Platelet Interaction with Bacteria

I. Reaction Phases and Effects of Inhibitors

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Interactions between human platelets and several common species of bacteria have been studied *in vitro* by the technic of recording nephelometry. Results of this investigation have established the following parameters of platelet-bacterial interaction: (1) Bacteria are potent stimuli of the platelet-aggregating reaction. (2) The platelet-bacterial interaction proceeds through four distinct phases which are separable by varying the platelet-bacterial ratios or adding appropriate chemical inhibitors. (3) The degree and rate of the platelet response is proportional to the ratio of bacteria to platelets. (4) Direct contact between platelets and bacteria, physiologic levels of divalent cations and nucleotide release from platelets are essential for aggregation of platelets by bacteria. (5) By adding equivalent numbers of six species of microorganisms to platelet-rich plasma, their potency as initiators of the platelet reaction was determined, with *Staphylococcus* being the most powerful. (Amer J Path 65:367–380, 1971)

PLATELETS ARE KNOWN principally for the critical role they play in forming hemostatic plugs and coagulating blood. Another aspect of platelet function, their capacity to interact with particulate matter, has been recognized nearly as long as their participation in hemostasis. Yet, the emphasis on hemostasis has largely overshadowed the possibility that these cells might be involved in host defense against infection. In 1901, Levaditi [1] observed that platelets clumped with *Vibrio cholerae* that had been introduced into the circulation of rabbits. Since that report, a number of investigatiors [2,3] have noted similar reactions of platelets *in vivo* with a spectrum of foreign particles. Inert colloids, antigen-antibody complexes, viruses and bacteria also stimulate the platelet release reaction and aggregation *in vitro*.[4–8]

Platelet-particle interaction may be an integral factor in host defense, assisting in the clearance of foreign material from blood by the retic-

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uloendothelial system. However, it is also evident that this facet of platelet reactivity may have adverse effects. Thrombocytopenia can be severe after bacterial invasion of the circulation; a significant fall in platelet count was reported recently in nearly 75% of the patients with proven sepsis.[9] Transient, adenosine diphosphate–induced thrombocytopenia and platelet aggregation can cause vascular damage in experimental animals.[10–12] A similar pathologic process may develop in the human microcirculation during the thrombocytopenic phase of a bacteremia. Immediate complications of septicemia, including vasculitis, occlusion of blood vessels, endocarditis and disseminated intravascular coagulation, may result from formation and deposit of septic platelet emboli. Aggregates of platelets and bacteria interacting with small vessels could be a factor in the genesis of delayed complications such as pyelonephritis, nephritis, brain abscess and pneumonitis. Such important clinical situations have focused our attention on the participation of platelets in host defense and injury to the microvasculature. The role of platelet-bacterial interaction in these phenomena is relatively unexplored. In order to define this domain of platelet physiology, basic features of platelet-bacterial interaction *in vitro* must be clarified. The present study was undertaken for this purpose.

Materials and Methods

Preparation of Platelets

Platelets were obtained from normal donors who had no medications in the 10 days prior to venipuncture. Blood was drawn by a two-syringe technic and immediately mixed with 3.8% trisodium citrate in a ratio of 10:1. Citrated platelet-rich plasma (CPRP) was separated by centrifugation at 100 *g* for 20 minutes at room temperature. Platelet-poor plasma (PPP) was obtained by sedimenting platelets from a portion of CPRP at 250 *g* for 20 minutes. The platelet count of the CPRP was determined and adjusted to 300,000/cu mm by dilution with PPP. In some experiments, the platelet count was adjusted to 500,000/cu mm. Complete details of preparing platelets for *in vitro* experiments and nephelometry were presented in previous publications.[13–15]

