Studies of the C3 Shunt Activation in Cobra Venom Induced Lysis of Unsensitized Erythrocytes[1] (36623)

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Recent studies have shown that utilization of the C3 shunt pathway by antigen–antibody complexes involves activation of a serum protein called C3 proactivator (1). This finding is of interest in that it demonstrates a convergence in the activation of the C3 shunt by cobra venom (2) and by immune aggregates. Of additional importance is the recognition that the C3 proactivator (C3PA) and the C3 shunt pathway are involved in antibody-mediated tissue damage and host defense mechanisms as reported by this and other laboratories (2–7). To further evaluate the immunopathologic potential of the C3 shunt mechanism of C activation, we considered that the design of a simple and valid hemolytic assay would be useful. This assay is based on several reports which show that the interaction of a purified beta-globulin from cobra venom (CVF) with C3PA in serum results in the lysis of unsensitized erythrocytes (E) (8–10).

Materials and Methods. Cobra venom factor. CVF was obtained from Cordis Laboratories, Miami, FL. Large pools of the lyophilized product were prepared and aliquots were stored at −70°.

Sera. Human serum from freshly clotted blood was centrifuged and stored at −70°. Guinea pig serum, rendered 0.02 *M* with respect to ethylenediaminetraacetate (EDTA) (pH 7.4), was used as a source of C-EDTA to supply the late complement (C) components, *i.e.*, C3 and C5 through C9. Prior absorption of the sera with guinea pig erythrocytes did not influence the outcome of these assays.

Erythrocytes. The experiments described below were performed with normal guinea pig erythrocytes (E). Although sheep E were also satisfactory, the guinea pig cells were preferred to minimize adventitious interaction with the guinea pig C-EDTA. Adult male guinea pigs were bled by cardiac puncture. The blood was thoroughly mixed with an equal volume of Alsever's solution prepared as in (11), but containing 100 μg each of penicillin and streptomycin/ml. The cells were stored at 4° for several days prior to use. For each experiment an appropriate volume of the cell suspension was centrifuged and washed three times in the cold with Veronal buffer containing 0.1% gelatin and 0.02 *M* EDTA (VBS-EDTA) (11). The cell suspension was standardized so that 1.0 ml of washed E added to 5.5 ml of cold distilled water yielded a lysate with an optical density of 1.30 at 4120 A in a Beckman DU-2 spectrophotometer equipped with cuvettes of 10 mm light path. Under these conditions 1.0 ml of the suspension contained 5×10^7 E.

Hemolytic assay of CVF activable factors ($CVFAH_{50}$). The procedure was carried out in two steps. In the first step, the CVF was incubated with several dilutions of the serum under assay for 30 min at 37°. The late C components in the form of C-EDTA were then added, and finally guinea pig E. The reaction mixtures were incubated for 60 min at 37°. The objectives sought in the first step were to use an amount of the CVF which would destroy the available C3, and retain the activable factor(s) in the test serum as

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the limiting constituent(s) as judged by the lysis of E. This was achieved through the use of 0.2 ml of the reconstituted CVF (100 units/ml) with 0.2 ml volumes of several serum dilutions. A paradoxical effect was often noted in that greater lysis resulted with more highly diluted than with more concentrated serum. To avoid this departure from linearity, the assays were initiated with a sixfold serum dilution.

The second step in the assay involved the addition of the late C components in the form of 0.3 ml of C-EDTA and 0.5 ml of the standardized suspension of guinea pig E in VBS-EDTA. This volume of C-EDTA provided a sufficient excess of the late C components, and the number of E an adequate degree of sensitivity and range for the lytic measurements. Prolongation of the second step beyond 60 min at 37° did not increase the degree of specific lysis. Following the second period of incubation, the tubes were chilled in an ice-water bath and diluted with 2.0 ml of chilled 0.15 *M* NaCl containing 0.1% gelatin. The tubes were thoroughly mixed, centrifuged and the lysates were analyzed for hemoglobin spectrophotometrically. The optical density values for complete lysis approximated 1.30 ± 0.05 while the "blank" values for the tubes lacking the CVF or the test serum had an optical density in the range of 0.2 to 0.3. Most of this color was contributed by the undiluted guinea pig serum used as the C-EDTA reagent. The results are plotted according to the von Krogh relationship, *i.e.*, a plot of the degree of lysis, $y/(1 - y)$, as a function of the serum volume yields a straight line of slope 0.5 ± 0.05 for human serum. The 50% intercept is taken as the end point and the hemolytic potency of the serum under test is expressed as $CVFAH_{50}$. A typical set of dose–response curves is given in Fig. 1.

