Title

Phospholipase A2 contamination of cobra venom factor preparations. Biologic role in complement-dependent in vivo reactions and inactivation with p-bromophenacyl bromide.

Journal

American Journal Of Pathology, 91(3)

ISSN

0002-9440

Authors

Shaw, JO
Roberts, MF
Ulevitch, RJ
et al.

Publication Date

1978-06-01

Peer reviewed

Phospholipase A_2 Contamination of Cobra Venom Factor Preparations

Biologic Role in Complement-Dependent In Vivo *Reactions and Inactivation With* p-*Bromophenacyl Bromide*

James O. Shaw, MD, Mary Fedarko Roberts, PhD, Richard J. Ulevitch, PhD, Peter Henson, PhD, and Edward A. Dennis, PhD

Cobra venom factor (CoF), the anticomplementary protein in *Naja naja* cobra venom, is usually purified by sequential ion exchange and gel filtration chromatography. CoF prepared in this manner contains small but significant quantities of phospholipase A_2 activity. This acyl hydrolase activity can be simply and efficiently removed on a large scale by treatment of CoF with *p*-bromophenacyl bromide (BPB), an irreversible modifier of the histidine residue in the active site of phospholipase A_2. BPB treatment does not alter the anticomplementary activity of CoF. *In vivo* experiments utilizing intratracheal injections of control and BPB-treated CoF, as well as pure phospholipase A_2, revealed that contaminating phospholipase A_2, and not the anticomplementary protein, was responsible for the observed acute neutrophil-associated lung injury. However, phospholipase A_2 had no effect on the hypotensive and thrombocytopenic effects of CoF injected intravenously into rabbits. Depletion of circulating C3–C9 by intraperitoneal injections of CoF was not altered by removal of phospholipase A_2 activity with BPB. (Am J Pathol 91:517–530, 1978)

AN ANTICOMPLEMENTARY PROTEIN isolated from *Naja naja* cobra venom, cobra venom factor (CoF), is widely employed as a tool for the elucidation of the role of the complement system in various biologic systems. This 145,000 molecular weight protein activates C3 and the terminal complement sequence by interaction with Factors B and D to form a C3 convertase.[1,2] Since this reaction proceeds at a relatively rapid rate,[2,3] CoF provides a single convenient reagent for *in vitro* and *in vivo* depletion of the complement components C3–C9[4] and has recently been utilized to investigate the pathophysiologic effect of brisk *in vivo* activation of the complement cascade in the circulation.[5,6]

Proper interpretation of results of experiments utilizing CoF depends on the availability of purified material, free of contamination by other

From the Department of Immunopathology, Scripps Clinic and Research Foundation, and The Department of Chemistry, University of California at San Diego, La Jolla, California.

Supported by Grants HLO-5229, HL 21565, AI 07007, and GM-20501 from the National Institutes of Health and by the Council of Tobacco Research. Dr. Roberts is a postdoctoral fellow on Grants GM-07,169 and GM-05,910 from the National Institutes of Health. Dr. Ulevitch is a recipient of USPHS HL 18376 Young Investigator Pulmonary Research Grant.

Accepted for publication February 6, 1978.

Address reprint requests to Dr. James O. Shaw, Department of Medicine, University of Texas, Health Sciences Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284.

0002-9440/78/0608-0517$01.00

proteins possessing biologic activity. The most widely used method of purification of CoF involves sequential ion exchange and gel filtration chromatography of lyophilized venom.[7] These procedures have been reported to separate CoF from other biologically important venom proteins,[8] including phospholipase A_2 (EC 3.1.1.4), a phosphatide acyl hydrolase. This enzyme has biologic significance by virtue of its ability to perturb cell membrane structure and thus potentially alter cell function.[9,10] Although phospholipase A_2 has a low monomeric molecular weight (11,000 to 15,000), it has a propensity to form aggregates [11] and has been reported to interact hydrophobically with the gel matrix in gel filtration procedures.[12] We[5] and others[13] have been unable to prepare phospholipase-A_2–free CoF by ion exchange and gel filtration chromatography, although Lachmann et al [14] have been able to remove most of the contaminating phospholipase A_2 by further purification steps. Recently, Waldmann and Lachmann [13] and Hall et al [15] described phospholipase A_2 contamination of gel-filtration–purified CoF and noted a direct effect of the enzyme in biologic systems designed to assess the anticomplementary activity of CoF.

Because we [16] previously found that the phospholipase A_2 from cobra venom (*Naja naja naja*) can be completely inactivated by treatment with *p*-bromophenacyl bromide (BPB), we have attempted to delineate the optimal conditions for BPB inactivation of the phospholipase A_2 contaminating CoF preparations. We wished to determine the role of contaminating phospholipase A_2 in mediating or modulating *in vivo* CoF effects that would otherwise be ascribed to the anticomplementary activity of this protein. Our results reveal that small amounts of phospholipase A_2 in CoF preparations can be biologically significant in at least one *in vivo* system but that removal of the acyl hydrolase activity can be accomplished easily with BPB treatment and without alteration in the anticomplementary activity of the CoF itself.

Materials and Methods

Rabbits

New Zealand white rabbits, 2 to 2.5 kg, of both sexes were used in the animal experiments. No food or water restrictions for the animals were utilized prior to the experiments.

