Purified Cobra Venom Factor: Effect on Blood Platelets[1] (36473)

W. Jean Dodds and Richard J. Pickering
(Introduced by Louis H. Muschel)
Division of Laboratories and Research, and the Kidney Disease Institute, New York State Department of Health, Albany, New York 12201

It has been shown by other investigators that antigen–antibody mediated release of vasoactive amines from rabbit platelets requires complement (1, 2) and that certain constituents of serum including IgM and complement components appear to be involved in the aggregation and retraction of washed human platelets by thrombin (3). Surfaces known to activate platelets can also activate complement when coated with gamma globulin (4) whereas complement inhibitors have been reported to prevent platelet aggregation and retraction (3, 5). These observations prompted us to examine the effect of a specific complement-reactive factor derived from cobra venom (CVF) on platelet function.

Materials and Methods. Venom from the cobra (*Naja haja*) was purified by DEAE-column chromatography and polyacrylamide gel electrophoresis as described previously (6). Following electrophoresis, the highly purified material with specific complement reactivity was eluted from the preparative polyacrylamide gel and was found to contain only one protein band on analytical polyacrylamide gel electrophoresis. The highly purified material effectively inhibited serum complement activity but did not contain phospholipase A or fibrinolytic activities as measured by egg yolk clearing, caseinolytic and fibrin plate assays, respectively. Neither the DEAE nor highly purified CVF preparations coagulated purified bovine fibrinogen or plasma from any of the species studied during a 3-hr incubation.

Platelet-rich plasma (PRP) was prepared from citrated whole blood of guinea pigs, rabbits, dogs, and humans by differential centrifugation (1500 rpm for 2–4 min) at 25°. For some experiments, platelets were washed three times according to Ardlie *et al.* (7) and resuspended. Their resuspending medium was modified to contain 1% human albumin in Tyrode's solution. Washed platelets resuspended in modified Tyrode's solution without divalent cation and in standard Tyrode's solution without albumin were investigated for comparison. Procedures were carried out at 25°. Similar studies were carried out using platelets from dogs with thromblasthenic-thrombopathia (8) and a patient with C3 deficiency (approximately 10% C3 activity).

Platelet aggregation was studied with adenosine diphosphate (ADP, 0.15 mg/ml, Sigma Chemical Co., St. Louis, MO) and thrombin (2 NIH U/ml, Parke–Davis, Detroit, MI) prepared in Tyrode's solution modified to exclude divalent cations. Purified CVF from polyacrylamide gels containing approximately 8400 U of C3 inhibiting activity/ml[2] was diluted 1:10 or 1:20 with imidazole buffered saline. Partially purified CVF obtained after DEAE chromatography, 2800 U/ml, was used in some experiments. Control solutions for venom studies were chromatographic or polyacrylamide gel eluates devoid of complement reactivity diluted 1:5, 1:10, or 1:20 in imidazole buffered saline.

[1] This work was supported in part by U.S. Public Health Service Grant No. HE-09902.

[2] 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° for 60 min. Each milligram of purified CVF contains approximately 300–500 U.

