

COMPLEMENT AND COMPLEMENT-LIKE ACTIVITY IN LOWER VERTEBRATES AND INVERTEBRATES*

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Cobra venom as a lytic and anticomplementary substance has been known since 1900 (1). More recently, Nelson (2) separated a nontoxic component of cobra venom and showed that it was selective in its action in that only C3c was destroyed. Nelson also recognized that a serum cofactor was essential to the inactivation of the complement system by the purified cobra venom factor (CVF)¹ and Müller-Eberhard and his associates (3) defined a proinactivator, a 5S pseudoglobulin of a γ -mobility in human serum essential to the inactivation of the complement system. Consonant with early work on the hemolytic action of cobra venom, Pickering et al. (4, 5) showed that this purified cobra venom factor eluted from a single protein band on polyacrylamide gel can produce lysis of guinea pig red blood cells in the presence of fresh serum. This lytic activity was shown to be a function of the terminal components of the complement system and was found not to involve any of the complement components which act in the earlier steps of the complement cascade when the system is activated by antigen-antibody complex. Subsequently Ballow and Cochrane (6) have confirmed these findings and Shin et al. (7) have reported that acting through the proinactivator of the complement system, the cobra venom factor inactivates C5, 6, 7, 8, and 9 in vitro.

These studies established that the cobra venom factor can utilize the entire terminal complement sequence and release chemotactic factors from C3 and C5 without engaging the earlier components. These observations are in keeping with studies by Gewurz et al. (8, 9) and Mergenhagen et al. (10) which had indicated that the complement sequence can be activated by separate pathways which differ from the classical immunological mechanism of activation. In the present study we have applied the cobra venom factor pathway to study the complement system in the

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¹ *Abbreviations used in this paper:* CVF, cobra venom factor; E^h, horse erythrocytes; E^r, rabbit erythrocytes; E^s, sheep erythrocytes; GGV⁺⁺, glucose gelatin veronal buffer with Ca⁺⁺Mg⁺⁺; GGV[—], glucose gelatin veronal buffer without Ca⁺⁺Mg⁺⁺; LIA, lysis-inducing activity.

sera of lower vertebrates and hemolymphs of critical evolutionary representatives of invertebrates.

The presence of C in lower vertebrates has been described by several investigators (11-15). However, complement in more primitive fishes (hagfish, lamprey) was not demonstrable by classical methods (16). Nelson (17), however, showed that lamprey serum possesses the 9th component. No evidence of the presence of C or C components has been previously described in the hemolymph of invertebrates. In the studies to be presented, we have shown that both lower vertebrates and invertebrates possess a lytic C system which can be activated via a pathway initiated by the cobra venom factor.

Materials and Methods

Vertebrate and Invertebrate Species Studied.—The vertebrate species studied were phylogenetic representatives of the Agnatha *Petromyzon marinus* (sea lamprey) obtained from the U.S. Bureau of Commercial Fisheries, Hammond Bay, Millersburg, Mich.; the *Eptatritus stoutii* (hagfish) from Pacific Biomarine, Venice, Calif.; the Chondrichthyes *Ginglymostoma cirratum* (nurse shark) from Miami Seaquarium, Miami, Fla.; the chondrosteian *Polyodon spathula* (paddlefish) from the South Dakota Division of Game Fisheries and Parks, Woonsocket, S. Dak.; and the teleost *Cyprinus carpio* (carp), Minn. Conservation, Division of Game and Fisheries, St. Paul, Minn. Two species of the amphibia *Rana pipiens* (leopard frog) and *Rana catesbeiana* (bullfrog) were included. Representative species of the reptilia were the *Chelydra serpentina* (snapping turtle) and *Naja haje* (Egyptian cobra). The frogs and turtles were obtained from Schettles, Stillwater, Minn. and the cobra serum from Ross Allen's Reptile Institute, Silver Springs, Fla. The chicken represented the Aves. For comparative purposes, the guinea pig was included in this study. The invertebrate species were selected from two marine stocks of the triploblastic phyla. The one stock leading to the insecta was represented by a primitive arthropod *Limulus polyphemus* (horseshoe crab) and a representative of the Sipunculoidea *Golfingea* sp (sipunculid worm). Of the second stock leading to the vertebrata, *Asterius forbesi* (starfish) represented the Echinodermata. The invertebrate species were obtained from the Marine Biological Laboratories, Woods Hole, Mass.

