Platelet Aggregation by *Streptococcus pyogenes*

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Heat-killed group A *Streptococcus pyogenes* induced platelet aggregation in platelet-rich plasma. Aggregation was dependent upon the ratio of platelets to bacteria, with maximal aggregation occurring at 0.8 platelets per bacterium (final concentration, 300,000 per µl). Inhibition of the reaction by 3 mM EDTA indicated it was a true aggregation and not merely adhesion and agglutination. Lactic acid dehydrogenase assays indicated lysis of platelets did not occur during a 6-min incubation period. Aggregation was inhibited in a dose-dependent manner by acetylsalicylic acid (100 µM to 10 mM) and quinacrine (15.6 to 250 µM), with no decrease in aggregation at the lowest concentration of inhibitor tested. *S. pyogenes* induced the release of [14C] serotonin, which was maximal (50%) at 2.4 min, when aggregation was nearly complete. Gel-filtered platelets were not aggregated unless fibrinogen (final concentration, 1.8 mg/ml) was included in the reaction mixture. *Staphylococcus aureus*, a group B streptococcus, and *Escherichia coli* were unable to induce aggregation in platelet-rich plasma under the conditions used for *S. pyogenes*.

Thrombocytopenia, thrombophlebitis, and coagulation defects are associated with infectious organisms which initiate the platelet release reaction. In this reaction, stores within platelet granules are released and contribute to fibrin deposition, platelet aggregation, and thrombus formation. Although in some instances these effects are due to autoimmune or immune complex phenomena which develop secondary to the infection, platelet activation and disturbances of the coagulation system by bacteria have been noted to contribute to the morbidity and mortality associated with the diseases they cause (20).

Direct platelet-microbe interactions which result in platelet aggregation and release stem from three mechanisms. Due to the presence of receptors for complement component C3b on platelets, agents which activate the complement system stimulate the platelet release reaction. Tuberculin (19), bacterial lipopolysaccharide (15), *Aspergillus* spp. antigens (22), and *Listeria monocytogenes* (16) need complement or a heat-stable plasma component presumed to be complement before they activate platelets. Strains of *Staphylococcus aureus* which possess protein A in their cell wall provoke the platelet release reaction (9) by virtue of their ability to combine with immunoglobulin G, which binds to the platelet Fc receptor. A number of microbial hemolysins, including the lecithinase of *Clostridium welchii*, the alpha-toxin of *S. aureus*, and both streptolysin S and streptolysin O, cause platelet degranulation by virtue of their lytic attack on mammalian cell membranes (3).

In this report, we describe the activation of platelets by *Streptococcus pyogenes*. Under the conditions used in this study, platelet activation relied on the presence of fibrinogen. Acetylsalicylic acid (ASA) and quinacrine, both of which are known inhibitors of the cyclooxygenase pathway of arachidonic acid metabolism, also inhibited platelet aggregation.

**MATERIALS AND METHODS**

**Bacterial cultures.** The hemolytic strain of *S. aureus* and the group B streptococcus used in this study were clinical isolates supplied by the Department of Pathology of this institution, and the group A *S. pyogenes* and *Escherichia coli* were stock cultures from the Department of Microbiology. The cocci were grown for approximately 42 h and the *E. coli* for 24 h at 37°C in brain heart infusion broth before being heat-killed by exposure to 56°C for 1 h. The cells were collected by centrifugation, washed three times, and suspended in sterile saline. Nephelometry calibrated against viable cell counts was used to establish the cell density.

**Chemicals.** [2-14C] Serotonin (side-chain 2.14C, 58 mCi/mmol) was purchased from Amersham Corp., Arlington Heights, Ill., and stored as a 100 µCi solution in 0.15 M NaCl. The reagents used in the lactic acid dehydrogenase assay, ASA, quinacrine, fibrinogen (human grade 1), EDTA, and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, Mo.

**Preparation of human platelets.** Human blood from drug-free donors was used as a source of platelets. Nine volumes of blood were drawn into one volume of...
TABLE 1. Aggregation of human platelets in PRP

<table>
<thead>
<tr>
<th>PRP incubation with:</th>
<th>Incubation time (min)</th>
<th>% Aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. pyogenes</td>
<td>6</td>
<td>87.5</td>
</tr>
<tr>
<td>S. pyogenes plus 3.1 mM EDTA</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>S. pyogenes plus 0.67 mM EDTA</td>
<td>4</td>
<td>80.0</td>
</tr>
<tr>
<td>Group B streptococci</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>S. aureus</td>
<td>30</td>
<td>0</td>
</tr>
<tr>
<td>E. coli</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>1.5 μM ADP (positive control)</td>
<td>4</td>
<td>93.8</td>
</tr>
<tr>
<td>Saline (negative control)</td>
<td>30</td>
<td>0</td>
</tr>
</tbody>
</table>

*The heat-killed bacterial cultures were used at 2.4 × 10⁷ cells per μl; platelets, at 3 × 10⁹/μl, were coincubated at 37°C for the times indicated. Positive aggregation tests were irreversible during the time periods stated.

a 3.8% sodium citrate solution in a plastic syringe. Platelet-rich plasma (PRP) consisted of the supernatant fluid derived from centrifuging the blood plasma in a plastic tube at 150 × g for 10 min. The PRP was collected into plastic tubes, held at room temperature, and used within 4 h.