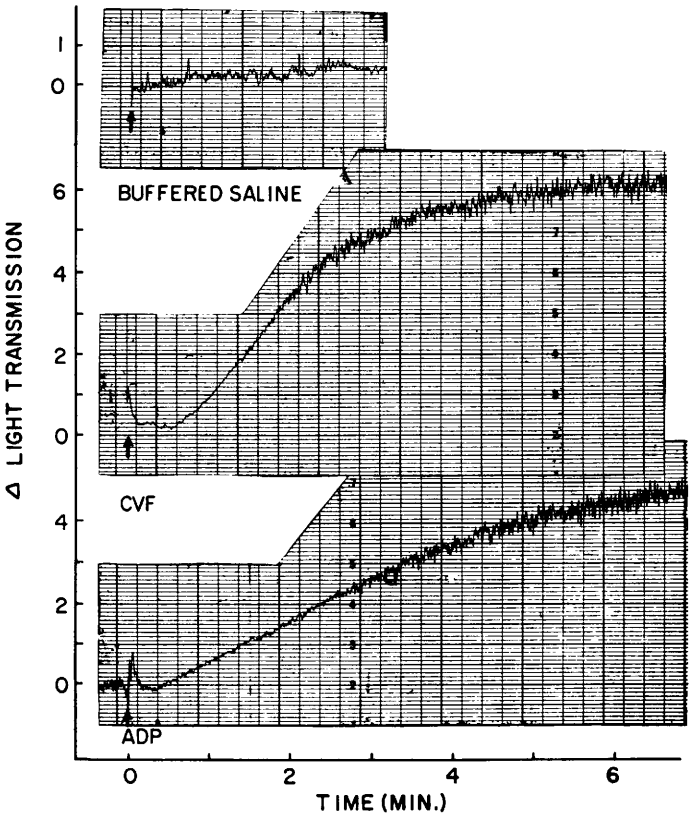

FIG. 1. Platelet aggregation at 25° in guinea pig platelet rich plasma (PRP) with undiluted partially purified cobra venom factor (CVF) and adenosine diphosphate (ADP). The final concentration of CVF and ADP/ml of PRP was 1400 U and 3 μg, respectively.

Aliquots of PRP (0.5 ml) or washed platelet suspensions were incubated with 0.5 ml of diluted CVF or buffer for 5, 15, and 30 min at 25° with continuous stirring. Parallel experiments were performed with ADP (0.05 ml) and thrombin (0.05 ml) diluted to 0.5 ml in buffered saline and added to equal volumes (0.5 ml) of PRP. The thrombin concentration used in these experiments aggregated platelets but did not visibly coagulate the plasma during the period of study. In all species, platelet aggregation was recorded visually as 0–4+ by phase-contrast microscopy with silicone-coated slides and coverslips. In guinea pig PRP, platelet aggregation at 25° was sufficiently active to facilitate recording by turbidometric technique (7, 8). Clot retraction in PRP was evaluated (8) and indicated as 0–4+. A modified Stypven time assay (9) was used to measure released platelet factor 3 activity (PF3) in supernatants of the incubated platelet reaction mixture obtained by centrifugation for 20 min at 1400*g*. Supernatant adenine nucleotide content was determined according to Mueller-Eckhardt and Lüscher (10). Percent release of PF3 and nucleotides was calculated with buffer-incubated platelets as a zero reference, and with sonicated, buffer-incubated platelets as a maximum; standard curves were made by serially diluting sonicated extracts in platelet-poor plasma (PPP). Total nucleotide levels in experiments with ADP-induced aggregation were corrected for exogenous ADP; PF3 measurements after thrombin-induced aggregation were corrected for exogenous thrombin. The latter was accomplished by incubating ap-

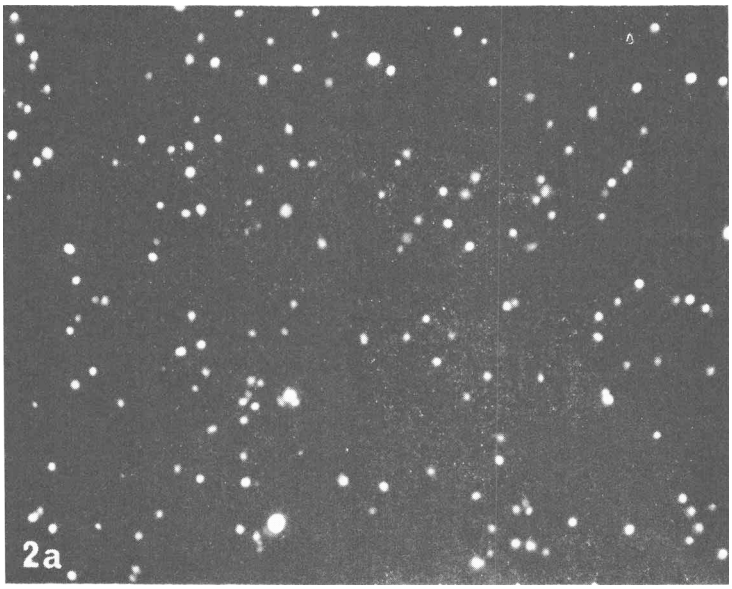

FIG. 2a. Phase-contrast photomicrograph of thrombopathic dog PRP after 8 min incubation with ADP (3 μg/ml PRP).

propriate concentrations of ADP and thrombin with PPP from the same species and subtracting the values obtained. Platelet factor 4 activity (PF4) was measured as described by Niewiarowski and Thomas (11) and also as modified for thrombin-induced aggregation by Thomas *et al.* (12). PF4 was calculated by standard curves prepared from Triton X-100 extracted platelets as described (11).