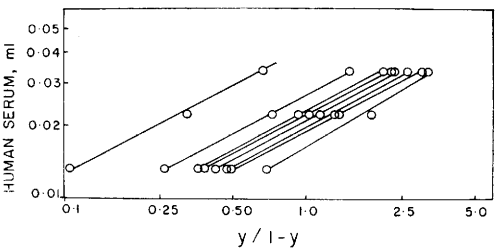

FIG. 1. Dose–response curves for lysis of E following CVF–serum interaction. The results obtained with sera from 8 different individuals are plotted according to the von Krogh equation, in which $y/(1-y)$ represents the degree of lysis (11).

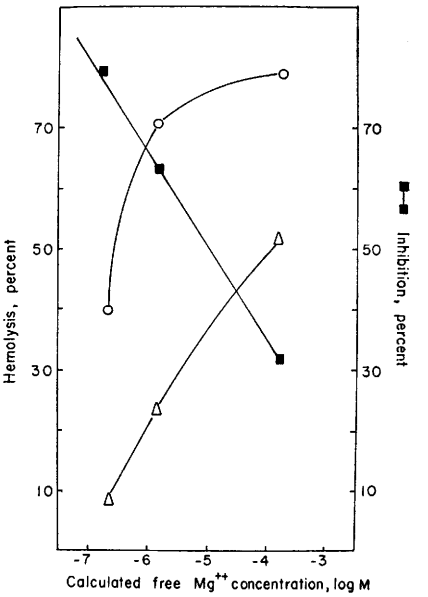

FIG. 2. Plot of the data in Table I for free magnesium concentrations in reaction mixtures containing 0.017 ml of serum. (○) Percentage lysis in presence of 0.017 ml serum plus 5×10^{-3} *M* EGTA and varying Mg^{2+}. (△) Percentage lysis in presence of 0.017 ml serum, 5×10^{-3} *M* EGTA, 2.5×10^{-3} *M* Ca^{2+} and varying Mg^{2+}. (■) Percentage inhibition of △ compared to ○. The free Ca^{2+} levels in △ were $< 10^{-9}$ *M*.

Results. Divalent cation requirement. Previous studies of the C3 shunt pathway demonstrated that magnesium served as metal cofactor (12–14). We have now extended these findings to the reaction system of CVF induced lysis and show that calcium, when added in the presence of magnesium depresses the extent of lysis. Accordingly, the diluent used in this study was fortified with 1×10^{-3} *M* magnesium in Veronal buffered sodium chloride containing 0.1% gelatin. The conclusion that Mg^{2+} is the sole divalent cofactor for this lytic process was based on calculations of free cation levels after the addition of EGTA, metals and serum, as given in Fig. 2 and the legend of Table I. The inhibi-

tory effect of calcium is readily apparent.

Effect of heparin. In view of the markedly destructive effect of heparin on the constellation of late C components (15) the effect of this compound on the present assay was evaluated. The result of repeated experiments indicated that heparin inhibits the lysis of E which follows cobra venom–serum interaction (Fig. 3). Further studies suggest that the site of heparin inhibition may be localized on the E membrane rather than in the sequence of fluid phase events. Thus, heparin acts equally well whether added during the first or second steps of this assay. More significantly, the dose–response curves for this system remain parallel in the absence or presence of 1 to 100 μg of heparin. Polyanions like heparin interact with membranes in other cellular systems such as the binding of reaginic immunoglobulins to human leukocytes (16).

Effect of C3 and anti-C3. Supplementation of the test serum–CVF reaction mixtures with relative large quantities of C3 failed to change the hemolytic titers. To cite one example, a serum with 29 $CVFAH_{50}$ units/ml yielded an identical activity titer after incorporation 350 units of C3 (Cordis Laboratories, Miami, FL). Furthermore, removal of the C3 in the test serum by pretreatment with an excess of heated goat anti-human C3 (Cordis Laboratories) failed to diminish the titer.

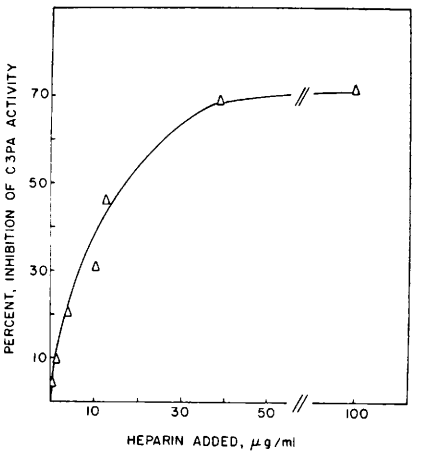

FIG. 3. Inhibition by heparin of CVF induced lysis of E.