Gel-filtered platelets (GFP) were prepared by passing 2.0-ml portions of the PRP preparation through a Sepharose 2B column (13). This column consisted of a 10-ml plastic pipet packed to a height of 20 cm over a nylon retaining screen with a mesh of 40 μm and had a flow rate of approximately 15 ml/h. The gel-suspending and eluting fluid consisted of Tyrodes solution in which the Ca²⁺ was replaced with Sr²⁺. The buffer also contained 200 μg of apyrase per ml, 0.1% glucose, and 0.35% bovine serum albumin. Fractions of 0.5 ml were collected. The GFP eluted in the void volume.

Platelet numbers were determined and adjusted to 300,000 per ml by the addition of platelet-poor plasma when necessary (4, 5). Platelet-poor plasma consisted of the supernatant fluid of PRP after centrifugation at 1,640 × g for 10 min.

Platelet aggregation. A single-channel Chronolog Aggregometer (model 300-1; Chrono-log Corp., Havertown, Pa.) attached to an Omniscribe Recorder (model 5112-1; Houston Instruments, Austin, Tex.) was used to measure and record platelet aggregation (16). In each experiment, platelet-poor plasma was used to adjust the instrument to 100% transmission to represent 100% aggregation. A mixture of 450 μl of either PRP or GFP and 50 μl of the Sr²⁺ Tyrodes buffer (13) was used to represent 0% transmission (aggregation).

In the actual experiments, 450 μl of PRP or GFP was added to the cuvette, which was placed in the aggregometer and allowed to warm to 37°C. Then 50 μl of the bacterial suspension was added, and the zero point was readjusted due to the density of the bacterial suspension. Platelet aggregation was followed for a minimum of 4 min. In a companion experiment, an addition of 50 μl of ADP to achieve a final concentration of 1.5 μM was used as a positive control for suitability of the platelet preparation.

In the inhibition experiments, ASA, quinacrine, and EDTA were preincubated with the PRP for 10 min at room temperature before being transferred to the 37°C environment in the aggregometer. When this temperature was achieved, the bacteria were added to initiate the experiment. In the fibrinogen experiment, the GFP and fibrinogen were held together for 10 min at room temperature. Otherwise, the experiment was conducted as with the inhibitors.

Serotonin and lactic acid dehydrogenase release. Platelet aggregation and serotonin and lactic acid dehydrogenase release were measured over the same time period from a common platelet preparation. Serotonin within platelets was labeled by incubation of PRP with 1 μM [³H]serotonin for 30 min at room temperature. After addition of the bacterial suspension, aggregation was recorded as usual. From a separate tube containing a larger volume of an identical mixture held in a 37°C water bath, 450-μl portions were removed at intervals of approximately 1 min and added to 75 μl of 933 mM formaldehyde containing 51 mM EDTA in 1.5-ml centrifuge tubes held in ice. The tubes were centrifuged in a Beckman model B microfuge at approximately 12,000 × g for 2 min. Duplicate 200-μl samples were removed and added to 10 ml of Brays solution and subsequently analyzed in a Packard 460 CD liquid scintillation counter. Total radioactivity in the samples was determined from uncentrifuged controls. Release of radio labeled serotonin was calculated from the formula:

\[
\% \text{ release} = \frac{S \text{ test} - S \text{ control}}{T \text{ control} - S \text{ control} \times 100}
\]

where S equals the radioactivity in the supernatant fluid and T equals the total radioactivity measured as disintegrations per minute (11).

Lactic acid dehydrogenase released from platelets during these experiments was determined by a standard method (2). A 10% solution of Triton X-100 was used to lyse platelets to determine the total amount of intracellular lactic acid dehydrogenase.

RESULTS

When E. coli, S. aureus, a group B streptococcus, and a group A S. pyogenes were each tested at approximately a 1:1 ratio with human
platelets, only S. pyogenes was able to catalyze platelet aggregation. This organism caused full platelet aggregation within 6 min. The E. coli was totally inactive during a 15-min incubation period, and the remaining two organisms were inactive over a 30-min test period (Table 1) under the conditions used in these experiments. Platelet aggregation by S. pyogenes was totally inhibited by 3.1 mM but not by 0.67 mM EDTA. ADP used as a positive control in all experiments usually produced 85 to 95% aggregation, whereas the saline control was invariably negative.

A titration of the bacterial dose dependency of platelet aggregation by S. pyogenes created the data presented in Fig. 1. When the bacterial concentration exceeded $5 \times 10^4$ per µl, a sharp increase in the aggregative response developed which was nearly maximal at a level of $8 \times 10^4$ per µl. Since no inhibitory effect was noted with a higher population of bacteria, the cells were used in a 0.8:1 ratio with platelets in further studies. In absolute numbers this is $2.4 \times 10^5$ bacteria to $3 \times 10^5$ platelets per µl.