Preparation of Bacteria

Suspensions of *Staphylococcus aureus*, strain 502A, were prepared from cultures grown 18–20 hours in Penassey broth (Difco). The bacteria were removed from the broth, washed twice with distilled water by repeated centrifugation at 460 *g* for 20 minutes and resuspended in Hanks' balanced salt solution (HBSS). The final HBSS suspension was diluted to 3×10^9 bacteria/ml, determined by triplicate counts in a Petroff-Hausser chamber, by quantitative plate culturing and/or by standardized optical density measurement. Cellfree media were prepared from the broth of an 18-hour culture of HBSS, after 1 hour of incubation, by centrifugation and passage through a 0.45 μ Millipore filter. *Streptococcus faecalis, Strep-*

tococcus pyogenes (type M57), *Escherichia coli* and *Diplococcus pneumoniae* (types 8 and 25) were prepared as stock suspensions of 3×10^9/ml in a similar manner after 18–20 hours of growth in appropriate broth media. Heat-killed organisms were prepared by incubating a stock suspension for 30 minutes at 60 C and were tested for nonviability by pour-plate culture. Stock bacterial cultures were maintained on agar plates held in cold storage and tested periodically for strain identification and contamination.

Nephelometry

Platelet-bacterial interaction was studied turbidimetrically on a model S201 Chrono-Log platelet aggregometer coupled to a Bausch and Lomb VOM-5 recorder. The baseline of the recordings was established with CPRP and the scale adjusted to give maximum deflection at the optical density of PPP. For some experiments the upper limit of deviation was set at an arbitrary high level with the gain control of the aggregometer in order to facilitate the detection of minor changes in light transmission. Stock suspensions of CPRP and washed bacteria were kept in a 37 C water bath during the experiment. Samples of 0.9 ml CPRP in the aggregometer were stirred at 1200 rpm with the temperature maintained at 37 C. A baseline was recorded for 1 minute and then 0.1 ml of the bacterial suspension was added to the sample. This gave a nominal ratio of one bacteria to one platelet. The platelet-bacterial reaction produced an increase in light transmission (decreased optical density) of the suspension which gave an upward deflection of the tracing on graph paper moving at 1 inch/min. The recording was continued until equilibrium was attained and no change in the tracings occurred for several minutes. Forming aggregates could be viewed in the CPRP through a small magnifying lens mounted on the aggregometer.

Platelet-Active Agents

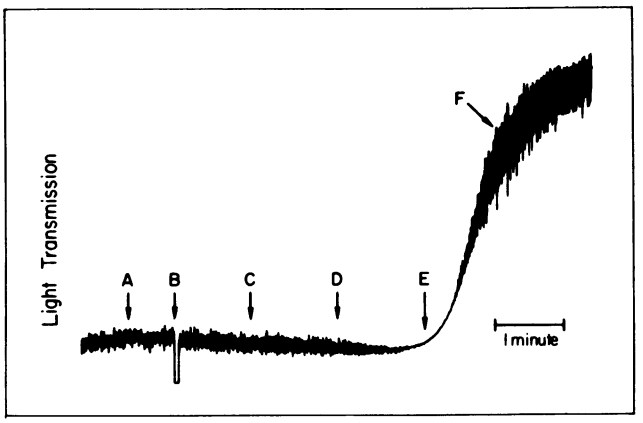
Platelet-bacterial interaction was compared with platelet aggregation tracings obtained by stimulation of matched aliquots of CPRP with adenosine diphosphate (ADP) and collagen. The influence of several chemical inhibitors of platelet reactivity upon the platelet-bacterial reaction was tested by adding the inhibitors to the CPRP prior to addition of the bacteria. Those agents reported in this study are adenosine, apyrase, and ethylenediaminetetracetic acid (EDTA). These substances were tested first at a level known to be fully effective in blocking platelet reactivity and then were titrated downward until no influence was detectable. Specific concentrations are stated in the results and figure legends.

Results

Basic Reaction of Platelets with Staphylococcus

Washed staphylococci mixed with platelet-rich plasma at a nominal ratio of one bacteria to one platelet produced a platelet reaction recorded as a reproducible sequence of events culminating in irreversible aggregation. This sequence could be divided into four phases which were distinguished by their characteristic appearance in the aggregometer tracings (Text-fig 1).