Cobra Venom Factor

CoF was prepared from lyophilized *Naja naja kaouthia* cobra venom (Biologicals Unlimited, Inc., Baltimore, Md., Lot ENnk-SCRF) by DEAE-Sephadex ion exchange and Sephadex G-200 gel filtration chromatography, as previously described.[7] The protein prepared in this manner is 98 to 99% homogenous when examined by analytic polyacrylamide gel electrophoresis. The anticomplementary activity of CoF was assayed with

normal human serum using sensitized sheep erythrocytes and was expressed as units of activity, as noted previously.[7] In all preparations, 1 mg of CoF contained 300 to 400 units of activity. The CoF was stored at 4 C in 0.01% azide and was stable without loss of activity for up to 4 months. Protein concentration was determined by the micro-Kjeldahl method.[17]

Phospholipase A_2

The purified enzyme was prepared from lyophilized *Naja naja naja* cobra venom as described previously.[11,16] Phospholipase A_2 activity was measured by the pH-stat technique using 6 mM egg phosphatidycholine in mixed micelles within 24 mM Triton X-100 and 10 mM $CaCl_2$.[18] The specific activity of the enzyme from several preparations was 800 to 900 μmole min^{-1} mg^{-1}.

C3 Determination

Rabbit serum C3 concentration was determined by radial immunodiffusion with goat antirabbit C3 antiserum.[19] Serial samples were measured in individual rabbits and expressed as a percentage of the baseline (preinjection level).

Inactivation of Phospholipase A_2

Phospholipase A_2 contamination of CoF preparations was determined in 25-, 50-, and 100-μl samples by the pH-stat technique.[18] Inactivation of phospholipase A_2 in CoF was accomplished by incubation with *p*-bromophenacyl bromide (BPB) (Aldrich Chemical Co., Milwaukee, Wis.). The BPB was dissolved in acetone at a concentration such that after 1 : 20 dilution the final concentration of acetone in the reaction mixture was 5%. CoF (1.25 mg/ml), buffered to the desired pH by addition of one-tenth volume 0.5 M potassium phosphate buffer, was incubated in a closed tube with BPB (0.05 to 0.4 mM) at 25 C and 37 C. At several intervals, 25 or 50 μl of this mixture was removed and assayed for phospholipase A_2 activity. At the end of the total incubation period, each sample was immediately dialyzed against normal saline for 12 to 24 hours and assayed for anticomplementary activity. Inactivation rates, $k_{inactivation}$, of phospholipase A_2 by BPB were derived from least-squares fit of the data to the following pseudo-first-order equation:

$$\log \frac{A}{A_0} = -k_{inactivation}$$

where A/A_0 is the fractional enzyme activity after BPB treatment for time, t.

^{14}C-*p*-Bromophenacyl Bromide

^{14}C-BPB was prepared with a specific activity of 44,000 cpm/μmole.[15] The labeled compound dissolved in acetone was incubated at 25 C with 1 mg CoF at concentrations of 0.1 and 0.4 mM at both pH 6 and pH 8. After 30 minutes the reaction mixture was dialyzed against 1% SDS overnight to remove any noncovalently bound ^{14}C-BPB. The incorporation of ^{14}C-BPB in the CoF preparation was determined by counting the dialyzed protein in a mixture of toluene (1758 ml), Triton X-100 (1000 ml), and Liquifluor (New England Nuclear, Boston, Mass.) (242 ml) in a Packard Tricarb scintillation counter. Binding of ^{14}C-BPB to CoF was expressed as mole/mole of CoF protein, assuming a molecular weight of 145,000 for the CoF.[4]

Biologic Activity of CoF

Intratracheal Injection

Rabbits were gently restrained in the supine position. With the rabbits under local anesthesia with 1% novocaine, a cannula was inserted in the femoral artery for blood

sampling.[5] The rabbit was tranquilized with intravenous methohexital (Brevital, Eli Lilly and Co., Indianapolis, Ind.), and the trachea was cannulated percutaneously immediately inferior to the thyroid cartilage. CoF or phospholipase A_2 diluted in 2 cc saline was injected slowly into the trachea with the rabbit raised to a 60° angle. This elevation was maintained for an additional 5 minutes before the animals were returned to the supine position for the remainder of the experiment. Five hours later, animals were killed with an overdose of pentobarbitol; the lungs were removed and fixed in 10% formalin; and sections were stained with hematoxylin and eosin. Histologic evidence of edema and red blood cell (hemorrhage) and neutrophil accumulation in alveoli was graded on an arbitrary scale of 1+ (trace, minimal change), 2+ (mild, scattered alveoli involved), 3+ (most, but not all, alveoli involved), and 4+ (changes in confluence in alveoli of involved areas). Animals tolerated the procedure well and saline-injected animals demonstrated normal lungs histologically at the time of death.

Intravenous Injection

Two base line arterial blood pressure recordings (Statham SP1400 Blood Pressure Monitor and Statham P37B Pressure Transducer) were obtained from supine restrained rabbits with a catheter in a femoral artery and femoral vein, and arterial blood samples were taken 5 minutes apart. The animals then received a rapidly injected intravenous bolus of CoF; blood pressure measurements plus duplicate 0.5 ml blood samples, one anticoagulated with 2 units of heparin, were obtained at various times after injection.[5] Alteration in circulating platelets was assessed as described previously[19] by changes in circulating ^{51}Cr-labeled rabbit platelets injected 18 hours prior to the experiment.