Anticomplementary Substances.—Equal volumes of the anticomplementary substances were incubated with equal volumes of test sera. The mixtures were incubated for 1 hr at 30°C and tested for anticomplementary activity. Endotoxin: 0.1 mg/ml (Difco product lipopolysaccharide *S. typhosa*, Batch 3124-10 [Difco Laboratories, Inc., Detroit, Mich.]). Antigen-antibody complexes were made with BSA and rabbit anti-BSA at the zone of equivalence. The resulting precipitate was washed several times with isotonic saline and resuspended to give approximately 2 mg protein/ml.

Salicylaldehyde, Congo red, hydrazine, and EDTA were obtained from Eastman Organic Chemicals, Rochester, N.Y. Zymosan was activated as described by Mayer (18) and purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, Batch No. 5818.

Purification of Cobra Venom.—Crude cobra venom *Naja haje* was obtained in the lyophilized state from the Miami Serpentarium, Miami, Fla., and purified as described by previous investigators (2). The final product was analyzed by disc electrophoresis and shown to have anticomplementary and lytic activity with guinea pig serum. This product was shown not to contain any phospholipase activity.

Measurement of Total Hemolytic Complement in Vertebrate Sera.—Gelatin veronal buffer, and glucose gelatin veronal buffer with and without Ca^{++} Mg^{++} (GGV^{++} , GGV^{-}) were

prepared as described previously (19). Sensitization of erythrocytes from sheep and from rabbit, depending on the species used and the measurement of CH_{50} , has been described earlier (15).

Assay System for Measuring Anticomplementary Activity in Lower Vertebrates.—0.1 ml of test serum was preincubated with 0.1 ml of purified CVF for 18 hr at 0°C; after this period, 0.8 ml GGV^{++} was added and the dilutions of the mixture were made in 0.5 ml volume. 0.5 ml of respective indicator cells sensitized with rabbit amboceptor or natural antibody, depending on the species tested (15), were added and the mixture incubated for 1 hr at 30°C or for 18 hr at 0°C. To determine the per cent lysis, 6.5 ml saline were added. The mixtures were centrifuged and the supernates read spectrophotometrically at 412 $m\mu$. The CH_{50} was determined by interpolation in the usual way.

Measurement of the Lytic Activity of CVF.—Dilutions of sera were made in 0.5 ml volume in GGV^{++} . To each tube 0.1 ml of CVF was added. 0.5 ml of sheep erythrocytes (E^s), rabbit erythrocytes (E^{rb}), or horse erythrocytes (E^h) were added next. The tubes were incubated for 1 hr at 30°C, diluted, and the CH_{50} measured.

Complex Formation.—To test for complex formation in the sera of different animals studied, equal amounts of CVF and serum or hemolymph were incubated for 2 hr at 30°C. This phase is defined as step I (Fig. 1). The lytic activity of the complex was tested by adding 0.1 ml of complex to tubes containing dilutions of fresh serum in 0.05 M EDTA in the presence of erythrocytes. This phase is defined as step II. The lytic activity of the complex was of a greater magnitude than the lytic activity of the control CVF-normal saline mixture at that molarity of EDTA. The latter mixture had either very low activity or was not present at all.

Interaction of CVF with Hemolymphs from Invertebrates.—Hemolymph from the sipunculid worm was obtained from the coelomic cavity. From *Limulus polyphemus*, the fluid was withdrawn by inserting the syringe under the carapace into the dorsal sinus, and from the starfish by inserting the needle into the ambulacral canal (groove). In bleeding the starfish greater efficiency was obtained when the animal was tilted toward the astro. Hemolymphs from the starfish and horseshoe crab were centrifuged at 10,000 rpm for 20 min in a Sorvall ultracentrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) Superspeed RC2-B, and the supernates were used for study. Dilutions of hemolymph was made in GGV^{++} in 0.5 ml volume. 0.1 ml of CVF was added to each tube followed by 0.5 ml of E (E^s or E^{rb}). The mixtures were incubated for 1 hr at 30°C and the tubes were left overnight for 18 hr at 0°C and treated as above for the determination of CH_{50} .