The first phase of the recorded reaction was a delay or *contact phase* in which there was no change in the broad baseline produced by



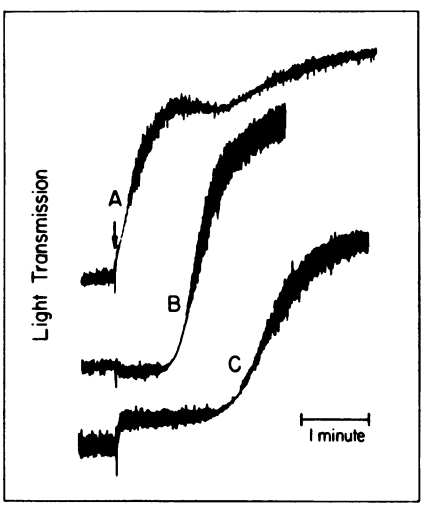
TEXT-FIG 1—Aggregometer tracing of platelet-bacterial interaction. The tracing records variations in light transmission (optical density) with time through a 1.0 ml sample of human platelets and bacteria. Citrated platelet-rich plasma (CPRP) was stirred at 1200 rpm and 37 C to establish the baseline, *A*. The breadth of the baseline is due to rapid oscillation of the tracing which is characteristic of stirred discoid platelets. *Staphylococcus* 502A was added to the CPRP at point *B* in a ratio of one microbe to one platelet. The response of the platelets to the bacteria was then recorded in four distinct phases: The *contact phase* (*C*) extended from the addition of bacteria at *B* until the tracing began to narrow at *D*. As the platelets became irregular in form the tracing narrowed from *D* to *E*, indicating the *shape-change phase*. When the phase of *early platelet aggregation* commenced, there was an increase in light transmission and the tracing begins to rise at *E*. As the aggregates increased in size, the tracing widened and finally plateaued (*F*), indicating that maximal aggregation had occurred and the final phase of *irreversible aggregation* was attained.

stirred discoid platelets. At the usual platelet concentrations (300,000/cu mm), the contact phase lasted from 1 to 4 minutes. The end of this first phase was signaled by rapid narrowing of the tracing width, a change associated with transition in the bulk of the platelets from discoid to irregular shape. The onset of the second phase, *platelet shape change*, was followed closely by a rise in the tracing indicating the phase of *early aggregation*. The tracing gradually widened as it rose and finally plateaued as the reaction proceeded into and through the phase of *irreversible aggregation*. The form and proportions of the aggregometer recordings of the platelet-bacterial reaction reflected a platelet aggregation response closely resembling that promoted by collagen. The tracing of platelet response to bacteria is compared to those of reactions with collagen and ADP in Text-fig 2.

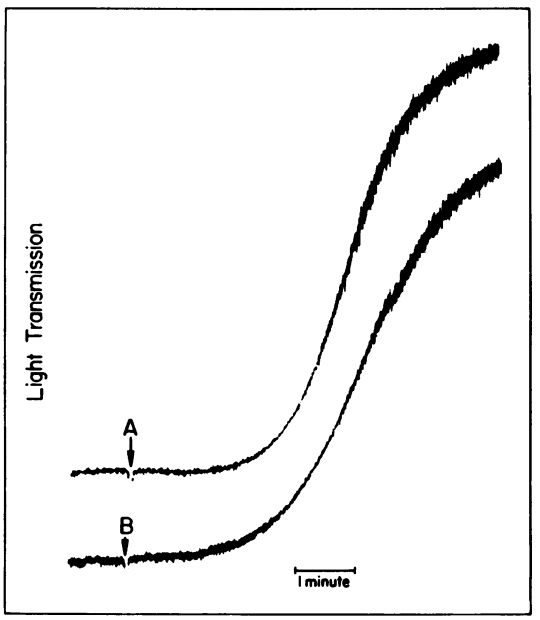
To dissect the platelet-bacterial reaction and to define the events underlying the recorded phases of response, the system was subjected to a number of modifications. The hypothesis that direct contact between bacteria and platelets was essential to stimulate the platelet response was tested in several ways. Broth from an 18-hour culture of

S aureus was centrifuged and filtered to remove the bacteria. A sample of the HBSS in which washed bacteria had been incubated for 1 hour at 37 C was similarly cleared of organisms. Neither of these media produced a recordable response when 0.1 ml of either was added with stirring to 0.9 ml of CPRP on the aggregometer. Equal numbers of live or heat-killed *S aureus* gave identical reactions with aliquots of the same CPRP, indicating a passive role for the bacteria (Text-fig 3). If bacteria were added to the platelet suspension without stirring, no change in optical density was observed.

