Inhibition of Endotoxic Lipopolysaccharide-mediated Platelet Aggregation by Cobra Venom Anticomplementary Factor

By Jack S. C. Fong, James G. White, and Robert A. Good

Aggregometry studies on endotoxic lipopolysaccharide (LPS)-mediated rabbit platelet aggregation were performed. Different preparations of LPS showed characteristic aggregometry profiles, and LPS with potent anticomplementary activities generally had a more vigorous platelet aggregation function than did LPS preparations with lesser anticomplementary functions. Cobra venom anticomplementary factor (CVF) inhibited LPS-platelet interaction, and the inhibition was both time and dose dependent. Dose-response curves of CVF inhibition on LPS or zymosan-mediated platelet aggregation were essentially identical. In vitro and in vivo studies showed that CVF inhibition persisted even when hemolytic complement activities reached more than 70% of those originally present. At the critical time of days 5 or 6 following CVF administration, the lack of platelet responses towards LPS could be restored by addition of fresh plasma from normal or C6-deficient rabbits, but not with plasma that had been treated with antigen-antibody complexes, zymosan, or heating at 56°C for 30 min. The experimental data indicate that serum protein(s) other than the terminal complement components are involved in LPS-platelet interaction. It seems most likely that the factor(s) perturbed reside in the mechanisms involved in activation of the alternate pathway. Furthermore, it appears quite possible that LPS-platelet interactions can be inhibited by manipulating the humoral factor(s) involved rather than by altering the platelets themselves.

AN INFLUENCE OF immunologic mechanisms on circulating blood platelets was suggested by the work of Achard and Aynaud when injections of antigens into sensitized animals were followed by thrombocytopenia. Their findings were supported by the demonstration of in vitro aggregation of platelets exposed to antigen antibody complexes, particular antigen-antibody complexes, homocytotropic antibody and antigens, or heterologous antiplatelet antibodies. Participation of the complement system in immune aggregation of platelets is well established.

The action of endotoxic lipopolysaccharide (LPS) was considered similar to that of antigen-antibody complexes in triggering platelet aggregation and release. LPS, a particulate substance, was expected to mediate platelet aggregation in the presence of complement components, the minimal requirement being C1–C5. Thus, the use of cobra venom anticomplementary factor
(CVF), which activated the terminal complement components C3-C9 (C3-t), rendering them unavailable, could regularly inhibit platelet aggregation triggered by antigen-antibody complexes or LPS. When LPS was injected parenterally into a rabbit, platelet levels fell precipitously. However, a platelet-sparing phenomenon was observed if the rabbits were treated with CVF prior to LPS administration.

Published literature appeared to support the concept that C3 unavailability caused by CVF was responsible for inhibition of LPS-platelet interaction. However, the instantaneous inhibition of LPS-platelet interaction conferred by CVF raised doubts about this concept because a significant quantity of complement components would still be available at the time of inhibition. Furthermore, only a limited amount of divalent cation was available in citrated plasma to support CVF activity which is dependent on divalent cation. In the present study the mechanisms of CVF inhibition on LPS-platelet interaction have been further evaluated. Results indicate that a factor or factors other than components of complement are also involved in platelet-LPS interaction.

MATERIALS AND METHODS

Animals

Male and female albino rabbits, weighing about 2 kg were used. Rabbits congenitally deficient in the sixth component of complement were raised locally from the Freiburg strain. These animals had free access to water and Purina rabbit pellets throughout the experiments.

Blood

Samples of blood were collected from punctures of the rabbit ear artery directly into polyethylene tubes containing 1:9 volumes of 0.1 M citrate buffer at pH 6.5 and containing 2.5%, (w/v) dextrose.

Platelet-poor Plasma

Platelet-poor plasma (PPP) was prepared by centrifugation of the blood at 1000 g for 20 min at 4°C.

Platelet-rich Plasma

Platelet-rich plasma (PRP) was obtained by centrifugation of the blood at 300 g for 20 min at room temperature. Plastic pipettes were used for transfer of plasma.

