significantly from one another. The ratio of free fraction values (treated/control) for the 10 matched pairs was 1.06 ± 0.27 (mean ± *SD*), and the correlation of these values between members of each pair was strong (Fig. 2).

Figure 3 is a plot of dicumarol free fraction values in serum against the serum concentration of albumin or total protein. The pronounced interindividual differences in serum protein binding of dicumarol were not related to corresponding differences in the concentration of serum proteins. Similar results were obtained previously for warfarin (2). Also consistent with previous observations (6) was the strong correlation between the free fraction values for dicumarol and warfarin in individual animals ($r = 0.937$, $p < 0.001$, $n = 20$ in this study).

The phenobarbital treatment regimen used is sufficient to cause pronounced enzyme induction, as reflected by the increased total clearance of warfarin[1] and dicumarol (8) and by the increase in relative liver weight. This treatment had no significant effect on the serum protein binding of warfarin and dicumarol. This result could mean that the concentrations of endogenous inhibitors in serum were not affected by enzyme induction or that the interindividual differences in serum protein binding of the coumarin anticoagulants are due to conformational differences of albumin, with enzyme induction having no effect on the steady-state concentration of that particular protein.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XXV: Warfarin–Ibuprofen Interaction in Rats

JOHN T. SLATTERY, AVRAHAM YACOBI, and GERHARD LEVY [x]

Abstract □ The effect of ibuprofen on the pharmacokinetics and anticoagulant action of racemic warfarin was determined in a crossover study on male Sprague–Dawley rats. At average plasma concentrations of 24–83 mg/liter, ibuprofen decreased the biological half-life and increased the total clearance of warfarin. It also increased the anticoagulant effect produced by a given plasma concentration of total (free and protein-bound) warfarin. These effects of ibuprofen appear to be a consequence of its displacing effect on warfarin in plasma.

Keyphrases □ Ibuprofen—effect on pharmacokinetics and anticoagulant action of warfarin, rats □ Pharmacokinetics—warfarin, effect of ibuprofen, rats □ Anticoagulant action—warfarin, effect of ibuprofen, rats □ Warfarin—pharmacokinetics and anticoagulant action, effect of ibuprofen, rats □ Anti-inflammatory agents—ibuprofen, effect on pharmacokinetics and anticoagulant action of warfarin, rats

The widely used nonsteroidal anti-inflammatory agent ibuprofen is a weak acid extensively bound to plasma proteins (1). As such, it may be expected to displace coumarin anticoagulants such as warfarin from plasma protein binding sites. This type of drug interaction can modify the distribution and elimination kinetics of warfarin and produce transient potentiation of its anticoagulant effect (2–4). Nevertheless, several clinical investigations failed to detect any effect of ibuprofen, in doses of up to 2.4 g/day, on the anticoagulant action of the coumarin drugs phenprocoumon and warfarin (5–8).

In view of the recent tendency to use doses of ibuprofen larger than 2.4 g/day for the treatment of inflammatory disease, it is important to determine *in principle* if ibuprofen interacts with warfarin under appropriate conditions (*i.e.*, at plasma ibuprofen concentrations sufficient to displace warfarin from protein binding sites). Such an investigation was carried out on rats dosed with ibuprofen at a rate producing ibuprofen concentrations moderately higher than those commonly encountered clinically.

EXPERIMENTAL

Male Sprague–Dawley rats[1], 250–300 g at screening time and 350–450 g during the interaction study, were maintained on a standard diet[2] with unrestricted access to water.

To determine the *in vitro* effect of ibuprofen on warfarin binding to rat serum protein, blood was obtained from the abdominal aorta of 11 ether-anesthetized rats and the serum was separated. To serum samples from individual rats were added 0.7–0.8 μg/ml of racemic ^{14}C-warfarin[3], 76 μCi/mg, and 0–150 μg/ml of racemic ibuprofen[4]. The free fraction of warfarin in serum (free/total warfarin concentration) was determined by equilibrium dialysis (9).

Sixty-three rats were screened for the *in vivo* interaction study by collecting 3 ml of blood from the tail artery under light ether anesthesia, separating the serum, and determining the free fraction of warfarin by equilibrium dialysis. Fourteen rats with widely differing warfarin free fraction values (0.0023–0.0157) were selected from this group for the

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Charles River Formula 4RF.
[3] Amersham/Searle Corp., Arlington Heights, Ill.
[4] Supplied by The Upjohn Co., Kalamazoo, Mich.