When decreasing numbers of staphylococci were added to constant amounts of CPRP, the contact phase showed the greatest change (Text-fig 4). This phase was prolonged two- to threefold as the ratio of bacteria to platelets was reduced from 1:1 to the order of 1:10. At these levels, after the lengthened contact phase the reaction proceeded to complete aggregation at about the same rate as with the 1:1 ratio. When the bacterial ratio was further reduced to 1:50, the



TEXT-FIG 2—Comparison of aggregometer tracings produced in matched samples of CPRP after addition (*below arrow*) of (*A*) 2 × 10^{-3} M ADP; (*B*) standard collagen suspension; (*C*) *Staphylococcus*. Tracing A reveals the separation of the platelet response into two phases. The first wave was due to exogenous ADP. Partial reversal took place before endogenous products secreted by the platelet stimulated the second, irreversible wave of aggregation. Slightly higher concentrations of exogenous ADP produced a single massive wave of irreversible aggregation similar to the tracings obtained after the addition of collagen (*B*) or bacteria (*C*). The onset of response to ADP was abrupt. In contrast, the platelet response to collagen or bacteria was characterized by a delay or contact phase before shape change and aggregation began. Secretion of endogenous ADP during the platelet release reaction was essential for irreversible aggregation in each case (see Text-fig 5).



TEXT-FIG 3—Comparison of aggregation produced in samples of CPRP by (*A*) living and (*B*) heat-killed *S aureus* at ratios of one bacterium to one platelet. The reactions were identical, indicating that living organisms were not essential for stimulation of the platelet response. In this and subsequent figures, bacteria were added to the system at the points below the vertical arrows.

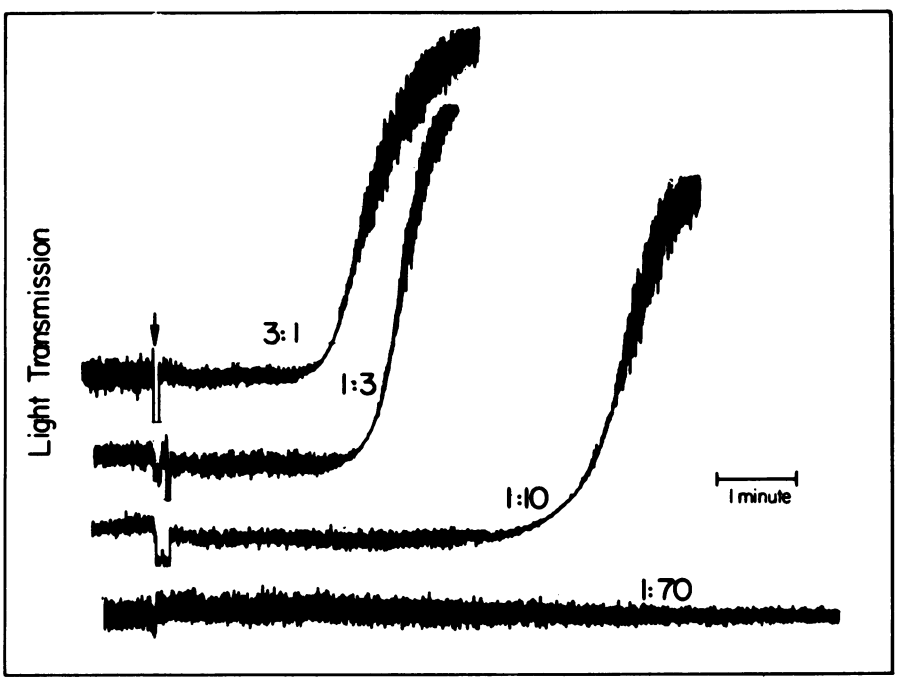
reaction was greatly prolonged, and only a partial rise in the tracing and few very small aggregates were obtained. Further reduction in numbers of bacteria produced a recording of platelet shape change which occurred after an extended contact phase, but aggregation was neither visible nor recorded for up to 30 minutes. Thus the threshold of shape change could be reached without progression to detectable aggregation. At still lower ratios of about one microorganism to 100 platelets, no reaction at all was present as evidenced by a completely unchanged tracing for 30 minutes.