RESULTS

Lysis-Inducing Activity of Purified Cobra Venom Factor on Vertebrate Sera and Invertebrate Hemolymphs and Complement Consumption of Vertebrate Sera by Purified Cobra Venom Factor.—Table I represents observations on the lysis-inducing activities (LIA) of purified cobra venom factor on various vertebrate sera and invertebrate hemolymphs. As indicated in the Table, LIA was present in the sera of the guinea pig and chicken, and was extremely marked in the frog. Further LIA was observed with hagfish serum and in two of the three invertebrate hemolymphs, i.e., horseshoe crab and sipunculid worm. By contrast LIA was absent in sera from turtle, cobra, carp, paddlefish, nurse shark, and lamprey, and from starfish hemolymph. In the horseshoe crab, LIA was labile even on storage at -70°C . The anticomplementary activity of purified CVF was present in mammalia (guinea pig), aves (chicken), reptilia (turtle,

cobra), amphibia (marine frog, bullfrog), and in one species of the chondrichthyes (nurse shark). No anticomplementary effect on sera of teleosts (carp) and chondrosteian (paddlefish) was found. Measurement of total complement activity in the above species has already been described (15).

TABLE I
Lysis-inducing Activity of Purified Cobra Venom Factor on Vertebrate Sera and Invertebrate Hemolymphs and C Consumption of Vertebrate Sera by Purified CVF

Category	Species	Total hemolytic C activity (CH ₅₀)*	Lysis-inducing activity (LIA ₅₀)‡	C-consuming activity§
<i>Vertebrates</i>				
Mammalia	Guinea pig	1500-2000	40-80	>95
Aves	Chicken	52-90	110-160	>95
Reptilia	Cobra	150-200	<5	>95
	Turtle	2000-3000	<5	>95
Amphibian	Frog	300-500	1000-1500	>95
Osteichthyes	Carp	100-150	<5	0
	Paddlefish	50-75	<5	0
Chondrichthyes	Nurse shark	300-400	<5	>95
Agnatha	Lamprey	<2	<5	—
	Hagfish	<2	20-40	—
<i>Invertebrates</i>				
Arthropod	Horseshoe crab	<2	12-24	—
Echinoderm	Starfish	N.D.	>5	—
Annelid	Sipunculid worm	<2	2.5-5.0	—

* Reaction mixtures were incubated for 1 hr at 30°C, 6.5 ml of saline was added and, after centrifugation, the hemoglobin released was determined spectrophotometrically. The CH₅₀ was determined by interpolation.

‡ Lysis-inducing activity of purified cobra venom factor. Test sera were diluted in glucose gelatin buffer containing Ca⁺⁺ and Mg⁺⁺ ions. 0.1 ml of purified CVF was added to each tube. 0.5 ml of erythrocytes was added next. The mixtures were incubated at 30°C for 1 hr. When invertebrate hemolymph was used, an additional incubation at 0°C for 18 hr was included. The dilution and release of hemoglobin was determined as for CH₅₀.

§ Complement consumption of vertebrate sera by purified cobra venom factor (CVF). Equal volumes of CVF and test sera were preincubated for 1 hr at 30°C, and the residual CH₅₀ determined.

Effect of Preformed Complexes (CVF + Frog Serum) on Frog Serum in EDTA.
—To further elucidate the nature of LIA in the lower vertebrates, we used the frog as a model. Since we cannot at present measure terminal complement components in frog serum directly, experiments were set up to test further the nature of the influence of CVF in promoting lysis of indicator cells in frog serum. Complex was produced by reacting CVF with frog serum for 2 hr.

When this complex was reacted with frog serum in 0.05 M EDTA in the presence of sheep erythrocytes (E^s), lysis was obtained. In Fig. 1, the formation of the complex is defined as step I and the reaction of the complex with C'EDTA as step II. These results would indicate that terminal complement components are necessary for red cell lysis by CVF in frog serum.

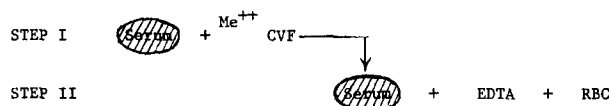


FIG. 1. Steps involved in the lysis-inducing activity of purified cobra venom factor (CVF) and their relation to serum factors and divalent cations (Me^{++}).

TABLE II

Effect of Known Inhibitors of Mammalian Hemolytic Complement Activity upon Lysis-inducing Activity in the Frog

Agent	Final concentration	Total hemolytic C activity	Lysis-inducing activity		
			Total LIA ₅₀	Step I	Step II
Zymosan	30 mg/ml	>90	>90	<5	>90
Heat	56°C, 30 min	>90	>90	90	>90
CVF	1 mg/ml	>90	>90	<5	>90
Hydrazine	0.07 M	>90	20	<5	20
Lipopolysaccharide	25 mg/ml	>90	>90	<5	>90
Salicylaldehyde	0.1 M	>90	>90	<5	>90
Congo red	20 mg/ml	>90	>90	<5	>90
EDTA	0.04 M	>90	80	90	—
Antigen-antibody	2 mg/ml	60	70	<5	70

The figures represent percentage inhibition of lysis-inducing activity by C inhibitors. Test sera were preincubated with known concentrations of inhibitors at 30°C for 1 hr and the residual CH_{50} and LIA₅₀ were determined. Wherever total C was inhibited, LIA was also inhibited except when hydrazine was used.