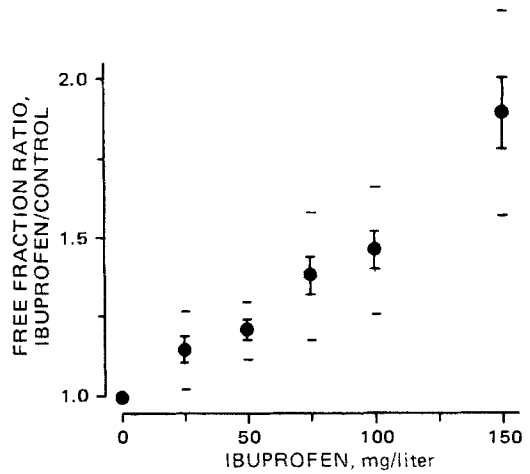

Figure 1—*Effect of ibuprofen on protein binding of warfarin in rat serum. Shown is the relationship between the warfarin free fraction ratio (with ibuprofen/control) and the ibuprofen concentration. Each point is the mean of the free fraction values in individual serum samples from 11 animals. Bars = ±1 SE; dashes = ±1 SD. The concentration of racemic warfarin was 0.718 ± 0.095 mg/liter, and the control free fraction value ranged from 0.00482 to 0.0149.*

interaction study, which was initiated 30 days after the screening experiment.

All animals received racemic ^{14}C-warfarin, 0.45 mg/kg iv. Some animals also received ibuprofen, 40 mg/kg ip at −1 hr and 20 mg/kg ip every 3 hr thereafter; the others received injections of normal saline solution at the same intervals. A crossover experiment was carried out 3 weeks later. The warfarin and ibuprofen injection solutions were freshly prepared by dissolving the drugs in a small amount of sodium hydroxide solution and diluting to the desired volume with isotonic pH 7.4 phosphate buffer.

Blood samples (0.20–0.45 ml) were obtained at frequent intervals from the tail artery (10). Plasma was separated and used for determination of prothrombin complex activity (10), warfarin concentration (11), and ibuprofen concentration (12). Ibuprofen was separated from the plasma extract during TLC separation of warfarin. Ibuprofen reference spots were located on the TLC plate after development by spraying the appropriate region with 0.1% bromocresol purple in water (13). A band of silica coating corresponding to the R_f of ibuprofen[5] was then scraped off,

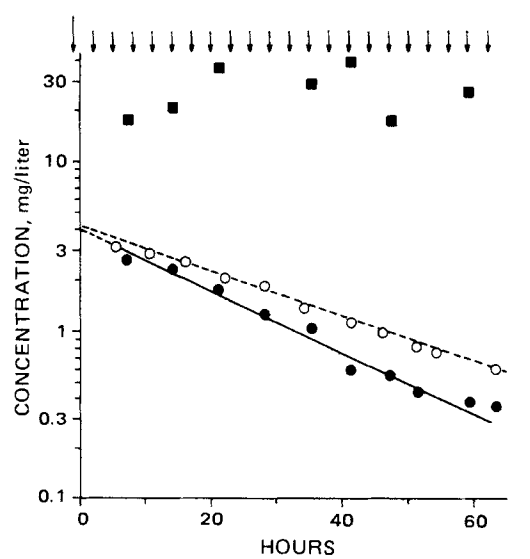

Figure 2—*Effect of ibuprofen administration on the elimination of warfarin in Rat 11. Shown are the warfarin concentrations in serum during the control experiment (○) and during ibuprofen administration (●) 3 weeks later. The ibuprofen concentrations in serum (■) were produced by an initial injection of 40 mg/kg at −1 hr (long arrow) followed by 20 mg/kg every 3 hr (short arrows).*

[5] The R_f of ibuprofen in this system is about 0.35; that of warfarin is about 0.7.