When CPRP was preincubated 1 minute with 1×10^{-3} M adenosine, the reaction of the platelets to bacteria at a 1:1 ratio was blocked. Titration of the adenosine downward failed to reveal a level at which a shape change and narrowing of the tracing could be obtained without aggregation. At the threshold of influence of adenosine, the delay or contact phase was prolonged; however, once begun, aggregation proceeded to completion at nearly the same rate as in the controls (Text-fig 5).

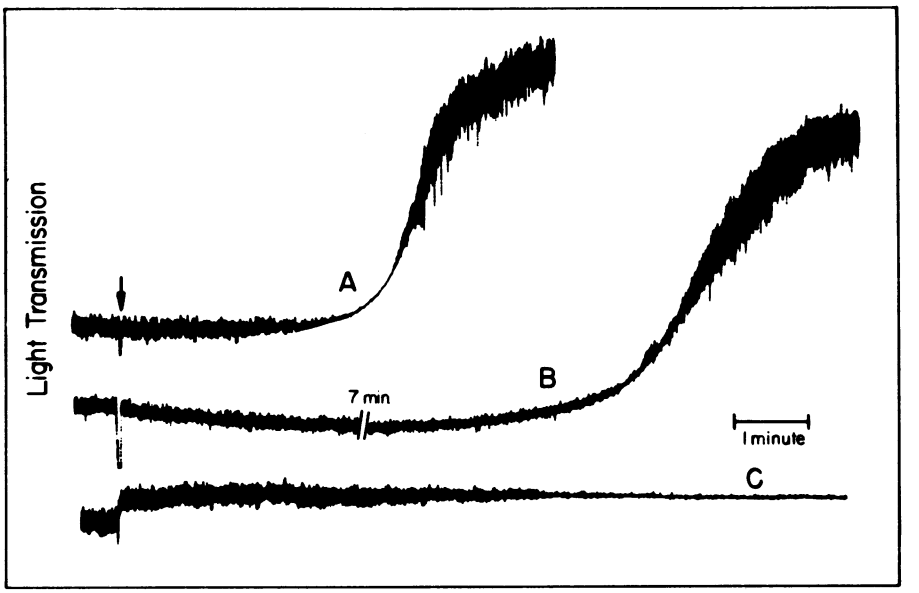
Preincubation of CPRP with apyrase (5 mg/ml) for 5 minutes produced a response to bacteria that was limited to lengthened contact and shape change phases (Text-fig 5). No aggregation was seen and there was no rise in the tracing. EDTA added to CPRP at a final concentration of 0.75 mg/ml gave a reaction tracing similar to that of apyrase. Higher levels of the chelating agent completely blocked the platelet response to bacteria (Text-fig 6).

Reactions of Platelets with Other Bacterial Species

Human platelets as CPRP were tested as above with *Streptococcus faecalis, Escherichia coli, Streptococcus pyogenes,* and *Diplococcus pneumoniae* (Text-fig 7). The responses of these organisms were compared to that evoked with S *aureus* in approximately equal num-