Intraperitoneal Injection

Rabbits were depleted of circulating C3–C9 by intraperitoneal injection of 600 units CoF or BPB-treated CoF administered in four divided doses over 24 hours.[5,19] Plasma C3 levels were assayed by radial immunodiffusion before, during, and after the injection series.

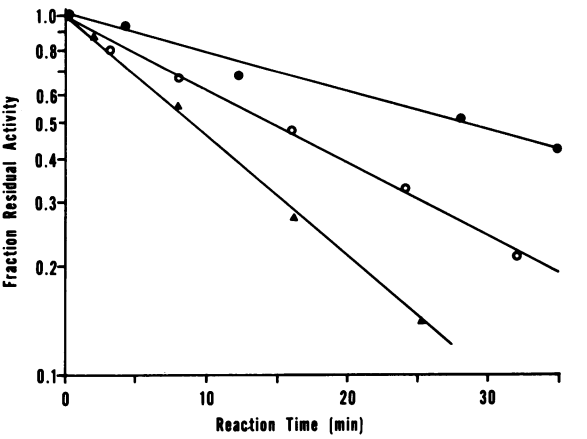
Scanning Electron Microscopy

Lungs from rabbits injected with phospholipase A_2 were fixed by intratracheal inflation of 25 cm H_2O pressure with 2% glutaraldehyde in Sörensen's phosphate-buffered saline for 45 minutes at 22 C. Blocks were dehydrated in ethanol, followed by Freon 113; critical-point dried in a Bomar-SPC-900/EX apparatus; and coated with gold–palladium in a Hummer Technics sputter coater. Specimens were viewed and photographed with a Hitachi S-500 scanning electron microscope.

Results

Phospholipase Activity in CoF

We first sought to determine the amount of phospholipase A_2 in CoF preparations. Examination of CoF prepared by ion exchange and gel filtration chromatography revealed small, but significant, quantities of contaminating phospholipase A_2, when assayed by the pH-stat technique.[18] Levels of enzyme activity from different CoF preparations were very similar, with a mean ± SEM (μmole min^{-1} mg^{-1} protein) of 3.0 ± 0.2 for seven separate CoF preparations (range, 2.5 to 3.9). This represents less than 0.4% phospholipase A_2 contamination of CoF, assuming similar



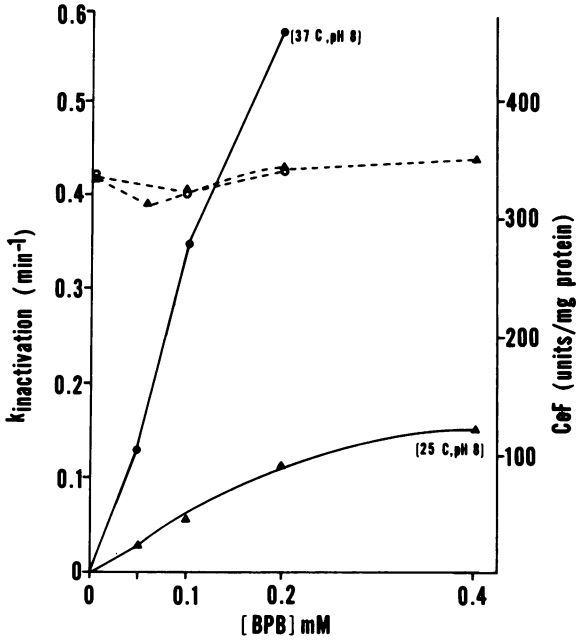
TEXT-FIGURE 1—Disappearance of phospholipase A_2 activity from CoF as a function of pH. CoF (1.25 mg/ml) was reacted with 0.1 mM *p*-bromophenacyl bromide (BPB) in 5% acetone at 25 C in 0.05 M potassium phosphate buffer at the desired pH. At the appropriate time, 50-μl aliquots were removed and assayed for phospholipase A_2 activity. Fractional residual activity was calculated based on the enzyme activity just prior to BPB addition. *Solid circles*, pH 6; *open circles*, pH 7; *triangles*, pH 8.

specific activity between this enzyme and that of the purified *Naja naja naja* phospholipase A_2.[11]

BPB Inhibition of Contaminating Phospholipase A_2

We examined the characteristics of the reaction of BPB with the phospholipase A_2 in CoF and any effects this reagent might have on the anticomplementary activity of the CoF itself. When CoF was incubated with BPB, phospholipase A_2 activity disappeared in a temperature- and pH-dependent manner. As demonstrated in Text-figure 1, at 25 C and with a large excess of BPB over protein the inactivation reaction was pseudo-first-order and pH-dependent. The inactivation rate constant, $k_{inactivation}$, determined over a range of inhibitor concentrations (0.05 to 0.4 mM) at pH 8, was clearly concentration-dependent. This was markedly accentuated when the reaction temperature was raised from 25 C to 37 C (Text-figure 2). All samples from the reactions depicted in Text-figure 2 had less than 10% of the original phospholipase A_2 activity (except 0.05 mM BPB, 25 C, 40% activity remaining) after 60 minutes of incubation. In sharp contrast, however, was the lack of any effect of BPB on the anticomplementary activity of CoF (Text-figure 2) even when 0.4 mM inhibitor concentration was utilized at 37 C, pH 8 (data not shown). Our current phospholipase inactivation reactions for all CoF preparations are performed at pH 8, 37 C, for 30 minutes with 0.1 mM BPB in 5% acetone. Under these conditions, in over 15 experiments, all detectable phospholipase activity has been removed without alteration in the anticomplementary titer of the CoF.