TABLE I. Magnesium Dependency of Cobra Venom Induced Activation of the C3 Shunt as Judged by Lysis of Unsensitized Guinea Pig Erythrocytes.[a]

Mg^{2+}	Ca^{2+}	Lysis (%); serum in test (ml):		
$M \times 10^{-3}$		0.033	0.017	0.008
0	0	21.3	6.0	2.9
0.2	0	80.8	39.9	11.7
1.0	0	90.7	70.8	29.7
5.0	0	89.8	78.9	51.0
0	5.0	10.1	9.2	8.2
0.1	2.5	81.0	8.2	5.9
0.5	2.5	92.4	23.5	8.6
2.5	2.5	95.1	51.6	17.7

[a] All reaction mixtures were 5×10^{-3} *M* with respect to EGTA (1,2 bis(2-dicarboxymethyl aminoethoxy) ethane), and as indicated, with respect to added magnesium and/or calcium. The data obtained with 0.017 ml of serum are plotted in Fig. 2 as a function of free magnesium based on the binding constants for the two divalent cations with EGTA.

Role of B2-glycoprotein II (GBG). The serum of a patient studied by Alper and his associates (17, 18) which lacked GBG exhibited no activity in our hands.[3] Furthermore, the supernates obtained after incubation of normal sera with an excess of specific antiserum to GBG (Behring Diagnostics, Woodbury, NY) were devoid of CVF inducible lysis. When the test sera were thus depleted, restoration of lytic activity was not observed after the addition of 400 units of purified C3.

Effect of immune aggregates. Pretreatment of human sera with immune aggregates containing 50 μg N of guinea pig γ-1, rabbit or horse immunoglobulins reduced the extent of lysis which followed the addition of CVF by 50 to 80%. It is noteworthy that no lysis ensued when immune aggregates were substituted for CVF in the reaction mixtures.

Effect of inulin. As with heparin, earlier studies showed that incubation of fresh serum with inulin led to marked diminution of the

[3] We are grateful to Dr. Chester Alper for supplying this serum.

lytic ability of the late C components (15). The mechanism of this depletion was explored in the present assay in terms of the CVF reactive serum component(s). We have observed that incubation of human serum with inulin results in profound depletion of CVF induced hemolytic activity. This effect is noted after preincubation of inulin with the serum for 30 min at 37°. After centrifugation the supernate lost most of its capacity to interact with CVF and induce lysis of E. A 50% activity loss resulted from preincubating 0.5 ml of serum with 0.5 mg of inulin. Inulin, like the immune aggregates, therefore binds or destroys serum constituent(s) involved in the CVF-C3PA induced lysis of E. As observed with immune aggregates, substitution of inulin for CVF in the reaction mixture does not lead to lysis.

Effect of hydrazine. Götze and Müller-Eberhard (2) recently reported that N_2H_4 prevented the conversion by inulin of C3PA to C3 activator. We have observed repeatedly that incubation of serum with N_2H_4 for 120 min at 37° (1.0 ml serum plus 0.25 ml of 0.075 *M* N_2H_4) increases the CVF-mediated lysis. The serum titers are generally about 20 to 50% higher than the control preparations preincubated concurrently with buffer. To cite one example, serum aliquots incubated with N_2H_4 or with buffer yielded $C3PAH_{50}$ titers of 33 and 20.8 units, respectively. The effect of N_2H_4 in conserving the serum hemolytic potency from a time and temperature dependent decay was demonstrated more dramatically in the following type of experiments. Incubation of serum with immune aggregates or inulin markedly reduces the hemolytic activity inducible by CVF. This loss is completely prevented if the serum sample is incubated with N_2H_4 before addition of the immune aggregates or inulin (see Table II). On the other hand, the activity of serum samples pretreated with immune aggregates is not restored by the subsequent addition of hydrazine.

Discussion. The experiments outlined in this report are concerned with an assay for studying activation of the C3 shunt pathway following cobra venom interaction with one or more serum constituents. The availability of such a procedure should facilitate further evaluation of the role of the C3 shunt pathway in various physiologic and pathologic events. The reports that immunoglobulins which fail to activate the classic C sequence through C1 [*e.g.*, IgA, IgG_4 (2) and IgE (19)] may consume the late acting C components by way of the C3 shunt heightens interest in attempts to better delineate the pathogenetic significance of this pathway.