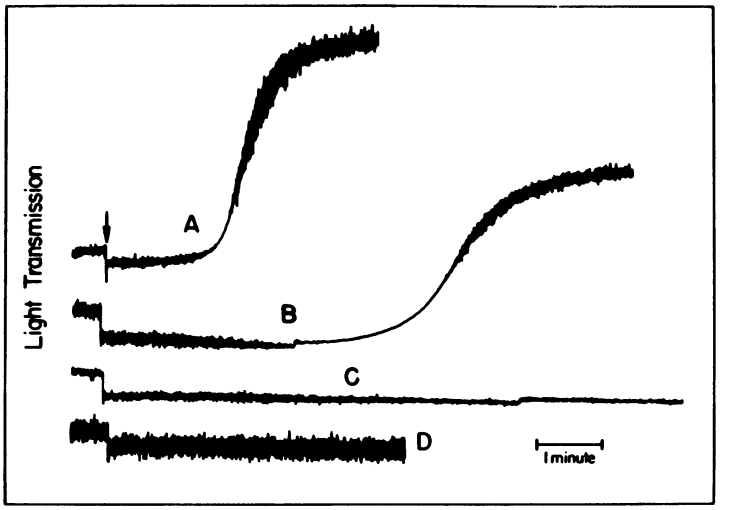
TEXT-FIG 4—Platelet responses to decreasing numbers of bacteria. Platelets were kept at a concentration of 300,000/cu mm and bacteria were added in numbers resulting in the indicated *bacteria* to *platelet* ratios. In the vicinity of 1 : 1 (*3 : 1* and *1 : 3*), the reactions were rapid and nearly identical to the phases show in Text-fig 1. As the bacteria were reduced (*1 : 10*), the contact phase was prolonged, but, once begun, aggregation proceeded at the same rate (indicated by the slope) as at 1:1. As the bacteria were further reduced, a point was reached, here at *1:70,* where the narrowing of shape change occurred but no aggregation took place. This last tracing was continued for another 27 minutes without an aggregation rise. At lower ratios, even the shape change response was absent.



TEXT-FIG 5—Effects of preincubation with adenosine or apyrase on platelet-bacterial interaction: (*A*) Control CPRP and *S aureus* 502A at 1:1. (*B*) Adenosine at 2×10^{-4} M was added to CPRP 5 minutes before the bacteria. The effect of adenosine was most marked in prolonging the contact phase (note 7-minute gap in tracing), but also produced a slight slowing of aggregation as indicated by the lower angle of rise. At higher concentrations of adenosine, the response was completely blocked. (*C*) Apyrase at 5 mg/ml added 1 minute before the bacteria prevented aggregation entirely by removing secreted ADP, but allowed a normal shape change with narrowing of the tracing.

bers when added to aliquots of the same platelet preparation. In each case, *Staphylococcus* proved to be the more potent stimulus of platelet aggregation. The reaction to beta-hemolytic streptococcus (*S pyogenes*) was nearly as rapid as the response to *Staphylococcus*. Platelets responded to *S faecalis* a good deal more slowly. The total reaction to this species was three to five times longer than that to *S aureus*, but it proceeded through complete irreversible aggregation. *E coli* was a poor stimulus of platelet aggregation. At the 1:1 ratio, the tracings of platelet interaction with *E coli* frequently went for 8–12 minutes without change and then proceeded through a very gradual phase of shape change. This was usually but not always followed by complete aggregation.

The response of platelets to type 8 pneumococcus was extended even beyond the response to *E coli* (Text-fig 7E). Protracted contact and shape-change phases were followed by very gradual early aggregation as indicated by the gentle slope of the tracing. At the 1:1 ratio, this total reaction with type 8 pneumococcus took 28 minutes.



