Nonimmune Chemotaxis In Vivo

Inhibition by Complement Depletion with Cobra Factor

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Wistar rats were depleted of complement components using purified cobra venom factor (CoF) administered over a 30-hour period by intraperitoneal injection. Complement depletion was confirmed by immunodiffusion assay, hemolytic assay and inhibition of the reverse Arthus reaction. Rats depleted to below 3% of their normal serum complement level showed marked inhibition of chemotaxis into inflammatory fluid produced in polyvinyl sponges 24 hours after implantation into the dorsal subcutaneous region. Subsequent studies were done to test the effect of high local concentrations of CoF on the microcirculation, the neutrophil and the connective tissue. Local exposure of these tissues and cells to CoF *in vivo* had no inhibiting effect on chemotaxis. Cobra venom factor did not affect neutrophil survival *in vitro* or *in vivo*. The most likely hypothesis is that CoF works by depleting complement and that *in vivo* chemotaxis of the nonbacterial, nonimmune type is complement dependent to the extent of 60 to 80%. The system described allows for quantitative determination of chemotaxis *in vivo* (Am J Pathol 73: 807–816, 1973).

THE MECHANISM of polymorphonuclear neutrophil chemotaxis has been attributed to several *in vitro* active chemotactic substances which are also present in inflammatory areas *in vivo*.[1] A simple quantitative system for evaluating the role of these factors *in vivo* is presented in this paper. Hill and Ward[2] have used histologic studies of tissue sections of myocardial infarcts in cobra venom factor (CoF)-treated rats and found that, "few if any, neutrophils accumulated in the infarcted tissue" at 24 hours. They believed that complement activation through cleavage of C3 to a series of chemotactic polypeptides by tissue proteases was the major *in vivo* chemotactic stimulus during the first 24 hours of inflammation. The role of such *in vitro* active substances as C567,[1] collagen fragments,[1] prostaglandins[3] and kinins[1] was not felt to

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be important in the sterile inflammatory reaction following tissue necrosis in the rat heart.

Polyvinyl sponge implantation [4] has been used for the last 17 years for the study of proliferating mammalian connective tissue physiology and biochemistry. Sponges have usually been removed after 14 to 28 days when they contained sufficient numbers of fibroblasts. During serial histologic [5] studies of implanted sponges during the first 2 weeks after implantation, we noted that the initial event during the first 24 hours was the filling of the sponge interstices with inflammatory fluid rich in polymorphonuclear leukocytes. In parallel with this migration of neutrophils into the sponges, the surrounding areolar connective tissue was also extensively infiltrated with such cells. Removal of sponge fluid by gentle compression after 24 hours has provided an abundant supply of neutrophils in 98% purity. Cell counts have usually ranged between 10 and 18 thousand cells/cu mm. We have found that this simple system provides an excellent quantitative method of measuring leukotaxis *in vivo*.

Materials and Methods

Ionagar No. 2 was a product of Consolidated Laboratories. Sheep red cells were obtained from the Animal Blood Center (Syracuse). Sheep cell hemolysin was a product of the Hyland Division of Travenol Laboratories Inc. Cobra venom (*Naja naja*) in 1-g vials was obtained from the Ross Allen Reptile Institute (Silver Springs, Florida). Anti-bovine serum albumin (BSA) serum was purchased from Pentex (Miles Laboratories), and crystalline BSA was obtained from Armour. Female Wistar rats weighing 200 g each were obtained from Charles River Breeding Labs. All other chemicals used were of reagent grade.

Complement Depletion

Two-hundred-gram female rats were assigned to four groups of 8 animals each. These were kept on an *ad lib* diet of Purina rat checkers and water. Animals were kept 4 to a cage. One group of rats (group A) was given 300 units/kg of purified cobra venom factor (CoF), prepared according to Ballow,[6] by six injections intraperitoneally over a 30-hour period. CoF was dissolved in .15 M NaCl at a concentration of 45 units/ml and was filtered through a .45-μ Millipore filter before use. Group B was given 250 units/kg, and group D was given 600 units of CoF/kg by an identical schedule. The control group (group C) was given six intraperitoneal injections of a rabbit serum protein solution (complement inactivated; 200 μg/ml) equivalent to the protein content of the injected CoF. Each group of animals was depleted and studied on different days. Purified CoF was kept frozen at −70 C, and an aliquot reassayed 1 day prior to use against freshly drawn human serum.[6] No loss of activity occurred over a 1-month period of preservation at −70 C.

Sponge Implantation and Measurement of Chemotaxis *in Vivo*

Forty-eight hours after the start of depletion, each rat was anesthetized with 37.5 mg/kg Nembutal® injected intraperitoneally. They were each implanted with four preweighed polyvinyl sponge squares (1.3 × 1.3 × .5 cm, wt 32 to

35 mg) in the dorsal subcutaneous fascial space through a 1.5-cm midline incision. The wounds were sutured by three to four interrupted 3-0 silk sutures and dressed with collodion and one layer of gauze bandage. A 1-ml cardiac blood sample was obtained prior to surgery.

