The Effect of Cobra Venom Factor on Hemostasis in Guinea Pigs

By W. Jean Dodds and Richard J. Pickering

Recent investigations have indicated an association between the coagulation and complement systems. Our present studies, designed to explore this association further, demonstrated that a purified anticomplementary fraction of cobra venom, cobra venom factor (CVF), produced significant changes in hemostasis when injected into guinea pigs. Some of the effects described may be secondary to the transient intravascular hemolysis produced by CVF. However, a more direct effect of purified CVF on platelet function is suggested by increased platelet factor 3 activity and inhibition of clot retraction and lysis. Furthermore, injection of purified CVF produced striking elevation of plasma fibrinogen without detectable fibrin or fibrinogen degradation products, altered plasminogen levels, and significantly reduced the activities of several extrinsic and intrinsic coagulation factors. These observations provide additional data to support the concept of a functional relationship between the mechanisms that initiate coagulation and activate complement.

INVESTIGATORS HAVE BEEN INTERESTED for some time in the possible interrelationships among platelets, blood coagulation, and the complement system. It is known, for example, that Hageman factor is involved through plasmin in the activation of the first component of complement, and that antigen-antibody mediated release of vasoactive amines from rabbit platelets requires complement. Recently, a defect of intrinsic coagulation was described in congenitally C6-deficient rabbits. Serum complement levels can be depleted in vivo by injection of material purified from cobra venom. This anticomplementary action of cobra venom factor (CVF) has been widely used to study the mechanisms of complement-related immunologic disease in experimental animals. Our studies examined the effect of purified CVF on platelet function and blood coagulation in guinea pigs.

MATERIALS AND METHODS

Venom from the cobra, Naja haja, was partially purified by DEAE-chromatography and then electrophoresed on polyacrylamide gels, as described previously. Multiple gels were cut into 1.5-mm sections and the active material was eluted. This highly purified material showed a single band when re-electrophoresed on polyacrylamide gel, contained the complement (C) inactivating property, and induced red blood cell lysis by fresh guinea pig serum. There was no detectable phospholipase or caseinolytic activity in either the DEAE or highly purified cobra venom fractions, and neither material lysed heated or unheated fibrin plates.
The guinea pigs used throughout our studies were from an inbred strain (Hartley), 4–5 mo old, and weighed 400–500 g; all the experiments were performed during a 3-wk period. Twenty control guinea pigs, ten of each sex, were injected intraperitoneally with 3 ml of saline, and used to establish baseline values for platelet function and coagulation tests. Twenty additional guinea pigs were injected intraperitoneally with an equal volume containing approximately 8400 C inactivating units* of DEAE-purified CVF, and were sacrificed in pairs, one of each sex, at 2, 4, 6, 8, 11, 14, 21, 24, 36, and 48 hr. The same experiments were repeated for the 2–14 hr period with another group of 12 guinea pigs that were sacrificed in pairs, one of each sex, after injection with approximately 8400 units of highly purified CVF material. In all injected animals, hemolytic complement levels were reduced to 10–15% of normal.

Control of CVF-injected guinea pigs were anesthetized by intraperitoneal injection of 60 mg of sodium pentobarbitol. The abdomens were opened and blood samples (8 ml) were drawn directly from the posterior vena cava into anticoagulant-filled syringes. Another sample (0.5 ml) was collected from the portal vein into a syringe containing 4.5 ml of cold buffered saline and used for clot retraction and lysis tests described below. Blood samples for serum were put into glass tubes, incubated 1 hr at 37°C, and centrifuged; serum was removed and recentrifuged to remove any red cells. Coagulation assays were performed on citrated plasma samples (1 part 3.8% trisodium citrate to 9 parts whole blood). Platelet-rich plasma containing 450,000–600,000 platelets/cu mm, was prepared by centrifugation for 2 min at 1500 rpm, and kept at room temperature; contamination with white blood

*The reciprocal of the dilution of CVF which will inhibit 50% of the hemolytic activity of 0.5 ml of a 1:2 dilution of guinea pig serum following an incubation at 37°C for 60 min.

