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ACKNOWLEDGMENTS AND ADDRESSES

Received July 6, 1976, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Buffalo, NY 14214.*

Accepted for publication August 13, 1976.

Supported in part by Grant GM 20852 from the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, MD 20014.

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Interrelation between Two Anticomplement Cobra Venom Factors Isolated from Crude *Naja naja* Cobra Venom

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Abstract □ Two moieties occurring in crude *Naja naja* cobra venom were found to possess anticomplement activity. Both materials possessed similar molecular weights and specific activities but dissimilar elution profiles upon ion-exchange chromatography. The anticomplement activities of these materials were maintained upon digestion with neuraminidase, and their elution profiles from cation-exchange chromatography became identical after this treatment. It was concluded that the differences between the two anticomplement materials were due to their different sialic acid contents.

Keyphrases □ Cobra venom—two anticomplement factors isolated by ion-exchange chromatography, effect of neuraminidase digestion □ Ion-exchange chromatography—isolation, two anticomplement factors from cobra venom □ Anticomplement activity—two factors isolated by ion-exchange chromatography from cobra venom, effect of neuraminidase digestion

Purified cobra venom factor is probably the single most valuable substance for lowering complement levels *in vivo*. The mechanism of action of cobra venom factor *via* one pathway was described previously (1). When the factor is administered in small doses to minimize the lysis of red blood cells, complement activity is depressed; this depression can be maintained for 3–4 days (1). However, an antibody eventually develops against the cobra venom factor, inhibiting its anticomplement activity.

The possible clinical usefulness of cobra venom factor in reducing the amount of infarcted tissue following coronary occlusion was reported previously (2). In experimental animals, the myocardium that can be salvaged is substantial, and the treatment may be started a number of hours following the occlusion and still be effective. Since acute myocardial infarction constitutes the most common cause of death in this country, any procedure that can salvage substantial quantities of the patient's myocardium after the patient's arrival at the hospital should be investigated. Animal studies showed that cobra venom factor diminishes the inflammatory response following ischemic damage and thus reduces the size of myocardial infarcts after coronary occlusion (3, 4). These conclusions were reached using various electrophysiological, biochemical, histological, histochemical, and electron microscope techniques.

During an investigation on the purification of cobra venom factor from crude *Naja naja* cobra venom, two anticomplement factors were obtained. This report concerns the identification of these materials and their interrelationship.

EXPERIMENTAL

All buffers were prepared from reagent grade materials according to American Chemical Society standards. Gel filtration media were equilibrated with starting buffer for 2 days or heated to 80° for 6 hr prior to column preparation. All absorbance measurements were made at 280 nm. Freshly drawn human blood was allowed to clot, and the resulting serum served as the complement source.

Anticomplement Activity—*Qualitative Assay*—The assay was based on previously reported methods (5–7). For scanning column runs, sensitized sheep erythrocytes, EA (8), were labeled by incubation with ^{51}Cr-labeled sodium chromate in barbital buffer, pH 7.4 (50 μCi/1 × 10^9 cells), at 37° for 45 min with constant agitation. The cells were washed (5 × 10 ml of barbital buffer at 4°), resuspended in buffer, and then standardized to a concentration of 1 × 10^9 cells/ml.

To a series of wells in a microtiter test plate was added 20 μl of human complement, C. To each well was added 20 μl of cobra venom factor or column sample, and the plate was incubated at 37° for 1 hr. Another 100 μl of buffer was added to each well, followed by 20 μl of the ^{51}Cr-primed sensitized sheep erythrocytes, EA, with constant agitation. The samples were incubated at 37° for 1 hr and centrifuged, and aliquots of 50 μl were removed from each well.

The amount of lysis was obtained by measuring the amount of ^{51}Cr-label released with a γ-counter. Controls consisting of sensitized cells alone, sensitized cells plus complement, and cells plus water were run concurrently with the described assay. The percentage of inhibition (I) of lysis was calculated from:

$$\%I = 100\left[1 - \frac{\text{cpm released by EA, sample, and C} - \text{cpm released by EA alone}}{\text{cpm released by EA and C} - \text{cpm released by EA alone}}\right] \quad \text{(Eq. 1)}$$