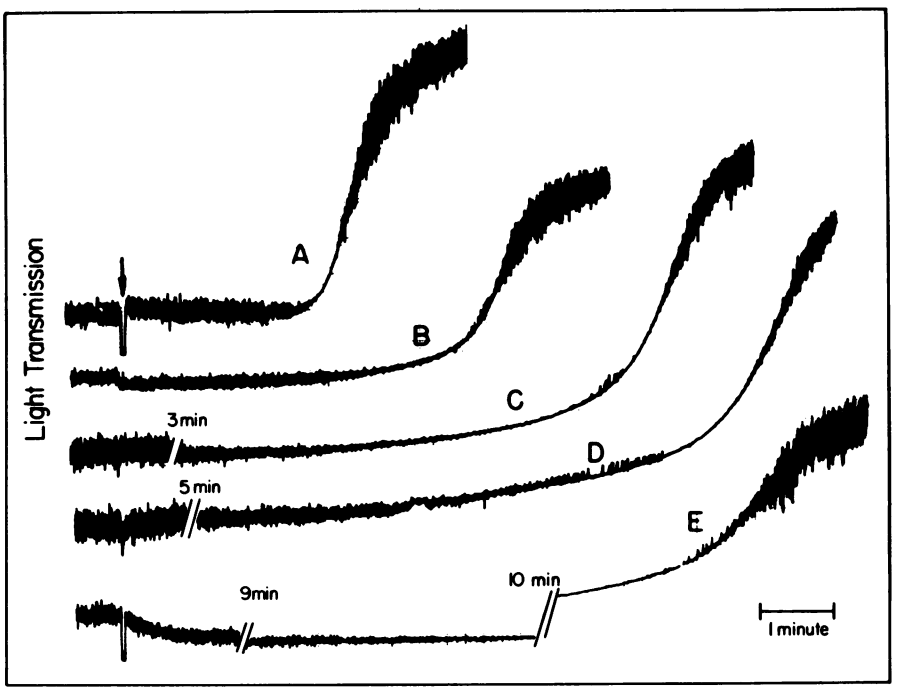
TEXT-FIG 6—Effect of varying concentrations of EDTA on platelet-bacterial interaction: (*A*) Control CPRP and *S aureus* 502A at 1:1. (*B*) 0.6 mg EDTA added to CPRP prior to the bacteria. The entire reaction was slowed but proceeded through irreversible aggregation. (*C*) 0.75 mg EDTA added to CPRP prior to the bacteria. There was a narrowing of shape change but no aggregation rise. (*D*) 1.0 mg EDTA completely blocked the response, demonstrating that the reaction depended upon physiologic levels of divalent cations.

In this same ratio and concentration, type 24 pneumococcus produced no recordable response when observed for over 20 minutes on the aggregometer.

Discussion

The results of this investigation demonstrate that several common species of bacteria are potent stimuli of platelet aggregation *in vitro*. The sequence of the reaction, as recorded on a platelet aggregometer, could be separated into four distinct phases which were reflected in specific alterations in light transmission through the platelet-bacterial mixture. These were the phases of contact, shape change, early aggregation and irreversible aggregation.

The contact phase of one to several minutes appeared to represent the time required for the number of platelet-bacterial encounters to exceed the threshold for initiation of a shape-change response in the bulk of the platelets. Several observations indicated that direct contact of bacteria with platelets was required to stimulate the platelet reaction. Stirring of the platelet-bacterial mixture was essential to obtain any change in light transmission indicative of shape change or aggrega-



TEXT-FIG 7—Comparison of platelet-bacterial interaction produced by different strains of bacteria. In each case, the bacteria to platelet ratios were 1:1. (*A*) *S aureus* 502A; (*B*) *Streptococcus pyogenes;* (*C*) *Streptococcus faecalis;* (*D*) *Escherichia coli;* (*E*) *Diplococcus pneumoniae* type 8. These tracings demonstrate a spectrum of potency among these organisms as stimuli of platelet aggregation.

tion. When culture broth or incubation media from washed bacteria were filtered free of organisms, these solutions failed to activate the platelets. This precluded any cell-free product of bacteria as the mediator of platelet stimulation. Similarly, results with washed platelets, as reported elsewhere,[16–18] demonstrated that plasma factors were not required and, therefore, could not be implicated as essential intermediaries in platelet-bacteria interaction. Morphologic evidence supports the need for contact and adherence of bacteria with platelets in order to stimulate the platelet response.[19,20]

The requirement for direct contact between platelets and bacteria was also supported by observations of the influence of bacterial concentration on the rate of response. The duration of the contact phase was a particularly sensitive and direct indication of bacterial numbers in a system with constant platelet concentrations. The contact phase and the rate of aggregation indicated by the slope of the tracing could be prolonged several-fold by a reduction of the ratios of bacteria

to platelets. When this ratio was of the order of 1:100, no reaction was detected for up to 30 minutes, even though there were still three million bacteria in the test system. Interaction between bacteria and a few platelets can take place even at this subthreshold ratio, as other methods have indicated.[20] However, the small number of platelet-bacterial encounters at the low ratio are too few in number to be detected by the aggregometer, which is sensitive only to the bulk changes in the reaction system. At the threshold of recordable response, significant numbers of platelets underwent shape change, but aggregation failed to develop to a degree detectable by nephelometry. A slight increase in bacterial numbers above the threshold resulted in a characteristic pattern of slow but progressive aggregation. These findings show that the platelet response stimulated by bacteria is not an all-or-none process. The state of activation necessary for completion of the reaction appears to depend upon a critical number of platelets reaching a particular stage of response at about the same time.[21]