^{14}C-BPB was incubated with CoF to determine the extent of BPB reaction with CoF itself. Under conditions too insensitive for detection of ^{14}C-BPB reaction with the small amounts of contaminating phospholipase



TEXT-FIGURE 2—Concentration-dependent phospholipase A_2 inactivation rates and effects on anticomplementary activity in BPB treatment of CoF. CoF (1.25 mg/ml) was reacted, at pH 8, with different concentrations of BPB at 37 C and 25 C, and samples were removed at appropriate times for phospholipase A_2 assay. Each data point (*solid lines*) represents the rate constant $k_{inactivation}$, determined (see *Materials and Methods*) from at least four points on the activity disappearance plot. After 60 minutes, samples were dialyzed against normal saline and assayed for anticomplementary (*dotted lines*) as described in *Materials and Methods*.

A_2, 0.9 to 1.2 (pH 6) and 1.8 to 2.3 (pH 8) moles ^{14}C-BPB were incorporated in each mole of CoF (two experiments at each pH).

Biologic Activity of Phospholipase in CoF

Since the contaminating phospholipase A_2 could be readily inactivated with BPB without alteration in CoF anticomplementary activity, we investigated the influence of this enzyme on the *in vivo* effects of CoF. CoF was introduced into rabbit lungs in an attempt to induce alveolar neutrophil accumulation through local activation of the complement cascade and generation of chemotactic fragments. When CoF was injected intratracheally, it induced a marked intrapulmonary inflammatory reaction characterized on light microscopy by neutrophil accumulation, edema, and hemorrhage within alveoli (Figure 1). This was accompanied by a blood neutrophilic leukocytosis (data not shown) and a fall in circulating C3 levels (Table 1). Heated CoF, which retained 85 to 90% of its phospholipase A_2 activity but was devoid of anticomplementary activity, retained its ability to cause lung injury. However, removal of phospholipase activity with BPB treatment of the CoF abrogated all lung proinflammatory potential of the CoF (Figure 2). The lung injury was reproduced by transtracheal injection of purified *Naja naja naja* phospholipase A_2 in amounts possessing hydrolase activity comparable to that found in

Table 1—Role of Phospholipase A_2 Contamination of CoF in the Induction of Acute Lung Injury

Reagent*	Phospholipase A_2† content	Histology‡ PMN accumulation	Hemorrhage	Edema	C3§ (% control)
Saline (4)	0	0	0	0	97 ± 2
CoF, untreated (6)	1.1	3+	2+	2+	54 ± 2
CoF, heated‖ (4)	1.0	3+	2+	2+	91 ± 6
CoF, BPB-treated ¶ (6)	< 0.05	0	0	0	56 ± 3
Phospholipase A_2 (6)	1.0	3+	2+	2+	ND

* All reagents diluted in 2 cc sterile saline-CoF, 100 units/animal; phospholipase A_2 *(Naja naja naja)* 1.2 μg/animal. Number of animals for each experiment is indicated in parentheses.
† μmole min^{-1} phosphatidycholine hydrolyzed by 100 units of CoF
‡ Graded as described in *Materials and Methods*
§ Quantitated by radial immunodiffusion; mean ± SEM for plasma values 5 hours after CoF injection
‖ 80 C for 2 minutes.
¶ 0.1 mM BPB, pH 8, 37 C, for 30 minutes, followed by dialysis
ND = not determined.

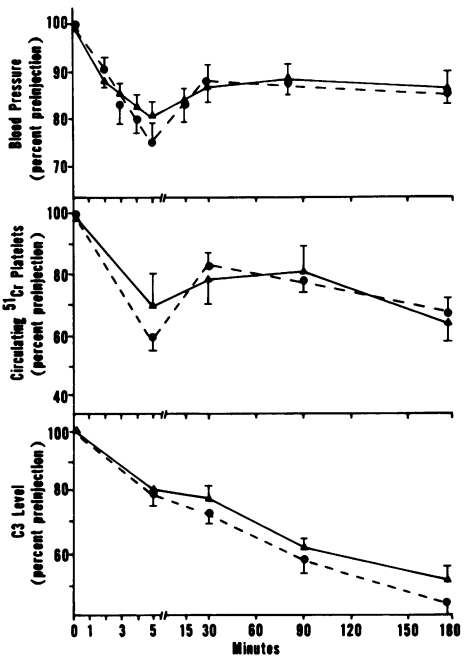
CoF (Table 1, Figure 3). Scanning electron microscopy demonstrated in this group the presence of fibrin strands in areas with intra-alveolar phagocyte and red cell accumulation (Figure 4).

Intravenous injection of CoF induces an acute reversible fall in blood pressure and circulating platelet levels.[5] As depicted in Text-figure 3, removal of all phospholipase A_2 activity in the CoF with 0.1 mM BPB failed to significantly affect the magnitude or kinetic relationship of the fall in systemic blood pressure and circulating ^{51}Cr platelet levels induced by the rapid intravenous in injection of 400 units of CoF. As expected, disappearance of circulating C3 was not affected by removal of phospholipase A_2 activity with BPB (Text-figure 3, lower panel).