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Males</th>
<th>Females</th>
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<tbody>
<tr>
<td></td>
<td>Mean ±2 SD</td>
<td>Mean ±2 SD</td>
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<tr>
<td>Hct (%/o)</td>
<td>40.7 ± 2.5</td>
<td>41.0 ± 2.8</td>
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<tr>
<td>Hb (g/100 ml)</td>
<td>12.3 ± 1.1</td>
<td>12.3 ± 0.8</td>
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<tr>
<td>WBC/cu mm</td>
<td>3487 ± 1312</td>
<td>2986 ± 1124</td>
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<tr>
<td>Platelets/cu mm</td>
<td>354,100 ± 110,528</td>
<td>419,400 ± 103,629</td>
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<tr>
<td>Clot retraction (% at 2 hr)</td>
<td>93 ± 10</td>
<td>98 ± 8</td>
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<tr>
<td>Clot lysis (hr)</td>
<td>11.6 ± 3.2</td>
<td>5.4 ± 2.2</td>
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<tr>
<td>PF3 (sec)—kaolin method</td>
<td>51.3 ± 3.1</td>
<td>53.2 ± 4.5</td>
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<tr>
<td>PF3 (sec)—Stypven method</td>
<td>23.7 ± 1.3</td>
<td>21.3 ± 1.7</td>
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<tr>
<td>Prothrombin consumption (sec)</td>
<td>128 ± 22</td>
<td>108 ± 16</td>
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<tr>
<td>PTT (sec)†</td>
<td>37.3 ± 3.6</td>
<td>38.0 ± 5.7</td>
</tr>
<tr>
<td>OSPT (sec)‡</td>
<td>31.1 ± 2.0</td>
<td>30.7 ± 2.5</td>
</tr>
<tr>
<td>Fibrinogen (mg/100 ml)</td>
<td>383 ± 82</td>
<td>370 ± 100</td>
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<tr>
<td>Plasminogen (CTA U/ml)</td>
<td>1.9 ± 0.6</td>
<td>3.3 ± 0.7</td>
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For all coagulation factors, standard curves were determined for each animal. The mean standard for each sex was arbitrarily assigned a value of 100%. The range was obtained by comparing each curve with the mean standard curve.

* Ten animals of each sex (see Materials and Methods).
† With purified human brain phospholipid.
‡ With human brain thromboplastin.
§ For all coagulation factors, standard curves were determined for each animal. The mean standard for each sex was arbitrarily assigned a value of 100%. The range was obtained by comparing each curve with the mean standard curve.
cells was minimal. Platelet-poor plasma, containing < 10,000 platelets/cu mm, was prepared by centrifugation at 5000 rpm for 30 min, and stored in ice. All samples were processed and stored in polycarbonate test tubes; no hemolysis was detected in any of the plasma samples from control guinea pigs. With the exception of assays for individual coagulation factors, all tests were determined immediately on fresh plasma. Plasma for specific coagulation assays was frozen at —90°C; assays were performed on 5 consecutive days after the samples had been frozen 6–8 wk. Partial thromboplastin times (PTT), one-stage prothrombin times (OSPT), prothrombin consumption times, thrombin times, and specific assays for factors V, VII, VIII, IX, X, and XI were performed as described previously.\(^7\) Factor VII was assayed with deficient canine plasma;\(^7\) factor VIII, IX, and XI assays used deficient canine plasmas in a one-stage PTT test.\(^8\) Other coagulation factors were assayed with deficient human plasmas, as no deficient canine substrates for these tests are available. Purified human brain phospholipid and human brain thromboplastin prepared in our laboratory were used in all coagulation assays.

Specific coagulation factor assays are reported as the per cent of activity compared with the reference standard of pooled plasma which was assigned a value of 100%, from the 20 control guinea pigs.