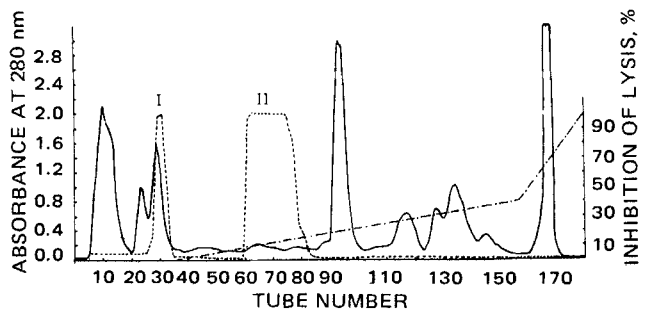

Figure 1—*Carboxymethylcellulose cation-exchange chromatography of dialyzed crude* N. naja *cobra venom. Key: —, optical density; - - -, anticomplement activity; and — · —, sodium chloride gradient (0.0 → 0.2 → 0.5 M).*

Quantitative Assay—The method was essentially the same as the qualitative one, except that serial dilutions of the anticomplement samples were assayed. To a series of wells in a microtiter plate was added 20 μl of human complement, to each of these was added the serially diluted sample, and the plate was incubated at 37° for 1 hr. Each well was diluted with 100 μl of buffer, and the microtiter plate was agitated during these additions. After incubation at 37° for 1 hr, the samples were centrifuged, aliquots of 50 μl were removed from each well, and the amount of lysis was ascertained by the ^{51}Cr-label released. Identical controls to those described were run concurrently.

The percentage of inhibition of lysis was calculated from Eq. 1 and was plotted against the dilution of the complement-consuming material. The dilution of material that produced 50% inhibition of lysis was considered equal to the number of cobra venom factor units in the original 20-μl aliquot of material. By this means, comparison could be made of the purification and activity of the various complement-consuming materials.

Isolation of Cobra Venom Factor—*Carboxymethylcellulose Chromatography*—One gram of lyophilized cobra venom[1] was suspended in 15 ml of 0.05 *M* sodium acetate buffer, pH 5.5, containing 0.02% sodium azide and then dialyzed overnight against two changes of 1 liter each of the same buffer. A column (1.5 × 25.0 cm) of CM-52 carboxymethylcellulose[2] was equilibrated with 0.05 *M* sodium acetate buffer, pH 5.5. The crude dialyzed cobra venom mixture, typically containing 560–760 absorbance units, was applied to the column; elution was performed using 0.05 *M* sodium acetate, pH 5.5.

When no further material was eluted from the column, as shown by absorbance measurements, a sodium chloride gradient was applied. The flow rate of the column was 20 ml/hr, and 4-ml fractions were collected. Each tube was assayed for anticomplement activity. The elution profile and the peaks of activity, I and II, are shown in Fig. 1.

Gel Filtration Chromatography—Lyophilized peak II, obtained from carboxymethylcellulose chromatography, was suspended in 3.5 ml of 0.015 *M* tris(hydroxymethyl)aminomethane–0.15 *M* sodium chloride buffer, pH 8.5, containing 0.02% sodium azide. This sample, containing 28 absorbance units, was applied to a column (1.5 × 85 cm) of Sephadex G-200 equilibrated in the same buffer.

The flow rate was adjusted to 15 ml/hr, and 2.5-ml fractions were collected. Three well-defined protein peaks were obtained (Fig. 2A). The major peak, containing all of the anticomplement activity eluted, possessed a molecular weight slightly less than 150,000, as shown by using human IgG1 as a molecular weight marker. The fraction containing the biological activity was pooled and then lyophilized.

Peak I, obtained from carboxymethylcellulose chromatography was treated in an identical manner as peak II. The elution and anticomplement activity profiles are shown in Fig. 2B.

Diethylaminoethylcellulose Chromatography—One gram of lyophilized cobra venom was suspended in 10.6 ml of 0.05 *M* barbital sodium buffer, pH 8.6, containing 0.05% sodium azide. This solution, containing 967 absorbance units, was filtered through a filtration membrane[3], 1 × 10^5 mol. wt. exclusion limit, and washed thoroughly with 0.05 *M* barbital sodium buffer, pH 8.6. The solution was then concentrated to 15.5 ml, and it possessed 688 absorbance units.

