, A. L., M. T. Thakur, A. Gottschalk, V. T. Andriole, and B. L. Zaret. 1979. Imaging experimental endocarditis with indium-111-labeled blood cellular components. Circulation **59**:336-343.
 41. Roberts, F. J. 1980. A review of positive blood cultures: identification and source of microorganisms and patterns of sensitivity to antibiotics. Rev. Infect. Dis. **2**:329-339.
 42. Roberts, R. B., A. G. Krieger, N. L. Schiller, and K. C. Gross. 1979. Viridans streptococcal endocarditis: the role of various species, including pyridoxal-dependent streptococci. Rev. Infect. Dis. **1**:955-965.
 43. Scheld, W. M., J. A. Valone, and M. A. Sande. 1978. Bacterial adherence in the pathogenesis of endocarditis. J. Clin. Invest. **61**:1394-1404.
 44. Semeraro, N., M. Colucci, and J. Vermeylen. 1979. Complement-dependent and complement-independent interactions of bacterial lipopolysaccharides and mucopeptides with rabbit and human platelets. Thromb. Haemostasis **41**:392-406.
 45. Sheth, N. K., V. P. Kurup, and B. A. Baron. 1980. The role of *Aspergillus fumigatus* antigens in blood coagulation and platelet function. Microbios **28**:91-96.
 46. Straus, D. C., S. J. Mattingly, and T. W. Milligan. 1977. Production of extracellular material by streptococci associated with subacute bacterial endocarditis. Infect. Immun. **17**:148-156.
 47. Tabak, L. A., M. J. Levine, I. D. Mandel, and S. A. Ellison. 1982. Role of salivary mucins in the protection of the oral cavity. J. Oral Pathol. **11**:1-17.
 48. Terleckyj, B., N. P. Willett, and G. D. Shockman. 1975. Growth of several cariogenic strains of oral streptococci in a chemically defined medium. Infect. Immun. **11**:649-655.
 49. Worner, P., and H. Patscheke. 1981. Antagonistic effects of *N*-ethylmaleimide on platelets treated with agents that are known to increase levels of cyclic AMP. Thromb. Res. **21**:201-206.
 50. Zimmerman, T. S., and H. L. Spiegelberg. 1975. Pneumococcus-induced serotonin release from human platelets. J. Clin. Invest. **56**:828-834.
 51. Zucker, M. B., and R. A. Grant. 1978. Nonreversible loss of platelet aggregability induced by calcium deprivation. Blood **52**:505-514.
 52. Zucker, M. B., and E. I. Peerschke. 1980. Specific binding of fibrinogen to human platelets: relationship to shape change and aggregation, p. 157-168. In A. Rotman, F. A. Meyer, C. Gitter, and A. Silberberg (ed.), Platelets: cellular response mechanisms and their biological significance. John Wiley and Sons, New York.