During the course of these experiments several parameters of C3 shunt activation by CVF–serum interaction were studied. Inhibition of the lytic reaction by heparin is one example. In our opinion, the parallelism of the dose–response curves in the absence or presence of heparin speaks for a membrane site of action [cf., however, (20)].

Considerations of the mechanism of CVF

TABLE II. Influence of Hydrazine on Cobra Venom Dependent Lysis of E.

Human serum[a] (ml)	Hydrazine added, 0.038 *M* (ml)	VBS[b] (ml)		PIA[c] (μg N/0.2 ml)	VBS (ml)		$C3PAH_{50}$ titer (units/ml)
0.2	0	0.2		0	0.2		28.5
0.2	0.2	0		0	0.2		36.4
0.2	0	0.2	Incubate 90 min	50	0	Incubate 30 min	15.6
0.2	0	0.2	at 37°	200	0	at 37°	15.2
0.2	0.2	0		50	0		31.3
0.2	0.2	0		200	0		31.4

[a] Fresh human serum. $C3PAH_{50}$ titer without preincubation = 32.3.

[b] Veronal buffered saline with 1×10^{-3} *M* Mg^{2+}.

[c] Washed preformed immune aggregates prepared at equivalence zone ratios with horse anti-pneumococcus Type I serum and Pn. I polysaccharide.

induced hemolysis must take into account the experiments with inulin and immune aggregates. Unlike CVF, the latter two reagents fail to mediate lysis when incubated with human serum, Mg^{2+}, C-EDTA and E. In our hands pretreatment with inulin or immune aggregates depletes the serum of factor(s) essential for CVF induced lysis. The hemolytic studies therefore pose the following question. Do inulin and immune aggregates interact with a reagent (properdin?) necessary for CVF generated lytic activity, or are there two different routes of activating the C3 shunt as suggested in (2, 20) and by the present data? Our findings show that inulin activation does not eventuate in lysis of E and that hydrazine exerts a dual effect. It destroys a factor necessary for inulin activation (20) but is inert with respect to CVF induced lysis.

The hydrazine experiments reinforce the notion that multiple serum factors participate in CVF-mediated lysis of E. As indicated above, preincubation of serum with hydrazine not only conserves the efficiency of a highly thermolabile factor needed for hemolysis, but, in fact, may even enhance this activity, generally, to an extent of about 10 to 25%. According to the sequence postulated in (20), hydrazine intervenes in the hemolytic system by conserving the integrity of the activable factor(s) such as C3PA which is required for CVF generated lysis of E. In the present assay system however, the union of CVF with serum factors proceeds very rapidly (13) thereby circumventing the destructive action of the C3PA convertase system.

There is still another facet of the action of hydrazine in these hemolytic events. As shown above, pretreatment of serum with inulin or immune aggregates decreases its ability to react with CVF. Incubation of the serum with hydrazine prior to admixture with inulin or immune aggregates completely abolishes the depleting potencies of these reagents (Table II). It is therefore apparent that a hydrazine sensitive factor(s) interacts with inulin and immune aggregates and that this factor(s) is not required for CVF induced lysis. However, when the CVF generating activity is depleted from serum by inulin or immune aggregates, its hemolytic function can no longer be restored by hydrazine. Since the interaction of serum with CVF differs from that with inulin or immune aggregates, it may be concluded that both the generation of lytic factors following CVF interaction with serum, and inhibition of this process by immune aggregates reflect the C3PA serum levels. This deduction is supported by the finding that the $CVFAH_{50}$ titers of human sera were proportionately diminished following the addition of varying amounts of anti-GBG serum and testing of the supernates. However, the validity of this conclusion cannot now be established in view of the evidence that a multiplicity of reactants partake in CVF elicited lysis of E. In addition two laboratories have recently provided further evidence to indicate that the activity attributed to the interaction of C3PA with CVF may require more than one serum component (21, 22). Further studies are obviously required to elucidate the role of the various factors in these phenomena. We are of the opinion however that the present assay may provide a useful means of delineating the individual reaction steps in the sequence which is initiated by CVF and which leads to lysis of unsensitized erythrocytes via the C3 shunt.

Summary. A procedure is described to evaluate the activation of the C3 shunt through cobra venom factor-mediated lysis of unsensitized erythrocytes. Data pertaining to several parameters of this assay are given. These include the requirement for magnesium, inhibition by heparin and the effects of hydrazine. The available evidence also suggests that the mechanism of cobra venom interaction with serum factors such as C3 proactivator differs from that of immune aggregates or inulin.

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