Table I—Concentrations of Ibuprofen in Plasma of Rats Used in the Warfarin Interaction Study[a]

Rat Number	Average Concentration (± *SD*), mg/liter	Number of Determinations
1	71.6 (±32.9)	7
2	68.1 (±27.8)	7
3	33.6 (±26.0)	12
4	38.7 (±31.9)	10
5	78.4 (±43.7)	5
6	71.6 (±27.2)	3
7	60.5 (±31.7)	6
8	48.8 (±28.6)	8
9	47.4 (±38.8)	9
10	82.7 (±40.3)	10
11	23.8 (±9.69)	7
12	42.9 (±32.9)	11
13	39.2 (±26.2)	8
14	54.8 (±28.3)	8
Mean	54.4	

[a] The rats received an initial ibuprofen dose of 40 mg/kg and 20 mg/kg every 3 hr thereafter.

placed in a glass centrifuge tube with 2.5 ml of 0.1 *N* sodium hydroxide, shaken for 20 min, and centrifuged. A 2-ml aliquot of the supernate was acidified with 0.5 ml of 6 *N* hydrochloric acid and extracted (20 min of shaking) into 5 ml of benzene, which was then separated by centrifugation; 4 ml of the benzene phase was used for GLC analysis (12). The recovery of ibuprofen was 90 ± 7.5% (mean ± *SD*, n = 14) and was independent of concentration in the 2–120-μg/ml range.

The effect of ibuprofen on the prothrombin complex activity (PCA) in the serum of otherwise untreated rats was determined by administering ibuprofen, 25 mg/kg ip, every 4 hr to six rats and obtaining blood samples at 12, 24, and 36 hr. The effect of ibuprofen on the rate constant for degradation of prothrombin complex activity, k_{deg}, was determined by administering a prothrombin complex activity synthesis blocking dose (12 mg/kg) of warfarin to 12 rats. Six received ibuprofen, 25 mg/kg ip, every 4 hr from −36 hr to the end of the experiment while the other six received normal saline injections at the same intervals. Blood samples for prothrombin complex activity determination were obtained every 3 hr for 9–12 hr after warfarin administration.

RESULTS

Ibuprofen, at concentrations of 25 mg/liter and above, significantly ($p < 0.001$) decreased the protein binding of warfarin in the serum of rats (Fig. 1). There was a statistically significant ($p < 0.001$) correlation between the control free fraction value and the free fraction value of warfarin at each ibuprofen concentration in individual animals.

Figure 2 shows the effect of ibuprofen administration on the pharmacokinetics of warfarin in a rat. The average plasma ibuprofen con-

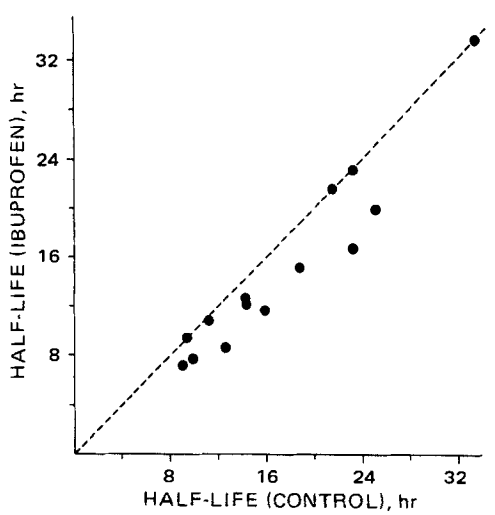

Figure 3—*Relationship between half-life of warfarin in the same animals in a control experiment and during ibuprofen administration in a crossover study. The stippled line has a slope of unity.*

Table II—Effect of Ibuprofen Administration on the Pharmacokinetics of Warfarin in Rats

Parameter	Control Experiment (C)	With Ibuprofen (I)	Ratio, C:I
Half-life, hr	17.3 (9.13–33.5)[a]	15.0 (7.17–33.5)[b]	1.19 (1.00–1.47)
Apparent volume of distribution, ml/kg	155 (98–212)	154 (118–197)[c]	1.01 (0.824–1.21)
Body clearance, ml/hr/kg	7.62 (2.36–13.4)	8.99 (2.56–16.2)[b]	0.851 (0.592–1.00)

[a] Mean (n = 14) and range of individual values. [b] Significantly different from control value by paired t-test ($p < 0.01$). [c] Not significantly different from control value.