Results. Figure 1 shows platelet aggregation tracings of guinea pig PRP after addition of buffer, undiluted partially purified (DEAE) CVF, and ADP. The interval between the addition of venom or ADP and platelet aggregation was about 40 sec. Diluted DEAE CVF (1:5) produced visible aggregation of guinea pig platelets in PRP or in washed platelet suspensions within 7–8 min, whereas 1:10 diluted DEAE CVF required 12 min. Highly purified (polyacrylamide) CVF at 1:20 dilution produced aggregation in guinea pig PRP and washed platelet suspensions within 12–15 min. Normal rabbit, dog, and human platelets were also aggregated by either partially or highly purified CVF. In contrast to guinea pig platelet preparations, platelets from these species did not show macroscopic clumping with CVF but were definitely aggregated after 7–15 min incubation when examined by phase-contrast microscopy. Buffer-incubated platelets were not aggregated during 30-min incubation as checked by direct microscopic observation. In all species studies, the interval between addition of CVF and platelet aggregation appeared directly proportional to the dilution of CVF. The relative response (in decreasing order) of platelets to venom was guinea pig > dog > rabbit > human.

Experiments with purified CVF and washed platelets resuspended in modified Tyrode's solution without divalent cation and in standard Tyrode's with and without albumin showed that CVF-induced aggregation required divalent cation but not albumin. However, the best platelet preparations contained both reagents in the resuspending medium.

Of particular interest was the finding that thrombopathic dog platelets were not aggregated by ADP or thrombin but were significantly altered during incubation with either preparation of CVF. Figure 2a shows thrombopathic platelets incubated 8 min with ADP. This figure also demonstrates the extreme variations of platelet size and the giant plate-

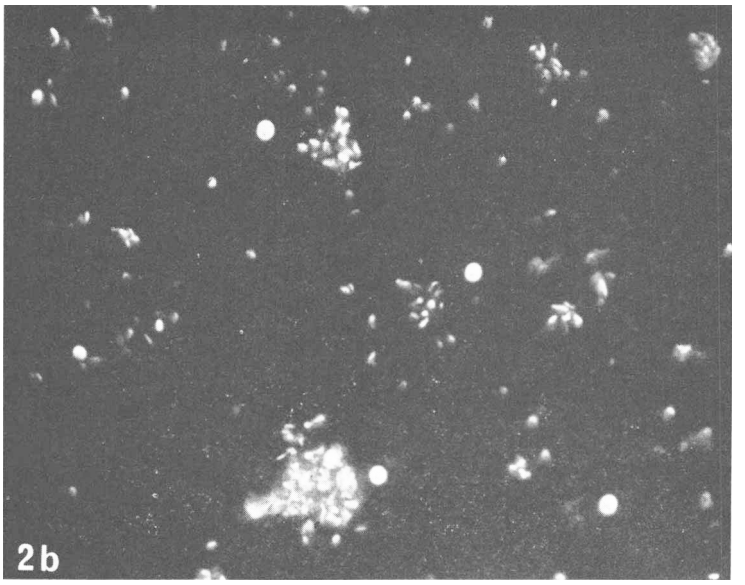

FIG. 2b. Phase-contrast photomicrograph of thrombopathic dog PRP after 8 min incubation with highly purified CVF (420 U/ml PRP).

lets characteristic of canine thrombasthenic-thrombopathia (8). Figure 2b shows the same platelet preparation after 8 min of incubation with highly purified CVF. Large platelet aggregates are present and many isolated platelets have changed from round or disc-shaped to sickled or elongated forms. Figure 2c shows the same thrombopathic platelets washed three times, resuspended, and incubated 10 min with highly purified CVF. Platelet aggregates were evident in these CVF-incubated samples, whereas washed thrombopathic platelets incubated with buffer were not aggregated. Platelets from a C3 deficient patient were also aggregated by ADP, thrombin, and both preparations of CVF.