Although not shown here, control and BPB-treated CoF preparations induced identical, reproducible circulating C3 depletion when injected intraperitoneally in rabbits in four divided doses over 24 hours. Both control and BPB-treated CoF groups (6 animals each) were greater than 95% depleted of serum C3 by 30 hours after the first injection.

Discussion

In this study we have confirmed other reports indicating that phospholipase A_2 is present in small (0.4%) but significant amounts in cobra venom factor prepared by ion exchange and gel filtration chromatography. Our inhibition studies revealed rapid efficient removal of the contaminating phospholipase activity by *p*-bromophenacyl bromide without alteration in the anticomplementary activity of CoF. Finally, we demonstrated that the phospholipase contamination is biologically significant in at least one *in*



TEXT-FIGURE 3—Effects of removal of phospholipase A_2 activity on hemodynamic, thrombocytopenic, and C3 depletion effects of intravenously injected CoF. CoF (400 units) previously reacted with 0.1 mM BPB in 5% acetone (*circles*) or with 5% acetone alone (*traingles*) for 30 minutes at 37 C, pH 8, and then dialyzed was rapidly injected intravenously into unanesthetized rabbits injected 18 hours previously with ^{51}Cr platelets. At appropriate times after injection, arterial blood pressure (*top panel*) was obtained from a femoral artery catheter and blood samples were removed for determination of platelet survival (*middle panel*) and C3 levels (*bottom panel*). Five animals received 5% acetone alone; six animals received 0.1 mM BPB in 5% acetone.

vivo model (CoF-induced acute lung injury) designed to investigate the anticomplementary activity of CoF.

Phospholipase A_2 Contamination of CoF

Contrary to a report by Pepys,[7] we [5] and others [13-15] have found small but biologically significant amounts of phospholipase A_2 activity in preparations of CoF purified by DEAE-Sephadex and Sephadex G-200 chromatography. Although gel filtration has proved useful for removing other contaminants from the 145,000 molecular weight anticomplementary protein,[6,7] it has not provided any major advantage in this study over the ion exchange step for separation from phospholipase, as the activity levels after gel filtration (3.0 μmole min^{-1} mg^{-1} protein) are similar to those reported earlier by one of us after DEAE Sephadex chromatography.[5] However, Lachmann et al have reported a 10-fold improvement in the ratio of CoF to phospholipase activity between the ion exchange and gel

filtration chromatography steps.[14] This discrepancy may possibly be explained by the different phospholipase assay techniques utilized in the latter study.

The inability of gel filtration to separate completely the 11,000 molecular weight enzyme from the 145,000 molecular weight CoF could be secondary to several factors. Cobra venom phospholipase A_2 has been shown to undergo concentration-dependent aggregation.[12,20] Although contaminating phospholipase is present in extremely low concentrations (approximately 4 μg/mg CoF), the relatively high (1 to 3 mg/ml) concentration of protein typically present in the anticomplementary activity peak in the Sephadex G-200 elution profile may cause the phospholipase to aggregate to higher order species.

The CoF purified by gel filtration retains phospholipase A_2 activity even after extensive dialysis (monomeric enzyme would be expected to pass through the dialysis membrane). Perhaps phospholipase A_2 has an affinity for the CoF itself, and it is this more specific interaction that is responsible for the contaminating phospholipase A_2 activity. Alternatively, the isozyme of phospholipase A_2 that is retained may be a much higher molecular weight form than is usually purified and examined, as has been suggested by Lachmann et al.[14] The aberrant elution of the enzyme during gel filtration could be due to hydrophobic interaction of the enzyme with the Sephadex matrix, a factor noted by Visser and Louw[12] to impede the separation of cobra venom cardiotoxin from contaminating phospholipase A_2.

Inactivation of Phospholipase With *p*-Bromophenacyl Bromide

p-Bromophenacyl bromide modifies a histidine residue in or near the active site of the enzyme and demonstrates half-site reactivity with pure cobra venom phospholipase A_2.[16,21] Our data revealed efficient pseudo-first-order inactivation of the phospholipase in CoF by BPB. Inactivation rates were pH- and temperature-dependent with optimal conditions for complete inactivation being 37 C and pH 8. At 25 C, reaction rates in the CoF preparations were approximately one third those seen with BPB and purified cobra venom phospholipase A_2.[16] The slower rates could be due to the previously mentioned enzyme aggregation in the presence of CoF and resultant inhibitor inaccessibility or just the presence of another protein.

The importance of BPB as a simple reliable agent for removing phospholipase A_2 activity in CoF is underscored by the data presented in Text-figure 2 demonstrating a lack of effect of this agent on CoF anticomplementary activity. This result is interesting in light of the ^{14}C-BPB indicating reaction of a small amount of the inhibitor with the CoF

protein itself. Thus, BPB treatment of CoF provides a rapid, efficient method for removal of contaminating phospholipase A_2 activity and circumvents the need for more laborious and less efficient purification procedures such as hydroxyapatite fractionation,[18] preparative polyacrylamide gel chromatography,[5] or preparative isoelectrofocusing.[14] For *in vivo* experiments, BPB can be easily removed from CoF by dialysis, whereas separation of ampholines from CoF prepared by isoelectrofocusing may prove more difficult.