centrations observed during the study ranged from 23.8 to 82.7 mg/liter in the 14 animals (Table I). Preliminary studies on six other rats yielded ibuprofen total clearance values from 139 to 220 ml/hr/kg and a half-life of 1.5–3.6 hr after a single 75-mg/kg iv dose. The effect of ibuprofen on the pharmacokinetics of warfarin in all 14 rats is summarized in Table II. Ibuprofen caused a small but statistically significant ($p < 0.01$) decrease in the biological half-life and increase in total body clearance ($p < 0.001$) of warfarin but had no apparent effect on the volume of distribution of the anticoagulant. There was a highly statistically significant correlation between the control and ibuprofen treatment values of half-life ($r = 0.957$, $p < 0.001$; Fig. 3), the apparent volume of distribution ($r = 0.980$, $p < 0.001$; Fig. 4), and the total body clearance ($r = 0.980$, $p < 0.001$; Fig. 5) for warfarin in individual animals.

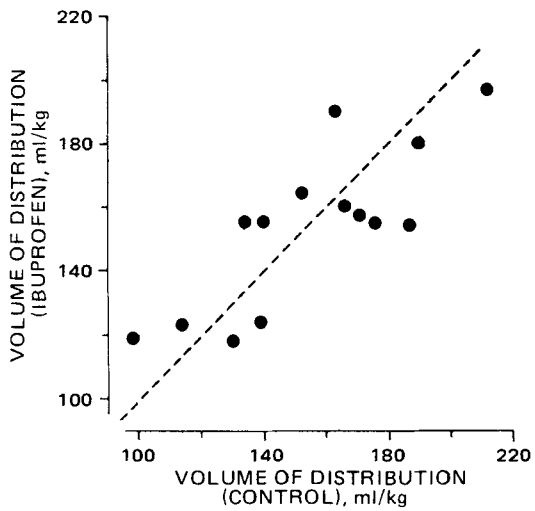

Figure 4—*Relationship between apparent volume of distribution of warfarin in a control experiment and during ibuprofen administration to the same animals in a crossover study. The stippled line has a slope of unity.*

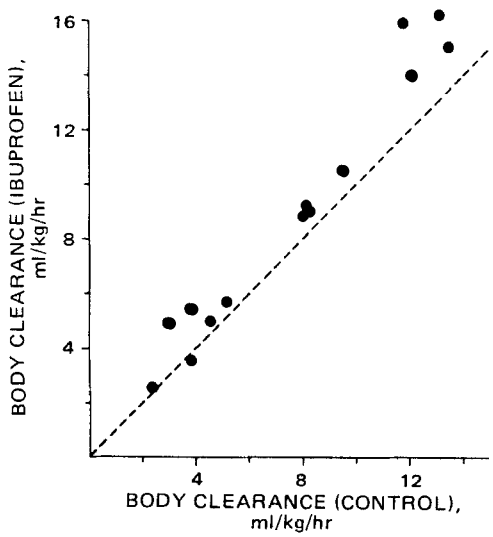

Figure 5—*Relationship between body clearance of warfarin in a control experiment and during ibuprofen administration to the same animals in a crossover study. The stippled line has a slope of unity.*

Table III—Effect of Ibuprofen Treatment[a] on Clotting Time of Plasma in Rats

Duration of Treatment, hr	Clotting Time, sec[b]
0	28.9 ± 0.88
0[c]	28.6 ± 0.66
12	29.3 ± 0.84[d]
24	29.6 ± 1.7[d]
36	28.9 ± 2.3[d]

[a] 25 mg/kg ip every 4 hr. [b] Mean ± SD, n = 8, except at 36 hr when n = 5 (three animals died). [c] Ibuprofen, 0.1 mg/ml, added *in vitro*. [d] Not statistically significantly different from zero-time value.

Ibuprofen, when added to plasma *in vitro* or when administered to the rats, had no apparent effect on the prothrombin complex activity, as reflected by a lack of significant change in the clotting time of plasma (Table III). Ibuprofen administration also had no statistically significant effect on the degradation kinetics of prothrombin complex activity (k_{deg} = 5.48 ± 0.42 days^{-1} in control animals and 5.99 ± 0.63 days^{-1} in ibuprofen-treated animals).