The effect of 1:10 diluted highly purified CVF on guinea pig and dog platelets is compared with that of ADP and thrombin in Table I. The data show that purified CVF caused platelet aggregation and inhibited thrombin-induced clot retraction. In addition, the CVF preparations produced a noticeable hazy or milky background in platelet suspensions after 5–10 min of incubation; the shape of these platelets as seen by phase-contrast microscopy was markedly altered. This change can perhaps be attributed to CVF-induced release of platelet constituents as shown in Table I. Nucleotide, PF3, and PF4 released during equivalent incubations with ADP and thrombin are included for comparison. Although thrombopathic platelets were not aggregated by ADP or thrombin, they were capable of releasing nucleotide, PF3, and PF4 when incubated with ADP, thrombin or CVF. Normal human platelets and those from a C3 deficient patient also released nucleotide, PF3, and PF4, following incubation with these three reagents.

The release of platelet constituents in our experiments varied with the length of incubation of platelets with CVF or aggregating agents. For example, after 5 min of incubation, release was moderate with thrombin, minimal with ADP, but zero with CVF. Maximum release was achieved with ADP and thrombin after 15 min incubation, and with CVF at 30 min. Our observations are in agreement with those of several investigators, especially Thomas *et al.* (12), who showed that release of adenine nucleotides and PF4 from platelets of man and several animal species occurred following incubation with various platelet-aggregating agents.

Discussion. We conclude that these puri-

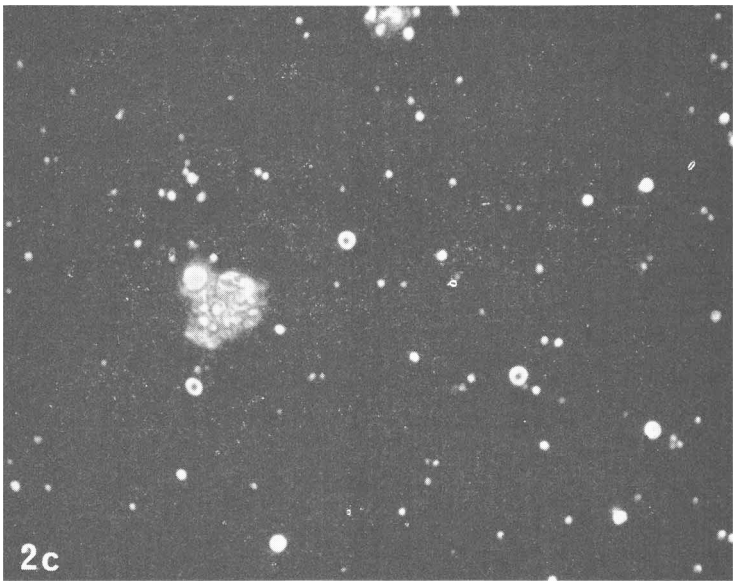

FIG. 2c. Phase-contrast photomicrograph of washed thrombopathic dog platelets after 10 min incubation with highly purified CVF (420 units/ml platelet suspension). The platelets were washed three times and resuspended with 1% purified human albumin in Tyrode's solution.

fied preparations of CVF alter platelet function perhaps by exerting an effect(s) on the platelet membrane. This may be a direct interaction of CVF with the membrane or an indirect effect mediated through serum proteins, such as CVF cofactor (13) and comple-

TABLE I. Platelet Aggregation, Release, and Clot Retraction Induced by Highly Purified Cobra Venom Factor (CVF), Adenosine Diphosphate (ADP) and Thrombin.[a]

Reagents	Aggregation	Release			Clot retraction
		Nucleotides (%)	PF3 (%)	PF4 (%)	
Guinea pig platelets					
Buffer	0	0	0	—	0
CVF (420 U/ml)	4 +	25–29	30–38	—	0
ADP (7.5 μg/ml)	3–4 +	—	24–34	—	—
Thrombin (0.1 U/ml)	4 +	—	65–82	—	4 +
Normal dog platelets					
Buffer	0	0	0	0	0
CVF (420 U/ml)	3 +	4.5–8	19–34	30	0
ADP (7.5 μg/ml)	3–4 +	14–16	36–65	27	—
Thrombin (0.1 U/ml)	4 +	25–52	20–80	25	4 +
Thrombopathic dog platelets					
Buffer	0	0	0	0	0
CVF (420 U/ml)	3 +	2–32	8–19	13	0
ADP (7.5 μg/ml)	0	7–18	15–47	13	—
Thrombin (0.1 U/ml)	0	20–46	15–68	32	0

[a] Experiments made with citrated PRP. Data from four experiments in each category. Release measured after 30 min incubation at 25°. (Mean and/or range of activity included.) Final concentration of reagents/ml of reaction mixture is indicated.