Platelet Aggregometry

A Chrono-log aggregometer coupled to a Bausch and Lomb VOM-5 recorder was used in the manner previously described. Briefly, 0.45 ml of PRP was added with a stirring rod to a cuvette and placed in the machine. Light transmission was adjusted to 10%; similarly, that for PPP was adjusted to 90%. Aggregating agents or CVF were added in 50-μl volumes. Platelet aggregation resulted in an increased light transmission and upward deflection of the recording pen.

Aggregating Agents

Collagen, ADP, and zymosan were purchased from Sigma Chemical Co., St. Louis, Mo. Collagen suspension was prepared as previously described. ADP was dissolved in normal saline to give a final concentration of 1 x 10⁻³ M. Zymosan was suspended in normal saline, boiled for 30 min, washed twice, and resuspended in saline at a final concentration of 1.75 mg/ml. CVF
was prepared from lyophilized cobra venom Naja haje as reported earlier. The CVF preparation used in these experiments was found to have 40 U of anticomplementary activity per ml. (One unit of anticomplementary activity is defined as the reciprocal of the dilution of the CVF preparation, 0.1 ml volumes of which reduced the hemolytic activities of 0.4 ml of a 1:20 dilution of normal human serum by 50% after an incubation period of 20 min at 37°C.)

Endotoxin

*Escherichia coli* LPS was prepared according to the method of Boivin et al. Additional LPS preparations were purchased from Difco Laboratories Detroit, Mich. These included *Salmonella typhosa* 0901 (c.555392), *Salmonella typhimurium* (c.551946), *Serratia marcescens* (c.535276), and *Escherichia coli* 0111:B4 (c553685).

Hemolytic Complement Assays

The preparation of reagents and cellular intermediates for total, Cl, and C3t hemolytic complement assays have been described previously.

LPS-Mediated Anticomplementary and Platelet Aggregating Activities

The anticomplementary activities of different LPS preparations were studied by incubating 100 µg of LPS with 0.1 ml of guinea pig serum at 37°C for 30 min. CH50 values of these samples, as well as controls that had no LPS added, were determined. Anticomplementary activities were expressed as the percentage inhibition on the total available hemolytic activities in 0.1 ml of guinea pig serum (43 CH50 U). Aggregometry studies were performed with these LPS preparations at 10 mg/ml.

CVF Inhibition of LPS-platelet Interaction

Two aspects of CVF inhibition were studied: (1) concentration of CVF required and (2) duration of incubation. First, CVF was serially diluted, added to PRP in 50-µl volumes, and incubated at 37°C for 10 min. Fifty microliters of LPS *S. marcescens* were then added immediately after placing the sample on the platelet aggregometer. Second, 50 µl of CVF at 2.5 U/ml were mixed with 0.45 ml of PRP at 37°C in the aggregometer cuvette. At different time intervals 50 µl of LPS *S. marcescens* were added and changes in light transmission recorded.

CVF Inhibition of Platelet Aggregating Agents

CVF at 40 U/ml was serially diluted with normal saline. For each aggregometry study, 50 µl of these CVF dilutions were mixed with 0.45 ml of rabbit PRP and incubated at 37°C for 10 min before 50 µl of an aggregating agent was added. These agents were LPS *E. coli*, ADP, collagen, and zymosan as described under the section for aggregating agents.

In Vivo Studies of CVF Inhibition on LPS-platelet Interaction

Four albino rabbits weighing about 2 kg each were injected intravenously with CVF at 100 U/kg at zero time. Blood samples were obtained prior to, immediately following, and daily after the injection for platelet aggregometry and hemolytic complement studies. For every sample, four aggregating agents, ADP, collagen, LPS *E. coli*, and LPS *S. marcescens*, were used to evaluate platelet functions. Furthermore, neutralization of CVF inhibition on LPS-platelet interaction was regularly tested by the addition of 50 µl of fresh plasma prior to the introduction of LPS.
RESULTS