Figure 6 shows the effect of ibuprofen treatment on the relationship between the relative synthesis rate of prothrombin complex activity and the plasma warfarin concentration in one rat. The linear logarithmic regression line can be characterized by its slope and by the concentration required to decrease the relative synthesis rate to one-half of normal (designated as the "effective concentration"). The results obtained in all animals are summarized in Table IV. Ibuprofen caused a significant decrease in the effective concentration and increase in the absolute slope value. There was a statistically significant correlation ($r = 0.974$, $p < 0.001$) between the effective concentrations of warfarin for individual animals in the control experiment and during ibuprofen administration (Fig. 7).

DISCUSSION

Ibuprofen can displace warfarin from serum protein binding sites. Since the total clearance of warfarin by the body is proportional to the free fraction of the drug in serum or plasma (14, 15) and since the anticoagulant action of warfarin is a function of the concentration of free drug

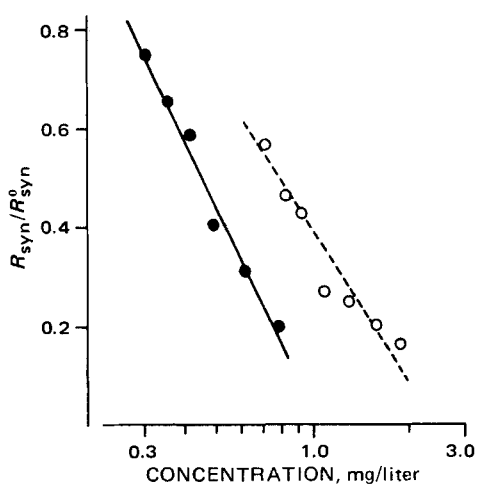

Figure 6—*Effect of ibuprofen administration on the relationship between the relative synthesis rate of prothrombin complex activity (R_{syn}/R^0_{syn}) and warfarin concentration in plasma. Data are from Rat 11 during a control experiment (○) and during ibuprofen administration (●).*

Table IV—Effect of Ibuprofen Treatment on the Relationship between Anticoagulant Effect and Plasma Concentration of Warfarin in Rats[a]

Parameter	Control (C)	Ibuprofen (I)	Ratio, C:I
Effective concentration (concentration when $R_{syn} = 0.5\ R^0_{syn}$), mg/liter	0.598 (0.146–2.03)[b]	0.414 (0.042–1.38)[c]	1.65 (0.730–3.48)
Slope of regression line for R_{syn}/R^0_{syn} *versus* log concentration	−1.01 (−0.455 to −1.69)	−1.31 (−0.851 to −2.06)[d]	0.830 (0.452–1.70)

[a] Crossover study on 13 rats (data on the 14th animal could not be obtained for technical reasons). [b] Mean (range). [c] $p < 0.005$ *versus* control (paired t-test). [d] $p < 0.05$ *versus* control (paired t-test).

(16), it follows that, all else being equal, displacement of warfarin from plasma proteins by ibuprofen should increase the total clearance of the anticoagulant and decrease the plasma concentration required to produce a given degree of anticoagulation. The results of this investigation are consistent with these considerations; ibuprofen treatment caused an increase in the total clearance of warfarin and a decrease in its effective concentration. Since ibuprofen has no apparent inductive effect on drug-metabolizing enzyme systems (1, 17), it is unlikely that the increased total clearance of warfarin was due to enzyme induction.

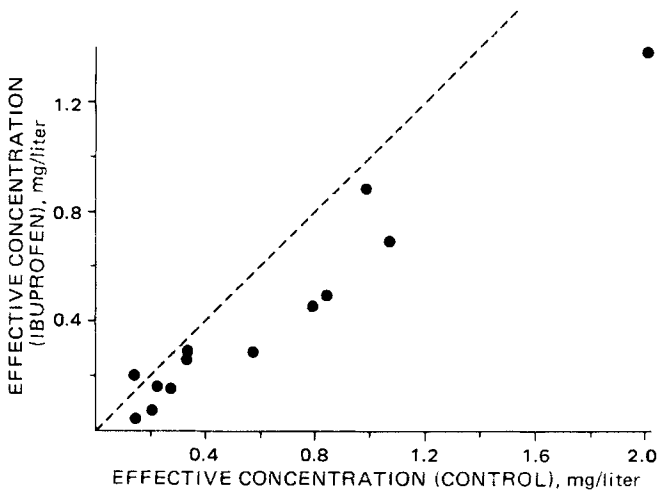

Figure 7—*Relationship between the effective plasma warfarin concentration in rats in a control experiment and during ibuprofen administration to the same animals in a crossover study. The effective concentration is the concentration of total (free and protein-bound) warfarin required to decrease* R_{syn} *to one-half of normal* (i.e., $R_{syn}/R^0_{syn} = 0.5$). *The stippled line has a slope of unity.*