Step I and Step II represent the two phases of lysis-inducing activity (Fig. 1). As shown, only heat and EDTA affected Step I, suggesting that during the formation of the complex, divalent cations are required and the serum factor(s) necessary to form the complex is (are) heat labile.

Inhibition by all C inhibitors except hydrazine was evident in Step II.

Effect of C Inhibitors on Lysis-Inducing Activity by Purified CVF and by Preformed Complex (CVF + Frog Serum).—In order to determine whether the lysis-inducing activity either by CVF alone or by preformed complex (CVF + frog serum) was C-dependent, experiments were set up with known complement inhibitors (Table II). As demonstrated in guinea pig serum, when frog serum was pretreated with a variety of inhibitors known to interfere with com-

plement function including Congo red, salicylaldehyde, endotoxin (*Salmonella typhosa*), EDTA, CVF, and heat, both classical C and lysis-inducing activities were inhibited. Hydrazine, however, which is known to inactivate the complement system by action at the 4th component, did not inhibit LIA either when total LIA was measured or at the Step II phase. With the exception of heat and EDTA, there was no effect of C inhibitors at the Step I phase.

Interchangeability of Invertebrate and Vertebrate Serum Factors.—The interchangeability of frog and invertebrate components in the induction of LIA by CVF are presented in Table III. Complexes were formed by using CVF and various vertebrate sera and invertebrate hemolymphs (step I). For step II frog serum in EDTA was provided. We observed that CVF-frog, CVF-limulus, and CVF-starfish complexes induced lysis of E^s in the presence of frog serum.

TABLE III
Interchangeability of Frog Sera and Invertebrate Hemolymph Components in the Induction of Lysis-inducing Activity by Purified Cobra Venom Factor

Species tested	Total LIA ₅₀ *	Step I† (frog 2 provided)	Step II‡ (frog 1 provided)
Guinea pig	40–80	<5	<5
Frog	1000–1500	>10	300–400
Starfish	<5	>10	<5
Horseshoe crab	12–24	>10	10–20

* Total lysis-inducing activity (as in Table I).

† Step I. Preformed complex formed with various species and reacted with erythrocytes and frog serum in 0.05 M EDTA.

‡ Step II. Complexes formed with frog serum and purified cobra venom factor and reacted with sera in EDTA from various species in the presence of red blood cells.

No lysis was obtained when guinea pig-CVF complex was employed. Most striking observations were observed when complexes formed with starfish hemolymph and CVF were reacted with frog serum in EDTA. The magnitude of lysis observed was similar to LIA when step I consisted of frog-CVF complex. Starfish hemolymph added to frog serum with E^s did not show lysis. At the molarity of EDTA used with frog serum, CVF alone was inactive. Moreover, the lysis was C-dependent as studied by use of inhibitors. Therefore, it seems that we were dealing with a complex of CVF and a component of starfish hemolymph capable of activating the terminal components of frog serum. When frog complex was provided to various sera and hemolymph in EDTA (step II), lysis was observed with frog serum and limulus hemolymph in the presence of EDTA and E^s.

DISCUSSION

Classical complement was measurable in sera of all the vertebrate species studied with the exception of the cyclostomes, but LIA and anticomplementary

activity of purified CVF was not always demonstrable. By contrast, in hagfish where classical C has never been found, LIA was observed. In sera of cobra, turtle, and nurse shark, anticomplementary activity but not LIA was present, and in carp and paddlefish, neither was demonstrable after treatment with purified CVF. A possible explanation is that an inhibitor to the lytic activity induced by CVF is present in sera of some species. It has been shown by previous investigators that in mammals CVF interacts with a serum 5S proinactivator forming a complex which in turn activates C3 and the terminal components (2, 3).