Role of Phospholipase A_2 in *In Vivo* Effects of CoF

Since venom phospholipase A_2 activity hydrolyses phospholipids at lipid water interfaces, it predictably has potent biologic effects on cell membranes, inducing membrane permeability changes and alteration of cell responses to external stimuli.[9,10] In addition to direct effects on membranes, phospholipase A_2 in CoF may interact with plasma components to produce biologically active substances.[13,15]

In our studies, CoF was injected transtracheally into rabbits in an attempt to activate the alternative complement pathway at the alveolar capillary membrane, generate chemotactic fragments locally,[22] and induce neutrophil accumulation and lung injury. Although CoF injections initiated pulmonary inflammation and depletion of circulating C3, our data (Table 1) indicate that the histologic changes noted in the lungs were not due to the anticomplementary activity of CoF but to the contaminating phospholipase A_2. This was illustrated by the proinflammatory potential of heated CoF and pure phospholipase A_2 and the abrogation of this activity when CoF was treated with BPB. The mechanism of phospholipase-A_2–induced intrapulmonary injury must remain speculative at this time, but preliminary experiments have failed to demonstrate neutrophil chemotactic activity for phospholipase A_2 incubated alone or with phospholipid-rich bronchial wash fluid.[23]

In contrast to the results obtained with transtracheal injection of CoF, intravenous injection produced hemodynamic and cellular changes which were not dependent on the presence of phospholipase activity. As indicated previously,[5] the rapid fall in blood pressure and circulating platelets requires a burst of circulating C3 activation. The data presented in Text-figure 3 and in an earlier report [5] clearly indicate the lack of any modulating effects of phospholipase A_2 on the pathophysiologic effects of rapid intravascular activation of the alternative complement pathway. The absence of any changes in circulating ^{51}Cr platelet kinetics in the presence of phospholipase is intriguing in light of recent reports of alterations in *in vitro* platelet physiology induced by venom phospholipase A_2.

However, large concentrations of enzyme (40 μg/ml), relative to the amount in CoF, were needed to induce the *in vitro* platelet changes.[10]

Since intraperitoneal injection of CoF is employed widely to deplete animals of complement components,[5,19,24] we compared the potential of intraperitoneally injected untreated and BPB-treated CoF to deplete circulating C3. As predicted from our other *in vitro* (Text-figure 2) and *in vivo* (Text-figure 3, Table 1) data, phospholipase A_2 removal had no effect on C3 depletion by the intraperitoneal route. In other studies, BPB treatment of CoF has not altered the CoF-induced decrement in splenic deposition of circulating antigen [25] or affected the ability of CoF to abrogate the pathophysiologic changes in an animal model of autoimmune myasthenia gravis.[26]

Therefore, our results confirm that CoF prepared by ion exchange and gel filtration chromatography is contaminated with small, albeit biologically significant, amounts of phospholipase A_2. This enzyme can be irreversibly inactivated by a brief incubation with *p*-bromophenacyl bromide with retention of full anticomplementary activity. *p*-Bromophenacyl bromide, thus, is a useful reagent for preparing biologically homogenous cobra venom factor.