Platelet factor 3 (PF3) activity was measured in platelet-rich plasma by kaolin\(^10\) and Stypven assays.\(^11\) Clot retraction and lysis were determined by the dilute whole blood technique of Fearnley et al.\(^12\) and Taylor and Müller-Eberhard.\(^13\)

Quantitative fibrinogen measurements were performed with 0.5 ml of plasma according to the method of Ratnoff and Menzie\(^14\) as modified by Nersky et al.\(^15\) The staphylococcal clumping test of Hawiger et al.\(^16\) was used to assay serum samples for fibrinogen and fibrin degradation products (FDP).

Quantitative plasminogen measurements were performed by the caseinolytic method of Johnson et al.\(^17\) after removal of antiplasmin and conversion of plasminogen to plasmin with urokinase.\(^17\)

**RESULTS**

Table 1 shows results obtained for hemograms, platelet function, and coagulation tests of 20 control guinea pigs, 10 of each sex. There was little difference between values obtained for normal males and females with the exception of clot lysis times and plasminogen levels.

Figure 1 shows data for blood counts and degrees of hemolysis in samples from guinea pigs injected with CVF fractions. The calculated limits of 2 SD above and below the mean are shown for the 20 control animals injected with an equal volume of saline. The injection of venom appeared to produce hemoco-agulation accompanied by marked leukocytosis and in vivo hemolysis. Platelet counts were not significantly altered except at 21 hr. Although hemolysis with hemoglobinuria seemed to be a consistent finding 6–14 hr after venom injection, it occurred only sporadically before and after this period. Data for the 24–48 hr period after injection of DEAE-purified CVF have not been included in Figs. 1–4 of this paper. Results during this period showed significant reduction in hematocrit, pronounced leukocytosis, normal platelet counts, and some intravascular hemolysis without hemoglobinuria.

The effect of CVF injection on platelet functions is shown in Fig. 2. CVF significantly inhibited both clot retraction and clot lysis. The delay of clot retraction and lysis generally accompanied the period of maximum hemolysis. PF3 activity was increased from as early as 2 hr, during the period of maximum hemolysis, and continued to be elevated from 24–48 hr. Clot retraction and lysis, however, had returned to normal during the 24–48 hr period.
Fig. 1. Injection of partially purified (DEAE) and highly purified (polyacrylamide) fractions of cobra venom into guinea pigs. DEAE venom is shown by hatched bars, open symbols, and dotted lines; polyacrylamide venom by solid bars, symbols, and interrupted lines. The limits of two standard deviations (95% confidence limits) above and below the mean for 20 control animals, 10 of each sex are shown (see Table 1). Four different animals were studied at each interval, two (one of each sex) with each venom preparation. The data points represent the average of the pairs at each interval; the range is included for all data points that fall beyond the defined limits for normal animals.

Figure 3 demonstrates prolongation of the OSPT and reduction of factor VII and X activities in venom-injected animals. Coagulation assays are expressed as the per cent activity compared with that of the standard pool of 20 control animals; the normal range of activity of these controls is also included. Prothrombin consumption was abnormal in CVF-treated animals at 6 hr but tended to rebound at 8 hr. Although the factor VII and X activities and prothrombin consumption were normal from 24–48 hr after purified CVF injection, the OSPT remained significantly prolonged.

Data from assays of intrinsic system coagulation factors are shown in Fig. 4. It is clear that CVF prolonged the PTT and significantly reduced plasma factor
Fig. 2. Platelet function tests on samples from the experiments described in Fig. 1.

VIII, IX, and XI activities. Factor VIII and IX activities appeared to increase during the first 2–4 hr after venom injection, then were markedly reduced at 6 hr as hemolysis began. Factor V activity, not shown here, was also reduced at 6 hr. The PTT continued within the normal range for the 24–48 hr interval after purified CVF treatment, but factor VIII and IX levels were significantly depressed. Factor XI activity, on the other hand, appeared to rebound above the normal limits at 36 and 48 hr.

Figure 5 shows results of quantitative fibrinogen determinations in venom injected animals as compared with those of normal controls. A marked increase in fibrinogen levels occurred with peak values at 36 hr. These findings are consistent with the acute-phase hyperfibrinogenemia that frequently accompanies severe physiologic injury in man and experimental animals. In addition to fibrinogen measurements, thrombin times performed on CVF-injected animals were within the normal range. Assay of the serum for FDP at 24, 36, and 48 hr revealed only a trace reaction in undiluted samples and no reaction at a 1:2 dilution.