The second phase of the reaction, the change from discoid to irregular shape in the bulk of the platelets, was signaled by narrowing of the tracing without deflection. The shape change could be evoked, independently of detectable aggregation, by reducing bacterial numbers, by careful titration of divalent cations with EDTA or by hydrolysis of secreted ADP with apyrase. Apyrase is known to remove ADP released from platelets; therefore, it prevents ADP-dependent platelet aggregation.[22,23] The experiments with adenosine, which blocks platelet response to exogenous or endogenous ADP, also support the ADP dependence of the platelet-aggregating response on contact with bacteria.

The final phases of platelet-bacterial interaction, the initial formation of platelet aggregates and irreversible aggregation, were marked by a rapid rise in light transmission and upward deflection of the aggregometer tracing. In the absence of inhibitors and in the presence of nearly equal numbers of bacteria and platelets, the rate of aggregation as indicated by the slope of the tracing was essentially the same in every sample, regardless of the duration of the contact phase. The onset of aggregation always occurred after the platelet shape change, although it sometimes began before the width of the tracing had narrowed to a single line. Prevention of aggregation by apyrase without affecting contact or shape change indicated that initiation of the platelet release reaction was necessary for platelet-to-platelet associations in this system. Once platelet clumping began, the process always proceeded to irreversible aggregation. Dis-

sociation of platelet-bacterial aggregates, which would have been indicated by a fall in the tracing toward the baseline, never occurred, even when samples were observed on the aggregometer for 30 minutes.

The response of platelets to bacteria strongly resembled the reaction of platelets to collagen. Addition of collagen to CPRP on the aggregometer is also followed by a short delay phase, shape change, formation of early platelet aggregates and irreversible aggregation. The adhesion of platelets to collagen during the delay phase is independent of calcium ions.[24] In the present study, titration of EDTA added to CPRP before bacteria were added indicated that divalent cations were essential for the aggregation of platelets by bacteria. However, the role of calcium ions in adhesion of platelets to bacteria in the contact phase could not be determined by the methods employed.

The results of the comparison of six strains of bacteria as stimuli of platelet aggregation demonstrated that there is a spectrum of influence. S *aureus* was the most potent, while pneumococcus was least stimulatory of the species tested. The differences in surface coats of these organisms, which probably account for differing influences upon platelets, are as yet undefined. It is intriguing to postulate that variations in surface coats that bear some correlation with infectivity and pathogenicity may also correlate directly or inversely with the capacity of these organisms to stimulate the platelet reaction.

The transient thrombocytopenia occurring after particulates are injected into the bloodstream prompted the studies of van Aken and Vreeken on the role of platelets in clearance of colloidal carbon by the reticuloendothelial system.[25] They concluded that small platelet aggregates that formed in the circulation after carbon particles were injected served as a vehicle for the transport and lodgment of the colloidal carbon in the vascular lumen at sites which could be cleared by phagocytic cells. We may speculate that this mechanism also serves in bacterial removal by the reticuloendothelial system. This would certainly agree with the findings of earlier workers who postulated such a role for platelets.[26,27] The present work lends further credibility to the concept of this platelet function by demonstrating clearly the propensity of platelets to respond vigorously and in a physiologic manner to contact with bacteria.

A major question raised by the present studies concerns the fate of the bacteria that encounter platelets and participate in the formation of platelet aggregates. Morphologic studies have shown that most of the bacteria are removed from the media by the platelets as they aggregate, and that these bacteria come to lie centrally in the mass

of adherent cells.[19,20,28] The presence of potential bacterial inhibitors or bactericidal agents within platelets has been demonstrated.[29–32] The existence of these platelets products suggests that platelets may be able to destroy bacteria. The fate of the bacteria in the *in vitro* system employed here has been explored and will be presented in a subsequent report.[18]

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