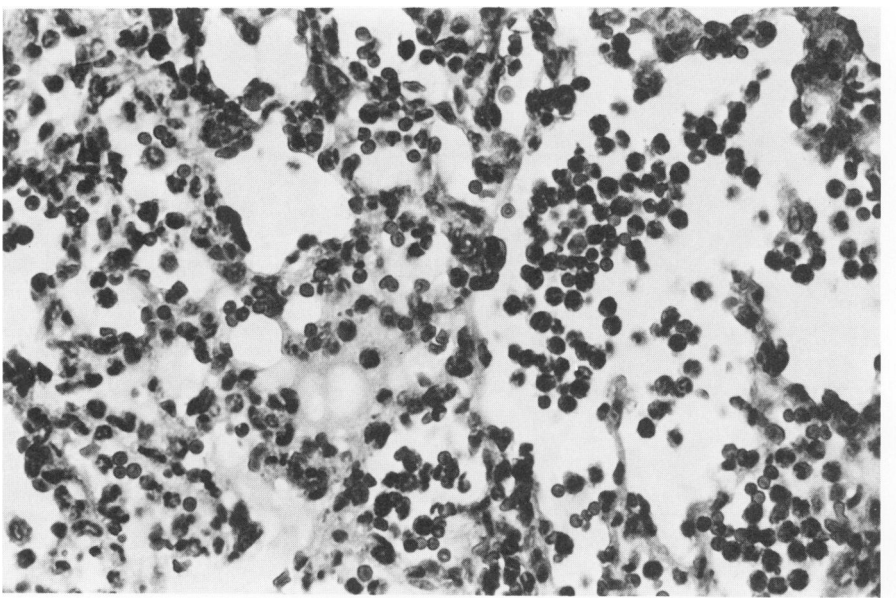
References

1. Cooper NR: Formation and function of a complex of the C_3 proactivator with a protein from cobra venom. J Exp Med 137:451–460, 1973
2. Dierich MP, Hadding U, König W, Limbert M, Scholrlemmer H-U, Bitter-Suermann D: Factor D in the alternate pathway of complement activation: Purification, physicochemical characterization and functional role. Immunochemistry 11:527–532, 1974
3. Cochrane CG, Müller-Eberhard HJ, Aikin BS: Depletion of plasma complement *in vivo* by a protein of cobra venom: Its effect on various immunologic reactions. J Immunol 105:55–69, 1970
4. Müller-Eberhard HJ, Fjellström K-E: Isolation of the anticomplementary protein from cobra venom and its mode of action of C_3. J Immunol 107:1666–1672, 1971
5. Ulevitch RJ, Cochrane CG: Complement dependent hemodynamic and hematologic changes in the rabbit. Inflammation 2:199–216, 1977
6. McCall CE, DeChatelet LR, Brown D, Lachmann P: New biological activity following intravascular activation of the complement cascade. Nature (Lond) 249:841–843, 1974
7. Ballow M, Cochrane CG: Two anticomplementary factors in cobra venom: Hemolysis of guinea pig erythrocytes by one of them. J Immunol 103:944–952, 1969
8. Pepys MB: Role of complement in the induction of immunological responses. Transplant Rev 32:93–120, 1976
9. Tu AT: Venoms: Chemistry and Molecular Biology. New York, John Wiley & Sons, Inc., 1977, p 52
10. Vincent JE, Zijlstra FJ: Biphasic effects of phospholipase A_2 on platelet aggregation. Effect of prostaglandin synthesis inhibitors and essential fatty acid deficiency. Prostaglandins 12:971–979, 1976
11. Deems RA, Dennis EA: Characterization and physical properties of the major form

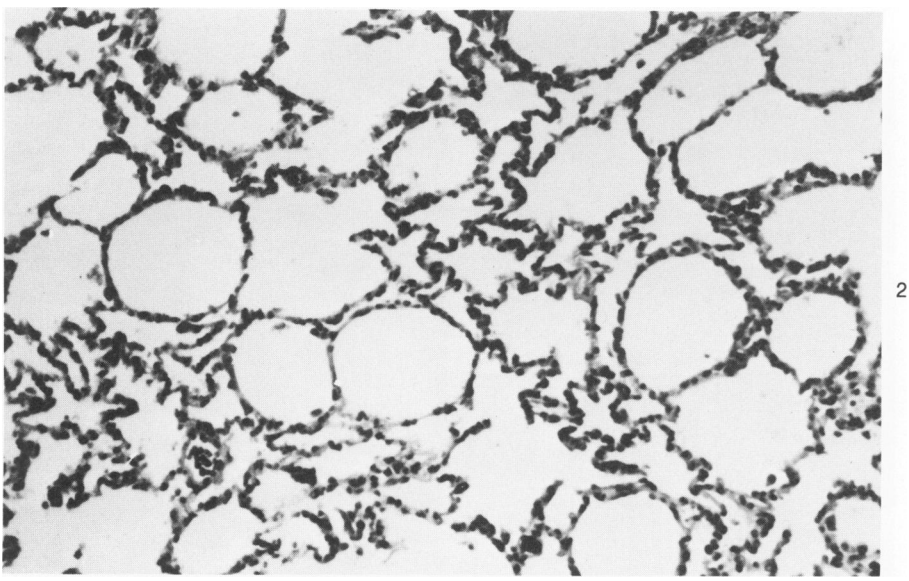
of phospholipase A_2 from cobra venom (*Naja naja naja*) that has a molecular weight of 11,000. J Biol Chem 250:9008–9012, 1975
12. Visser L, Louw AI: The importance of protein structure and conformation in the preparation of phospholipase-free cardiotoxin from snake venom. Biochim Biophys Acta 491:349–353, 1977
13. Waldmann H, Lachmann PJ: The failure to show a necessary role for C_3 in the *in vitro* antibody response. Eur J Immunol 5:185–193, 1975
14. Lachmann PJ, Halbwachs L, Gewurz A, Gewurz H: Purification of cobra venom factor from phospholipase A contaminant. Immunology 31:961–968, 1976
15. Hall JL, Rowlands DT, Nilsson UR: Complement-unlike hemolytic activity in lobster hemolymph. J Immunol 109:816–823, 1972
16. Roberts MF, Deems RA, Mincey TC, Dennis EA: Chemical modification of the histidine residue in phospholipase A_2 (*Naja naja naja*). A case of half-site reactivity. J Biol Chem 252:2405–2411, 1977
17. Ferrari A: Nitrogen determination by a continuous digestion and analysis system. Ann NY Acad Sci 87:792–800, 1960
18. Dennis EA: Kinetic dependence of phospholipase A_2 activity on the detergent Triton X-100. J Lipid Res 14:152–159, 1973
19. Ulevitch RJ, Cochrane CG, Henson PM, Morrison DC, Doe WF: Mediation systems in bacterial lipopolysaccharide-induced hypotension and disseminated intravascular coagulation. I. The role of complement. J Exp Med 142:1570–1590, 1975
20. Roberts MF, Deems RA, Dennis EA: Spectral perturbations of the histidine and tryptophan in cobra venom phospholipase A_2 upon metal ion and mixed micelle binding. J Biol Chem 252:6011–6017, 1977
21. Roberts MF, Deems RA, Dennis EA: Dual role of interfacial phospholipid in phospholipase A_2 catalysis. Proc Natl Acad Sci USA 74:1950–1954, 1977
22. Ruddy S, Austen KF, Goetzl EJ: Chemotactic activity derived from interaction of factor D and B of the properdin pathway with cobra venom factor or C3b. J Clin Invest 55:587–592, 1975
23. Shaw J: Unpublished observations
24. Henson PM, Cochrane CG: The effect of complement depletion on experimental tissue injury. Ann NY Acad Sci 256:426–440, 1975
25. Romball C, Ulevitch R, Weigle WO: The role of complement in the regulation of antibody production *in vivo*. (In preparation)
26. Lennon V: Myasthenia gravis: A prototype immunopharmacological disease. Proceedings of International Symposium on Organ Specific Autoimmunity. Verona, Italy, Menarini Foundation, 1977 (In press)

Acknowledgments

The authors would like to thank Kimie Hirabayashi and Raymond Deems for technical assistance during this study and Mrs. Margaret Stone for secretarial assistance during the manuscript preparation. We would also like to thank Gay Lee, Gerry Sandford, and Karen Prescott for preparation of the illustrations.