The ability of S. pyogenes to release serotonin from platelets simultaneous with aggregation is detectable after 1.5 min of incubation (Fig. 2). After 2 min of incubation, serotonin release is nearly at its maximum of 50% of the intracellular store. At this time, aggregation is nearly two-thirds complete. Aggregation continued to increase and was essentially complete at 3 min.

To ensure that the release of serotonin was not accompanied by platelet lysis, lactic acid dehydrogenase assays were performed at the termination of the release experiment, i.e., 4 min. The data in Table 2 indicate that the platelets contained the expected amount of the enzyme (866 ± 202 U), whereas the supernatant fluids from the saline control and bacterial platelet mixture had similar low levels, 153 and 206 U, respectively. This indicates that the bacteria did not lyse the platelets.

Two inhibitors of the cyclooxygenase pathway of arachidonate metabolism (10, 17) were examined for their ability to inhibit platelet aggregation by S. pyogenes. The concentrations of ASA (Fig. 3) and quinacrine (Fig. 4) were chosen on the basis of their ability to impair ADP-induced platelet aggregation in our laboratory. Both inhibitors were effective, ASA in the millimolar range with 5.0 mM producing about 75% inhibition and 31 µM quinacrine produced about 81% inhibition.

A requirement of S. pyogenes for fibrinogen to aggregate platelets was demonstrated by the supplementation of GFP with human fibrinogen. GFP alone or when supplemented with fibrinogen at 0.9 mg/ml we unaffected by the streptococcus; however, a 1.8 mg/ml concentration of fibrinogen supplement restored full aggregative susceptibility (Fig. 5).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Lactic acid dehydrogenase released (U/ml ± SE)</th>
</tr>
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<tbody>
<tr>
<td>S. pyogenes</td>
<td>206.7 ± 7.5</td>
</tr>
<tr>
<td>Saline (negative control)</td>
<td>153.3 ± 5.8</td>
</tr>
<tr>
<td>Triton X-100 (positive control)</td>
<td>866.7 ± 202.2</td>
</tr>
</tbody>
</table>

* Calculations were from three trials; $2.4 \times 10^5$ bacteria and $3 \times 10^5$ platelets per µl were incubated at 37°C for 4 min.
peptidoglycan (8, 18) can be ignored due to the negative data derived from the use of the group B streptococci. Although purified streptococcal lipoteichoic acid will bind to platelets, no evidence exists that intact cocci are capable of this. Even so, the teichoic acid-platelet reaction is cytolytic (1). The results of the lactic acid dehydrogenase assays conducted in PRP revealed that the streptococci caused the release of only 207 U of enzyme per ml compared with the negative control of 153 U/ml and the Triton X-100 positive control of 867 U/ml. Thus, little, if any, platelet lysis occurred.

In addition, the ability of S. pyogenes to use fibrinogen-treated GFP for the platelet reaction indicates that other serum proteins, such as complement which degranulate platelets via a lytic mechanism, are not required under the conditions tested. Indeed, the fibrinogen requirement coupled with the evidence that M and T proteins (12, 21) of group A streptococci bind fibrinogen indicates that fibrinogen receptors on the bacterial and platelet surface serve as catalysts for the activation reaction.

The interaction of S. pyogenes with platelet-bound fibrinogen is unique for bacteria, but the yeast phase of Histoplasma capsulatum activates platelets when both fibrinogen and immunoglobulin G are present (7).

Several Candida species adhere to preformed fibrin-platelet clots by virtue of a unique mannan on the yeast cell wall (14). This apparently does not involve fibrinogen or does so by a different mechanism than S. pyogenes since complement is required. The studies with Candida are of interest since vegetations on heart valves involved in Candida endocarditis form a matrix of yeast, fibrin, and erythrocytes. In addition, the yeast stimulated platelets released a heat-stable,

![Graph](image-url)
trypsin-sensitive, heparin-binding molecule which stimulates germ tube formation and yeast growth (23).

Although several microorganisms or their products are known to stimulate the platelet release reaction, little effort has been made to identify the biochemical pathway within the platelets which causes them to discharge their granules after a microbial stimulus. Only with *H. capsulatum* and *L. monocytogenes* has evidence about the contribution of the prostaglandin system been obtained relative to the platelet reaction. The *Listeria* system releases serotonin without platelet lysis, despite the need for a heat-labile serum factor (6). This system is resistant to inhibition by the cyclooxygenase inhibitors indomethacin and aspirin. In contrast, the fibrinogen-dependent platelet release reaction caused by *H. capsulatum* is blocked by indomethacin. The failure of platelets to aggregate in the presence of *S. pyogenes* when co incubated with ASA or quinacrine confirms that cyclooxygenation of arachidonic acid is necessary for the platelet reaction (10, 17).

ACKNOWLEDGMENT

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LITERATURE CITED