Plasminogen levels varied considerably between male and female control
Fig. 3. Serum prothrombin consumption and extrinsic system coagulation assays on samples from experiments described in Fig. 1. Coagulation assays are expressed as per cent activity compared with that of the mean standard curve obtained for the 20 control animals. The range of activity from these 20 controls is shown.

and CVF-injected guinea pigs (Table 1 and Fig. 5). These findings suggest a possible hormonal influence on plasminogen production. In CVF-treated female animals, plasminogen levels were markedly reduced during the first 12 hr but returned to the upper limits of normal at 24-48 hr. By contrast, CVF-injected male animals had no change in plasminogen until 24 and 36 hr when levels were significantly elevated.

It should be emphasized that some of the animals injected with CVF had platelet function and coagulation changes without obvious hemolysis, and most parameters had returned to normal by the 14-21 hr period in which hemolysis was no longer apparent. We attempted to simulate conditions of
CVF-induced intravascular hemolysis by intraperitoneal injection of guinea pig hemolysate (including red cell stroma). Studies of platelet function and coagulation were performed on a group of six test guinea pigs and six saline-injected controls. The only changes noted were a slight reduction in intrinsic blood coagulation activities with moderate reduction in prothrombin complex activity. No change in hemogram, fibrinogen, PF3, clot retraction, or lysis was observed; factor X activity was also normal.

Several guinea pigs injected with purified CVF showed small petechial and ecchymotic hemorrhages in the kidneys, liver, bladder, brain, and gastrointestinal tract. These lesions were most evident in animals sacrificed between 6–12 hr but some were present in animals sacrificed at 24–48 hr. Histologic sections of affected organs revealed focal hemorrhages with some hemoglobin
cast casts, and small areas of acute inflammatory change with necrosis; there was no evidence of thrombosis in these lesions.

DISCUSSION

Significant hemolytic complement depletion was detectable as early as 4 hr after intraperitoneal injection of partially or highly purified CVF and reached 10% or less of normal levels within 24 hr. There was no significant difference between the data obtained in experiments with either of these purified fractions...
of CVF. Intravascular hemolysis and hemoglobinuria were observed between the 4th and 14th hr following administration of purified CVF and were rarely seen after 14 hr. Some animals had small focal hemorrhages of the visceral organs when they were sacrificed. Within 10 hr of purified CVF injection, there was a pronounced leukocytosis and some animals showed a tendency to hemocoagulation as indicated by a transient rise in hematocrit (Fig. 1). Neutrophilia following injection of CVF into rabbits and rats has been observed by Cochrane et al.\textsuperscript{18}

Our studies showed a significant prolongation of the OSPT and PTT, beginning 4 to 6 hr following purified CVF administration. The prolonged OSPT was observed throughout the period of study, whereas the abnormal PTT continued only for as long as the 8th to 14th hr. The delay in measurable alteration in OSPT and PTT may explain why previous investigators\textsuperscript{18} did not observe changes in these parameters following CVF administration. Earlier studies with whole cobra venom demonstrated an inhibitory effect on certain blood coagulation parameters, particularly factor IX.\textsuperscript{22} However, these experiments cannot be compared with our study or that of Cochrane et al.\textsuperscript{18} because we utilized CVF, a purified material isolated from cobra venom for its specific reactivity with the complement system.

Striking elevations of fibrinogen occurred in our CVF-injected guinea pigs between 24 and 48 hr following injection (Fig. 5). However, we could not demonstrate significant amounts of FDP in these animals. Similar elevations in fibrinogen levels without FDP were reported by Fong and Good\textsuperscript{19} after injection of CVF into rabbits. Cochrane et al.\textsuperscript{18} noted a slight increase in fibrinogen in one CVF-treated rabbit but reduced fibrinogen levels in four guinea pigs following an intravenous injection of CVF. The changes in plasminogen levels in CVF-injected male and female animals are not understood. In addition, there appeared to be no logical relationship between data obtained from plasminogen, fibrinogen, FDP, and clot lysis measurements.