ment. The prolonged incubation required for diluted CVF to produce platelet aggregation and release suggests that the mechanism involves platelet membrane injury or interaction similar to that induced by immune complexes or collagen. This may be analogous to the red cell membrane injury induced by the combination of CVF and serum (6). However, our data are also compatible with an entirely different mechanism(s). For example, thrombopathic platelets which are definitely aggregated by CVF are not aggregated by ADP, thrombin, or collagen. The fact that purified CVF also reacted with washed platelets and platelets from a C3 deficient patient suggests that plasma complement components may not be essential. However, it has not been shown that these washing procedures for platelets do, in fact, remove all adsorbed complement proteins. In addition, since the plasma of our patient was relatively C3 deficient and we did not determine the status of platelet-adsorbed C3, we can only assume that either C3 is not a major component of this phenomenon, or that trace amounts are adequate.

Many investigators have reported multiple effects of snake venoms on blood coagulation and platelets, but these studies almost exclusively involved the use of crude venom preparations (9, 14). Our studies utilized only venom reagents purified to isolate the specific complement-reactive components. Although our purified preparations of cobra venom do not clot plasma or purified fibrinogen, it is possible that small amounts of thrombin generated by purified CVF caused a direct effect on platelets. This hypothesis should be ruled out, however, by the evidence of CVF-induced aggregation of thrombopathic platelets because these platelets are not aggregated by thrombin even at very high or low thrombin concentrations. In addition, Davey and Lüscher (9, 15) found poor correlation between the rate at which certain venoms coagulated purified fibrinogen and the extent of venom-induced platelet aggregation and nucleotide release. A specific fraction of *N. haja* venom with phospholipase A activity has been reported to lyse washed human platelets and abolish their clot retraction (14). However, lysis of platelets was not seen in our experiments with a highly purified preparation of CVF which did not contain detectable amounts of phospholipase.

Summary. A specific complement-reactive factor derived from cobra venom (CVF) inhibited complement activity, impaired clot retraction, and induced aggregation and release of intracellular constituents in platelet-rich plasma or suspensions of washed platelets from guinea pigs, rabbits, dogs, and humans. Platelets from thrombopathic dogs and from a complement deficient (C3) patient responded similarly when mixed with purified CVF; possible explanations for these observations are included.

We are indebted to Mr. Jack Newman, New York University Medical Center, for testing our CVF preparations for the presence of contaminating fibrinolytic enzymes.

1. Henson, P. M., J. Immunol. **104**, 924 (1970).
2. Henson, P. M., and Cochrane, C. G., J. Exp. Med. **129**, 167 (1969).
3. Taylor, F. B., Jr., and Müller-Eberhard, H. J., J. Clin. Invest. **49**, 2068 (1970).
4. Jobin, F., and Tremblay, F., Thromb. Diath. Haemorrh. **22**, 450 (1969).
5. Marney, S. R., Jr., J. Immunol. **106**, 82 (1971).
6. Pickering, R. J., Wolfson, M. R., Good, R. A., and Gewurz, H., Proc. Nat. Acad. Sci. U.S.A. **62**, 521 (1969).
7. Ardlie, N. G., Packham, M. A., and Mustard, J. F., Brit. J. Haematol. **19**, 7 (1970).
8. Dodds, W. J., Thromb. Diath. Haemorrh. Suppl. **26**, 241 (1967).
9. Davey, M. G., and Lüscher, E. F., Biochim. Biophys. Acta **165**, 490 (1968).
10. Mueller-Eckhardt, C., and Lüscher, E. F., Thromb. Diath. Haemorrh. **20**, 155 (1968).
11. Niewiarowski, S., and Thomas, D. P., Nature (London) **222**, 1269 (1969).
12. Thomas, D. P., Niewiarowski, S., and Ream, V. J., J. Lab. Clin. Med. **75**, 607 (1970).
13. Götze, O., and Müller-Eberhard, H. J., J. Exp. Med. **134**, 90s (1971).
14. DeVries, A., and Cohen, I., *in* "Recent Advances in Blood Coagulation" (L. Poller, ed.), p. 277. Little, Brown, Boston (1969).
15. Davey, M. G., and Lüscher, E. F., Nature (London) **207**, 730 (1965).

Received Dec. 17, 1971. P.S.E.B.M., 1972, Vol. 140.