LPS Anticomplementary and Platelet Aggregation Activities

These studies evaluated further the correlation of LPS anticomplementary and platelet aggregation activities. Platelet aggregometry results with five types of LPS are presented in Fig. 1. It was apparent that all preparations of LPS studied were capable of reacting with rabbit platelets. However, kinetics of the LPS-platelet interactions differed slightly for each type of LPS used. A few parameters were chosen to further characterize kinetics of these interactions: (1) lag period, which measured the interval of time between the addition of LPS to PRP and the first upward deflection of the tracing; (2) slope, which measured the maximal change in upward deflection over a 15-sec period; (3) delta-T, which represented the maximum change of light transmission secondary to platelet aggregation; and (4) reaction period, which measured the time interval between the addition of LPS to PRP and the plateauing of platelet aggregation. These data, as well as those concerning anticomplementary activities of LPS, are presented in Table I. No simple correlation between LPS anticomplementary and platelet-aggregation activities could be formulated when individual kinetics of aggregometry were considered. However, if aggregometry was considered as a whole, a vigorous LPS-platelet interaction appeared to associate with a potent anticomplementary activity.

CVF Inhibition of LPS-platelet Interaction

While all five preparations of LPS studied were capable of causing platelet aggregation, CVF was effective in inhibiting all these LPS-platelet interactions. A dose-response curve reflecting inhibition by CVF of *S. marcescens* LPS-induced platelet aggregation is presented in Fig. 2. All PRP samples were preincubated with different dilutions of CVF at 37°C for 10 min before the LPS was added. When the amount of CVF was equal to or greater than 0.5 U in a
Table 1. Kinetic Analysis of Platelet Aggregation Mediated by Endotoxin

<table>
<thead>
<tr>
<th>Endotoxin</th>
<th>Log*</th>
<th>Slope*</th>
<th>Delta-T*</th>
<th>Reaction Period*</th>
<th>Anti-C Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. typhosa</td>
<td>3.50</td>
<td>6.0</td>
<td>58</td>
<td>9.25</td>
<td>87</td>
</tr>
<tr>
<td>S. marcescens</td>
<td>1.25</td>
<td>11.5</td>
<td>42</td>
<td>4.00</td>
<td>85</td>
</tr>
<tr>
<td>S. typhimurium</td>
<td>5.00</td>
<td>1.0</td>
<td>12</td>
<td>9.50</td>
<td>32</td>
</tr>
<tr>
<td>E. coli</td>
<td>1.00</td>
<td>2.0</td>
<td>23</td>
<td>5.00</td>
<td>90</td>
</tr>
<tr>
<td>E. coli 0111:B4</td>
<td>3.00</td>
<td>0.5</td>
<td>9</td>
<td>9.50</td>
<td>14</td>
</tr>
</tbody>
</table>

*Log, time interval in minutes between the addition of LPS to PRP and the first upward deflection of the tracing; slope, maximal change in percent of light transmission over a 15-sec period; delta-T, maximal change in percent of light transmission as a result of platelet aggregation; reaction period, time interval in minutes between the addition of LPS to PRP and the plateauing of the tracing; anti-C activity, percentage of CH50 consumed in 0.1 ml of guinea pig serum by 100 µg of endotoxin following an incubation of 30 min at 37°C.

Fig. 2. Inhibition of endotoxin-mediated platelet aggregation by CVF of (1) 0.0625, (2) 0.125, (3) 0.250, and (4) 0.500 U. Mixtures of 0.45 ml of PRP and 50 µl of CVF were incubated at 37°C for 10 min before 50 µl of endotoxin S. marcescens at 10 mg/ml were added.

Fig. 3. Inhibition of endotoxin-mediated platelet aggregation by 0.125 U of CVF. Mixtures of PRP and CVF were incubated at 37°C for (1) 15 sec, (2) 2 min, (3) 4 min, and (4) 12 min prior to the addition of 50 µl of endotoxin S. marcescens at 10 mg/ml.
total volume of 0.55 ml, complete inhibition of LPS-platelet interaction was observed. Inhibition was noted when the CVF present was between 0.25 and 0.125 U. However, with CVF at 0.0625 U or less, no inhibition of the interaction of LPS and platelet could be recorded. A dose-dependent inhibition of LPS-platelet interaction by CVF was clearly demonstrated. With a constant amount of CVF, 0.125 U, inhibition on LPS-platelet aggregation as a function of time was studied. These results, presented in Fig. 3, showed that the degree of inhibition was directly proportional to the length of CVF and PRP incubation. Thus, a dose-, as well as time-, dependent inhibition by CVF of LPS-platelet interaction was demonstrated. With the use of larger amount of CVF, the incubation time required for inhibition could be dramatically reduced.