Our data, shown in Figs. 3 and 4, indicate that both extrinsic (factors VII and X) and intrinsic (factors VIII, IX, X, and XI) system coagulation activities were significantly affected by purified CVF injection. The initial increase in plasma factor VIII and IX activities, followed by a rapid decrease in these activities for the next 2 to 6 hr, suggests a direct activation by CVF with subsequent consumption or removal of activated coagulation factors by the reticuloendothelial system (RES). The role of RES in the clearance of activated coagulation factors has been demonstrated previously by Rabiner and Friedman\textsuperscript{20} and by Deykin et al.\textsuperscript{21} In addition, many substances, such as tissue thromboplastin, thrombin, trypsin, Russell's viper and other snake venoms, endotoxin, and hemolysates, are known to activate the coagulation mechanism when injected into experimental animals\textsuperscript{20,22}

The ability of CVF to inhibit retraction and lysis of dilute blood clots in vitro has been described previously by Taylor and Müller-Eberhard.\textsuperscript{19} We have recently confirmed their observations using blood from humans, rabbits, dogs, and guinea pigs incubated with purified CVF.\textsuperscript{23} Data from the in vivo experiments reported here (Fig. 2) demonstrated a similar inhibition of clot retraction
and lysis in blood drawn from CVF-injected guinea pigs. Among the most interesting observations in these studies has been the apparent direct effect of purified CVF on blood platelets in vivo. Although the platelet count was not significantly altered (Fig. 1), PF3 activity was increased after venom injection (Fig. 2). Correspondingly, in vitro experiments with guinea pig platelets have shown that purified CVF produces platelet aggregation followed by release of PF3, platelet factor 4, and nucleotides. On the basis of these observations, it seems reasonable to propose that the increase in plasma PF3 activity in animals following parenteral administration of purified CVF reflects the in vivo release of platelet thromboplastic materials. This effect on platelets may be brought about by a mechanism similar to that which gives rise in vitro and in vivo to CVF-induced erythrocyte membrane injury with resulting hemolysis. In addition, although we could not reproduce CVF-induced changes by injecting hemolysate, we cannot ignore the contribution of thromboplastic materials released from red cells that are also known to activate coagulation both in vitro and in vivo. However, whether the process whereby purified CVF induces the release of red cell and platelet contents is related to the effect of CVF on the complement system remains to be determined.

The marked delay of clot lysis produced by purified CVF may be related to a direct inhibition of fibrinolysis, as well as to the retarded clot retraction produced by altered platelet function. This concept is supported to some extent by the failure to demonstrate significant amounts of FDP in CVF-treated animals despite the marked elevation of plasma fibrinogen with coincident reduction in plasma procoagulant activities. These data do not exclude the possibility that CVF injection induces hypercoagulability, with subsequent consumption coagulopathy, and at the same time directly inhibits the fibrinolytic system. We were not able, however, to demonstrate thrombi in histologic sections of visceral organs from CVF-injected animals.

Our observations raise several questions of major importance to the relationship between the coagulation and complement systems. Do the coagulation changes induced by purified CVF reflect a primary effect on the coagulation mechanism rather than a secondary complement-mediated effect? If the effect of purified CVF on coagulation is independent of the complement system, the implication is that CVF contains at least one additional property distinct from but as yet not separated from the complement activator. Alternatively, if our data are interpreted to suggest a secondary effect through complement, this effect may be the result of platelet and erythrocyte membrane injury with release of intracellular constituents or may indicate a more direct, intimate relationship between several of the coagulation factors and complement components. Such a relationship has been implied recently by the demonstration that C6-deficient rabbits have significant coagulation defects which are eliminated in vitro by adding purified C6 to their plasma.

ACKNOWLEDGMENT
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REFERENCES


22. DeVries, A., and Cohen, I.: Hemorrhagic and blood coagulation disturbing ac-


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