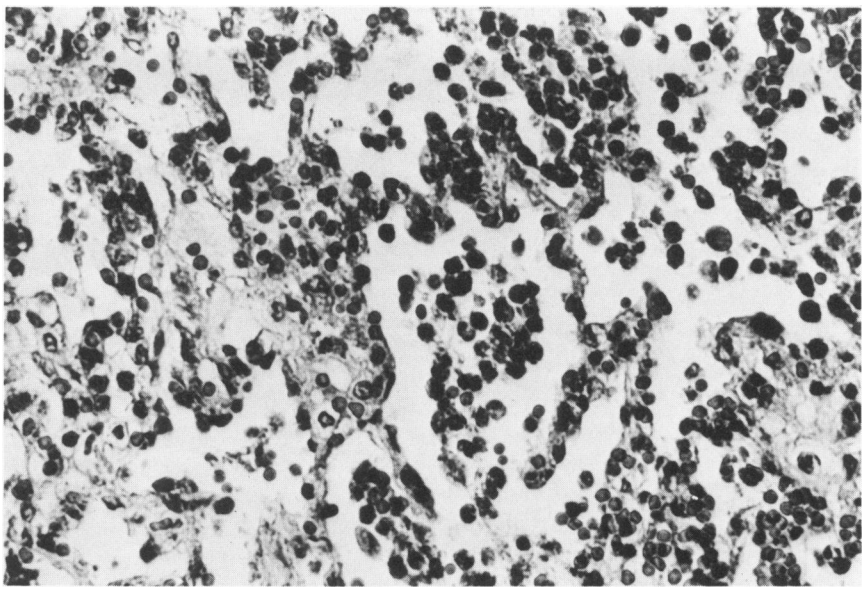


1



2

Figure 1—Neutrophil, red cell, and edema accumulation with air spaces in a rabbit injected intratracheally with 100 units of untreated CoF. (H&E, × 250) **Figure 2**—Findings typical of all lung fields in rabbits injected with 100 units of BPB-treated CoF. (H&E, × 100)



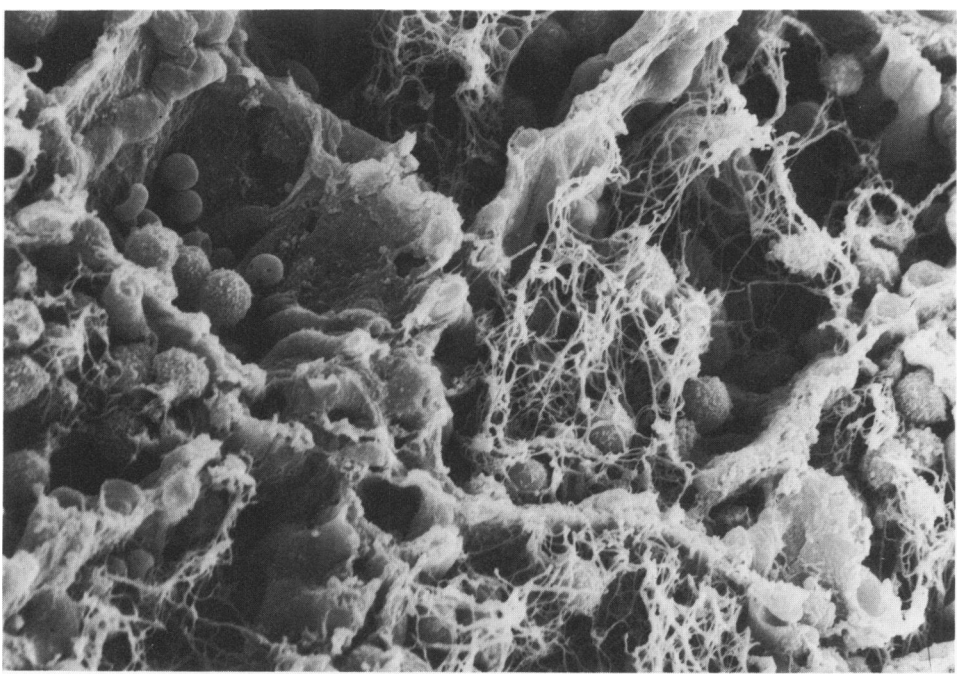


Figure 3—Lung inflammatory changes produced in rabbits injected with 1.2 μg *Naja naja naja* phospholipase A_2. (H&E, × 250) **Figure 4**—Scanning electron microscopic view of lung from a rabbit injected with phospholipase A_2, demonstrating fibrin strands in alveoli containing phagocytes and red cells. (× 800)