**CVF Anticomplementary Activities in Rabbit Plasma and Serum**

The quantity of CVF used in these experiments was designed to correspond to the amount necessary for inhibition of LPS-platelet interaction. Thus, a final concentration of CVF in the mixture of 2 U/ml was selected. Results of these studies are presented in Fig. 4 as percentages of residual complement hemolytic activities against time. It was apparent that a significant quantity of hemolytic complement activities was still present after 20 min of incubation when the LPS-platelet interaction had been fully inhibited. Furthermore, the drop in hemolytic complement activity produced by CVF in both citrated plasma and in serum was similar, indicating that citrate had little influence on the anticomplementary action of CVF.

**CVF Inhibition on Platelet-Aggregating Agents**

Following platelet and LPS interaction, aggregation was observed. The extent of aggregation was reflected by a net change of light transmission. Thus, the altered magnitude of the change in light transmission could be used as an index of inhibition when experimental and control tracings were compared.
Results of studies dealing with CVF inhibition on platelet-aggregating agents are presented in Fig. 5, where inhibition of aggregation was calculated as a difference between percentages of control (100%) and experimental aggregations. CVF did not inhibit aggregation induced by ADP, produced some inhibition on the influence of collagen, and produced a marked inhibition on both zymosan- and LPS-mediated aggregations.

**In Vivo Inhibition of LPS-platelet Interaction by CVF**

Representative tracings from studies on in vivo inhibition of LPS-platelet interactions by CVF are presented in Fig. 6. Prior to the injection of CVF, PRP from these rabbits reacted normally to LPS as depicted in tracing A. However, following CVF administration and for as long as 5–6 days afterwards, PRP samples from these animals were no longer reactive to LPS, although they had perfectly normal response to collagen and ADP (Fig. 6C and 6D). After 6 or 7 days following CVF treatment, partial interaction between platelet and LPS was observed (Fig. 6B), and from day 7 onwards PRP responses were similar to those of preinjection studies (Fig. 6A). Data on total, C1, and C3t hemolytic complement determinations are presented in Fig. 7 with percentage of activities plotted against time in days. The 100% total, C1, and C3t hemolytic activities, calculated as mean levels at day zero were 55, 850, and 340 CH50 U, respectively. It is apparent from these data that, even in the presence of 40% of C3t, platelets were still unresponsive to LPS. Furthermore, partial responses were observed when C3t level reached 75% of normal. Following the injection of CVF, it was possible to demonstrate the presence of circulating anticomplementary activities and to transfer inhibition activities on LPS-platelet interaction. However, these activities gradually diminished, so that by days 5 and 6 no anticomplementary activity or capacity to transfer inhibition could be
Fig. 6. Endotoxin S. marcessens-mediated platelet aggregation with PRP from rabbits that are normal or fully recovered (A), partially recovered (B), or exhibiting effects (C, D) from CVF treatment.

Fig. 7. Total hemolytic complement (---), Cl (-----), and C3t (-----) activities of rabbits injected with 100 U of CVF per kilogram of body weight at zero time.
Table 2. Restoration of Endotoxin-Mediated platelet aggregation in PRP from CVF-treated Rabbits by Various Plasma Preparations

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Restoration of LPS-platelet Interaction</th>
<th>CH50 Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>Normal</td>
<td>+</td>
<td>58</td>
</tr>
<tr>
<td>C6-deficient</td>
<td>+</td>
<td>0</td>
</tr>
<tr>
<td>56°C 30’</td>
<td>–</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Zymosan*</td>
<td>–</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Ag-Ab*</td>
<td>–</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

* Rabbit plasma and zymosan (20 mg/ml) or bovine serum albumin-rabbit antibovine serum albumin (2 mg/ml) were incubated at 37°C for 30 min. The anticomplementary agents were subsequently removed by centrifugation.

...demonstrated. At the same time, the lack of response towards LPS by PRP from rabbits treated with CVF could be corrected with in vitro addition of normal plasma.