The material was charged on a column (2.5 × 40 cm) of DE-52 diethylaminoethylcellulose[2] equilibrated with 0.05 *M* barbital sodium buffer, pH 8.6. The flow rate of the column was 35 ml/hr, and 5-ml fractions were collected. After the initial protein was eluted from the column, a sodium chloride gradient was applied. The elution and anticomplement activity profiles are shown in Fig. 3.

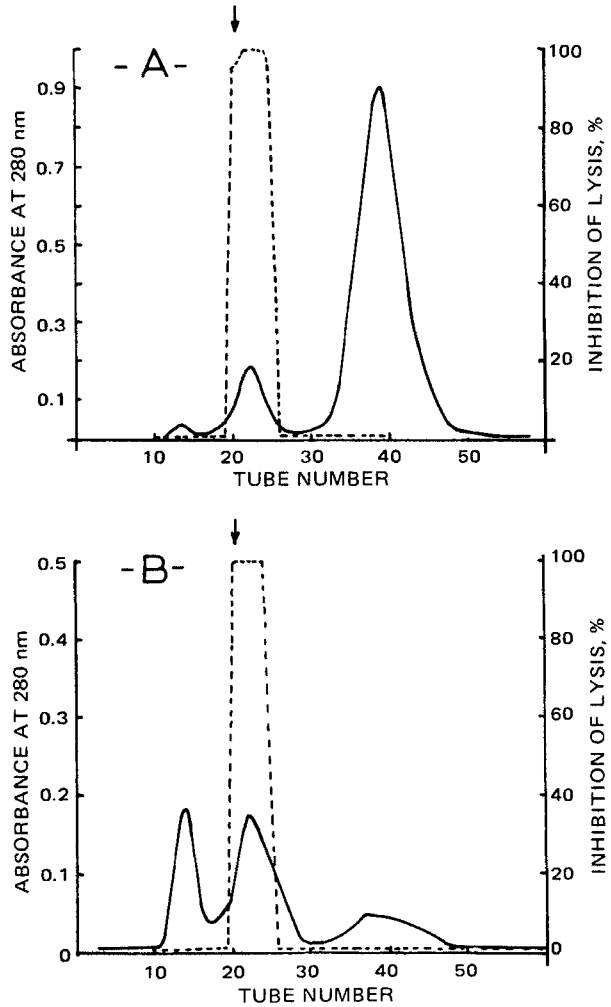

Figure 2—*Sephadex G-200 chromatography of peaks I and II obtained from cation-exchange chromatography (Fig. 1). Figure 2A corresponds to peak I and Fig. 2B to peak II. The molecular weight marker, human IgG1, is indicated by an arrow. Key: —, optical density; and - - -, anticomplement activity.*

Neuraminidase Digestion A—In separate but concurrent experiments, 4.2 ml of anticomplement active peak I, 5.46 absorbance units, and 4.4 ml of anticomplement active peak II, 2.66 absorbance units, obtained from the procedure described under *Carboxymethylcellulose Chromatography* were digested with insolubilized neuraminidase. To each sample in 0.05 *M* sodium acetate–0.02% sodium azide, pH 5.5, was added 1.7 units of insolubilized neuraminidase. Each mixture was incubated at 37° for 1 hr with shaking. At the end of the digestion, the solutions were cleared by centrifugation and the supernates were removed by decantation. Control samples of peaks I and II were treated identically except that buffer was substituted for neuraminidase.

Quantitative anticomplement assays of each peak after neuraminidase treatment and its appropriate control were performed. Peak I possessed 680 cobra venom factor units per absorbance unit without neuraminidase digestion and 640 after digestion. Peak II possessed 695 cobra venom factor units per absorbance unit without neuraminidase digestion and 642 after digestion. A further control to investigate the activity of the insolubilized neuraminidase was also performed. To 0.4 ml of a 4% solution of bovine mucin in 0.05 *M* sodium acetate–0.02% sodium azide buffer, pH 5.5, was added 1.7 units of insolubilized neuraminidase. The digestion was conducted for 1 hr at 37° with mixing and was arrested by removal of the insolubilized enzyme by centrifugation. The presence of sialic acid was determined using 2-thiobarbituric acid as reported by Warren (9) and modified by Aminoff (10).

[1] Ross Allen Reptile Farm, Silver Springs, Fla.
[2] Whatman, Maidstone, Kent, England.
[3] Diaflo.