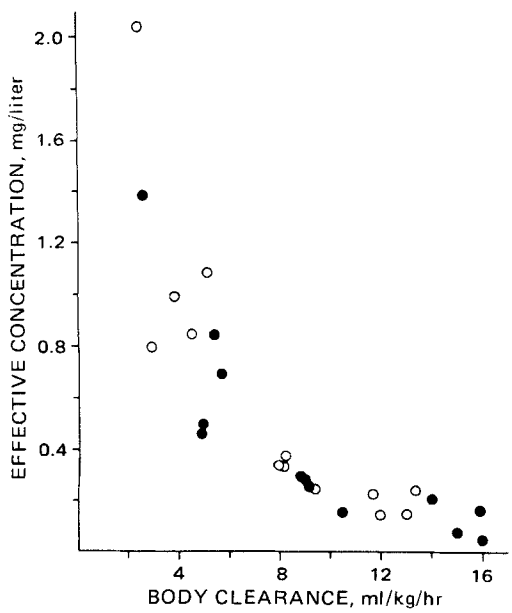

Figure 8—*Relationship between the effective plasma warfarin concentration and the body clearance of warfarin in individual rats in a crossover study. Key:* ○, *control experiment; and* ●, *during ibuprofen administration.*

Due to the short half-life of ibuprofen in rats, the concentrations of this drug fluctuated significantly during the 3-hr interval between injections. The timing of blood sample withdrawals was dictated primarily by the need to characterize the pharmacokinetics and time course of the anticoagulant effect of warfarin. Consequently, blood samples were not obtained at constant times after ibuprofen injection, and this magnified the intra- and intersubject variations in the observed plasma ibuprofen concentrations. For the same reason, the free fraction value of warfarin fluctuated within ibuprofen dosing intervals. These complexities and the need to keep the animals in good condition for the crossover experiment made it impossible to determine the time course of concentrations of free (not protein-bound) warfarin in serum during the *in vivo* interaction study.

It is feasible, however, to determine indirectly if displacement of warfarin from serum proteins is the sole or predominant mechanism of the observed warfarin–ibuprofen interaction or if additional factors contribute significantly to the interaction. Since both the body clearance and anticoagulant effect of warfarin are functions of the free fraction of drug in plasma, the relationship between body clearance and effective concentration should be the same in control and ibuprofen experiments if the interaction is due only to displacement of the protein-bound anticoagulant. As evident in Fig. 8, this appears to be the case. In support of this conclusion are the observations that ibuprofen has no apparent effect on the synthesis and degradation of prothrombin complex activity and that the average increase in total clearance of warfarin (Table II) is of similar magnitude as the increase in warfarin free fraction produced by the observed average ibuprofen concentrations (Table I and Fig. 1).

Based on pharmacokinetic data from humans (12), the steady-state plasma concentration of ibuprofen produced by a daily dose of 2.4 g is estimated to be 30–40 mg/liter. Peak concentrations of ibuprofen during a dosing interval will be appreciably higher, and an increase in the dosing rate will produce a corresponding increase in the steady-state concentration. It was estimated that the binding capacity of albumin in human plasma for ibuprofen is only 120 mg/liter (1). Inflammatory disease is often associated with hypoalbuminemia (18), resulting in a decreased binding capacity of plasma. For these reasons, it is desirable to determine the effect of various concentrations of ibuprofen on warfarin binding in normal and hypoalbuminemic human plasma.

This investigation showed that ibuprofen can, in principle, modify the elimination kinetics and anticoagulant activity of warfarin. These effects appear to be the consequence of decreased serum protein binding of warfarin in the presence of ibuprofen. Since this displacing effect of ibuprofen is concentration dependent, the clinical significance of these findings can only be assessed by determining the effect of therapeutic concentrations of the agent on the plasma protein binding of warfarin (19). Such determinations are in order also for other nonsteroidal anti-inflammatory agents that have recently come into clinical use (20).

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

BRIAN J. JOHNSON [x] and UMBERTO N. KUCICH [x]

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:

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