Further studies were carried out with PRP from rabbits at days 5 or 6 following CVF administration. To 450 µl of PRP, 100–200 µl of different plasma preparations were added, followed with 50 µl of LPS S. marcescens. The aggregometry studies are presented in Table 2. Plasmas from both normal and C6-deficient rabbits restored the LPS-platelet interaction.

**DISCUSSION**

Thrombocytopenia is a prominent feature in both clinical sepsis and experimental endotoxemia and is readily explained by in vitro demonstration that platelets aggregate in the presence of LPS. Such an interaction is believed to play a major role in the initiation of disseminated intravascular coagulation exemplified by the experimental model of generalized Shwartzman reaction where, following LPS administration, parallel development of thrombocytopenia, consumptive coagulopathy, and deposits of fibrin in glomerular and other vessels have been observed. The mode of LPS-platelet interaction resembles the influence of antigen–antibody complexes on platelet aggregation, platelet release, and consumption of complement components. A heat labile factor necessary for the LPS-platelet interactions has been described in earlier reports. The present study has examined the LPS-platelet interaction and its inhibition by CVF. That CVF inhibits platelet aggregation mediated by LPS is fully confirmed by the present study. Furthermore, indirect evidence that C3 proactivator (C3PA) and/or other protein(s) depleted by CVF probably accounts for the inhibition of LPS-platelet interaction is also provided. Serum protein(s) other than the terminal complement components are involved in LPS-platelet interaction.

It has been established that CVF interacts with a serum protein C3PA in the presence of ionized magnesium ions to form a complex which consumes the classic terminal complement components. The interactions are temperature and time dependent. Kinetic studies on hemolytic complement consumption by CVF, at a dose that inhibited LPS-platelet interaction, showed that even after 20 min of incubation, more than 60% of the hemolytic complement activities...
were still available. Furthermore, in vivo experiments with recovery of total or C3t hemolytic complement activities up to 40% were not associated with the return of platelet responses to LPS. However, when a small amount of fresh plasma which would not bring more than 10% of the original hemolytic activities was added, a prompt restoration of the platelet responses to LPS occurred. The data suggest that protein factor(s) other than components of the classic complement system are responsible for the CVF inhibition. Consumption of C3PA by CVF and its lack of availability for the reaction seems the mechanism most likely responsible.

Direct proof of this hypothesis must await purification and isolation of rabbit C3PA, but other results in our report provide support for this concept. First, experiments on restoration of platelet responses by different plasma fractions suggested that the factor(s) responsible was heat labile, consumed by antigen–antibody complexes, zymosan, and provided by normal or C6-deficient plasmas. Second, the inhibition conferred by CVF on LPS-platelet interaction was shown to be both time and dose dependent. This mechanism is compatible with an enzymatic process or, more specifically, with the activation of C3PA and consumption of C3t. Finally, dose-response curves of CVF inhibition on LPS- and zymosan-mediated platelet aggregation are essentially identical. The latter is known to activate the complement system through the alternate pathway and react with platelets via the complement system.424

When different preparations of LPS were used for our aggregometry studies, it was apparent that each sample had its own characteristic effect on platelet aggregation. When lag period, slope, and change of light transmission of the tracings were considered individually, no simple correlation could be drawn between any one of them and anticomplementary activities of the LPS. However, when these parameters were considered as a whole, LPS with greater anticomplementary activities did appear to have a more potent influence on platelet aggregation than did the LPS preparations with lesser anticomplementary action. These findings may explain, at least in part, the extreme diversities of biologic activities of different preparations of LPS that have been observed by many investigators.25

Irrespective of the type of LPS used, however, platelet aggregating activities can be inhibited by CVF. The results of the present study support our previous finding that platelets were spared in CVF treated rabbits when challenged with LPS.9 In contrast to other means of inhibiting platelet aggregation that necessitate an intrinsic alteration of platelet physiology,26 manipulation of LPS-platelet interaction can be implemented by changing humoral factor(s) without disturbing basic platelet function. Thus, platelets obtained from CVF-treated rabbits react to collagen and ADP in a consistently normal manner. The observation that LPS- and antigen–antibody-induced platelet aggregation is mediated by humoral factor(s) suggests the possibility of inhibiting immunity-mediated coagulopathy without tampering with the functions of the platelets themselves.

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REFERENCES

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