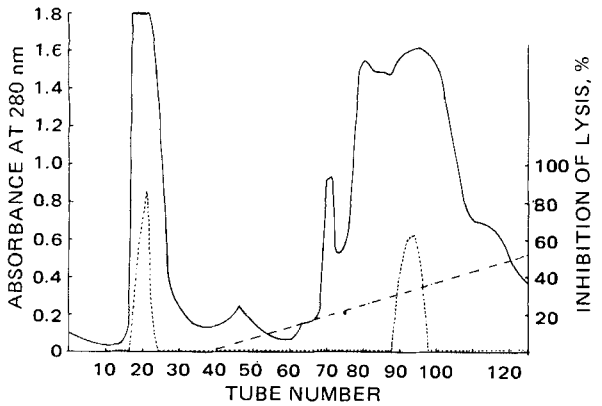

Figure 3—*Diethylaminoethylcellulose anion chromatography of crude* N. naja *cobra venom. Key: —, optical density; - - - , anticomplement activity; and — · —, sodium chloride gradient (0.0 → 0.25* M*).*

Neuraminidase Digestion B—Peaks I and II obtained from the carboxymethylcellulose ion-exchange chromatography were combined. The solution was concentrated using a filtration membrane[3], 30,000 mol. wt. exclusion limit, and then was dialyzed overnight against 1000 ml of 0.05 *M* sodium acetate–0.05% sodium azide buffer, pH 5.5. The solution was centrifuged to give 13.5 ml containing 13.5 absorbance units of material. This material was incubated with 3.4 units of previously calibrated insolubilized neuraminidase for 1 hr at 37° with agitation.

After this digestion period, the enzyme was removed by centrifugation. The supernate, 14.0 ml, 10.4 absorbance units, was chromatographed on a column (2.0 × 37.5 cm) of CM-52 carboxymethylcellulose. The flow rate of the column was 40 ml/hr, and 5.5-ml fractions were collected. Each fraction was assayed for anticomplement activity. Only one peak of anticomplement activity was detected, and it eluted from the column in an identical position to that of peak II before neuraminidase digestion.

RESULTS AND DISCUSSION

The complement fixation assays utilized the quantitation of ^{51}Cr-release by the action of complement on sensitized erythrocytes (5–7). The anticomplement activity of a pertinent sample was ascertained by its prior incubation with human serum and then quantitation of the unconsumed complement (8).

Dialysis of crude *N. naja* venom usually removed up to 25% of the available optical density. Chromatography of the dialyzed cobra venom through a carboxymethylcellulose column gave two peaks, I and II (Fig. 1), of anticomplement activity. Similarly, chromatography of crude *N. naja* cobra venom on diethylaminoethylcellulose gave two peaks of anticomplement activity. Other investigators reported the presence of only a single material with anticomplement activity (11, 12), mol. wt. 144,000 (12).

Each anticomplement material from cation-exchange chromatography (Fig. 1) was subjected to gel filtration chromatography on Sephadex G-200. Both materials eluted in identical positions (Fig. 2), and both had apparent molecular weights of slightly under 150,000. Furthermore, the specific anticomplement activities of each peak were very similar. The interrelationship between peaks I and II (Fig. 1) was shown using neuraminidase digestion.

In separate experiments, peaks I and II were digested with insolubilized neuraminidase (Digestion A); within experimental error, there was no change in the specific activity of either peak. In another experiment (Digestion B), fresh samples of peaks I and II were combined and then treated with insolubilized neuraminidase. Only one peak of anticomplement activity was detected when this digest was subjected to cation-exchange chromatography.

Neuraminidase cleaves sialic acid (*N*-acetyl- and *O*-acetylneuraminic acids) from suitable substrates. However, sterically hindered sialic acids are resistant (13). Neuraminidase did not cause either cobra venom factor I or II to lose anticomplement activity. However, it did change the elution profile of peak I on cation-exchange chromatography such that it eluted in an identical position to that of peak II. These results indicate that these anticomplement materials, peaks I and II, probably differ in their sialic acid compositions.

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ACKNOWLEDGMENTS AND ADDRESSES

Received July 2, 1976, from the *Department of Microbiology, University of Alabama Medical School, Birmingham, AL 35294.*

Accepted for publication August 25, 1976.

Supported by Grant AI 11970 from the National Institutes of Health.

* Recipient of an Institutional Research Fellowship, Award T32-AM07069, from the National Institute of Arthritis, Metabolism, and Digestive Diseases.

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