Striking results were obtained with LIA of CVF when frog serum was used. Indeed a higher titer of lytic activity was obtained when CVF was the activator than when sheep cells sensitized with the rabbit antibody were exposed to frog serum. Because this system for studying lytic function of cobra venom was highly reproducible we concentrated on the frog to determine whether the lysis of E^s produced with CVF was C-dependent. Our data shows that all inhibitors known to deplete total C activity depleted LIA also. These findings are similar to those previously reported in the guinea pig system when LIA was initiated with CVF.

Perhaps the most challenging observations are with those animals which do not seem to possess classical complement, i.e., the primitive cyclostome (hagfish) and invertebrates. LIA was demonstrable in hagfish serum and in the hemolymph of two of the three invertebrates studied. These findings suggest that components perhaps homologous to terminal C components of mammals may be recognizable when the alternate CVF pathway is used to activate this system.

We also showed in the frog that a complex formed with CVF and frog serum was essential, as in the guinea pig, to activate the lytic or "attack" components of the complement system. Indeed, preformed complex initiated lysis in the presence of frog serum treated with EDTA. This complex-induced lysis was also shown to be inhibited by known inhibitors of the complement system. Formation of the complex was independent of the complement system even though it required divalent cations. Complexes formed with hemolymph of the horse shoe crab and CVF induced lysis of E^s in the presence of EDTA-treated frog serum. This complex also induced lysis of E^s in EDTA-treated hemolymph of the horse shoe crab. CVF complex formed with frog serum induced lysis in EDTA-treated hemolymph of the crab. Starfish hemolymph treated with CVF induced lysis of E^s in EDTA-treated frog serum and the titer of the lytic reaction was comparable to that obtained when CVF-frog serum complex was introduced with E^s in EDTA-treated frog serum. The latter lytic reaction was also shown by complement inhibitors to be dependent on the terminal components of the frog complement.

It seems clear from the present studies that activities like those associated

with complement are present in lower vertebrates, e.g. the frog, and similar activities are present in representatives of several lines of invertebrates specifically chosen to be widely divergent phylogenetically.

Many studies have been performed to determine whether invertebrates have classical adaptive immune responses (20, 21). As yet, even though suggestive evidence has been obtained for the existence of cellular immunity in the earthworm (22), no clear evidence of immunoglobulin or true adaptive antibody responses have been presented for invertebrates. This conclusion involves the hazards of negative evidence, but from studies thus far the burden remains squarely on those who contend that invertebrates possess adaptive immunity comparable to that of mammals. Studies from our laboratories (23, 24) as well as by others (25-28) indicate that the complement system may play a role in inflammatory reactions under circumstances where initiation by the classical immune pathway is not involved. With this evidence and that from studies reported here, it is attractive to think of the complement system as being more primitive than the immune system itself. This primitive system which can be activated by the cobra venom factor may not even have been originally related to immunity as it is conventionally considered. Could it be, for example, that this more ancient complement system readily activated by CVF, especially when complexed with a serum factor, was a system involved in the bodily defense through its relation to inflammation and phagocytosis to which the more latterly developing classical immunity system was linked by the imposition of the earlier complement components C1, 4, and 2? Such a developmental mechanism would, indeed, be a natural mechanism for utilizing the primitive defense reactions of phagocytosis, inflammation, and vascular response to amplify certain of the specific combining characteristics of the adaptive immune responses.

SUMMARY

A purified cobra venom factor with C-inhibiting activity also promotes lysis of erythrocytes in fresh mammalian serum. Lysis-inducing activity of purified cobra venom factor was found in sera of lower vertebrates including the cyclostome hagfish and in invertebrates. Lysis-inducing activity was most effective with frog serum. Frog serum was found to be more hemolytic for E^s in the presence of CVF than when cells were sensitized with hemolysin. The hemolysis induced by CVF with frog serum, as in the higher vertebrates, was inhibited when sera were pretreated with known C inhibitors including heat, chelators, endotoxin, immune complexes, and CVF itself.

Complexes formed with CVF and either frog serum or invertebrate hemolymph promoted lysis of indicator cells in the presence of frog serum in EDTA. This lysis was most marked when the starfish-CVF complex was used and was C-dependent. Conversely, complex formed with frog serum and CVF promoted

lysis of E in the presence of invertebrate hemolymph (*Limulus*) in EDTA. Hence, serum components were to some degree at least interchangeable between vertebrate sera and invertebrate hemolymph. Lysis-inducing activity of purified CVF occurs in a wide range of species, has revealed activities resembling those of terminal C-components in lower vertebrates and invertebrates, and provides one means for study of C and C-like activities in primitive species.

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