Twenty-four hours later, the original skin wounds were opened without bleeding, and three sponges were carefully removed from each animal with minimal pressure, to prevent loss of fluid content. We have determined that 0.5 to 1 kg of force/sq cm is necessary to express fluid from the sponges. The sponges were then reweighed, and their contents expressed into small plastic cups containing EDTA to prevent cell clumping. Biopsies of sponge and adjacent capsule were obtained and stained with hematoxylin and eosin. Cardiac blood samples were again obtained and used for complete blood counts and for serum studies. All blood samples were allowed to clot for 1 hour at room temperature. The serum was obtained by centrifugation at 25 C for 20 minutes at 1500 rpm in an International Centrifuge and was made .01 M with respect to EDTA and frozen at −70 C in small aliquots. Cell counts on inflammatory fluid and blood were made by direct counting in a hemocytometer chamber in duplicate, and the results were confirmed by the Coulter counter. Agreement between the two methods was within 10%. Differential cell counts were made on Wright-stained smears of inflammatory fluid and blood. Cultures were taken from each sponge site in each animal to control for bacterial infection which stimulates chemotaxis.

If the total inflammatory fluid nucleated cell count (cells per cubic millimeter) was multiplied by the percent neutrophils found by differential counting and the total fluid volume of the sponges in cubic millimeters (obtained from the sponge weight), it was possible to calculate the number of neutrophils present per sponge. Neutrophils were present in 98 to 100% purity in the fluid. Differential counts of sponge sections before and after fluid removal showed the same differential counts. There was also no evidence of neutrophils sticking to the sponges, which could decrease their concentration in the expressed fluid. A small amount of fluid and cells remains behind but this is accounted for by the determination of sponge weight.

Assays for Complement Depletion

Assay for C3 was accomplished by immunodiffusion. Serum samples were diluted serially from 1:2 to 1:160 and subjected to immunodiffusion in agar against an antibody to rat C3. This antibody was prepared in the rabbit according to the method of Mardiney [7] using zymosan to differentially bind C3 from rat serum. Our antibody gave a single immunoprecipitin line against rat C3 on immunoelectrophoresis [8] against EDTA-treated normal rat serum. This antiserum consistently gave a single line with C3 by immunodiffusion up to a normal rat serum dilution of 1:120.

$C'H_{50}$ assays on all serums were performed after addition of calcium and magnesium to the EDTA-treated serum.

Reverse Arthus reactions [9] were readily produced in normal rats and sponge-implanted rats not treated with CoF. Each rat was given 12.5 mg of BSA by intravenous or intracardiac injection followed immediately by the intradermal injection of anti-BSA γ-globulin prepared from anti-BSA serum according to Sober.[10] This was done to remove complement components from the γ-globulin preparation. Arthus reactions were performed immediately after sponge removal and their development observed for 8 hours.

Serums to be assayed for complement activity were obtained prior to depletion, immediately before sponge implantation and 24 hours later at the time of sponge

removal. Seven days after depletion was begun a final serum sample was obtained.

Local Effects of CoF

Each member of a group of 6 rats was implanted with four polyvinyl sponge squares in the dorsal subcutaneous region of the back. The two sponges placed to the right of the midline contained .15 M saline, those placed on the left contained 5 units of CoF in .15 M saline. Sponges were removed and studied as above after 24 hours. Serum samples were obtained to determine the degree of complement depletion produced by CoF administration by this route.

In Vivo and *In Vitro* Survival of Neutrophils Exposed to CoF

Five rats were implanted with eight polyvinyl sponge squares each. These sponges were removed 20 hours later, and their contents expressed into a sterile beaker containing EDTA. The inflammatory fluid contained 10,400 neutrophils/cu mm in a volume of 9.4 ml. This was divided into two equal portions. To one portion CoF was added to a concentration of 20 units/ml. An equivalent amount of saline was added to the other portion of cells. Four sponges were then implanted into each of 6 new rats. The two sponges placed on the right had been soaked in the inflammatory fluid-saline mixture and those on the left in the inflammatory fluid-CoF mixture. Sponges were removed after 4 hours and the contents expressed. Cell counts, differential counts and trypan blue counts were made from the fluid expressed from each sponge. Cell counts and trypan blue counts of the original mixtures of white cells with saline and with CoF were also obtained after 2 hours *in vitro* at 25 C. All statistical determinations of means and standard deviations and Student *t* tests were done by computer calculations.

Results

Table 1 demonstrates that CoF-treated rats depleted to below 3% of normal C3 (groups A and D) levels show markedly impaired leukocyte chemotaxis *in vivo*. Accumulation of neutrophils in sponge fluid is inhibited 60 to 80%. Failure to deplete animals to a C3 level below 3% allowed a normal chemotactic response. The animals depleted to C3 levels below 3% also had complete inhibition of the reverse Arthus reaction and $C'H_{50}$ titers of 0. It was of interest that we have observed $C'H_{50}$ titers of 0 with 5 to 6% of normal C3 levels remaining. These animals usually have normal chemotaxis. This suggests that $C'H_{50}$ titers are not adequate to assay for levels of depletion necessary to impair chemotaxis. Table 1 also indicates that CoF-treated animals show an absolute increase in numbers of circulating neutrophils.

Variability of counts from the sponges obtained from each animal was less than the variability between animals. There was a highly significant difference between the control and experimental groups A and D.

Table 2 provides an explanation for the fact that group D, which received 600 units/kg showed less depletion than group A, which received

Table 1—The Effect of Complement Depletion on Chemotaxis In Vivo

Group*	Peripheral blood neutrophil counts (cells/cu mm)	C3 levels by immuno-diffusion (% of normal range)	$C'H_{50}$ (μ/ml)	Reverse Arthus reaction†	Inflammatory fluid neutrophil counts (cells/cu mm)	Inflammatory fluid neutrophil counts (cells/sponge $\times 10^{-6}$)	Percent inhibition	Significant difference‡ at
A (300 μ/kg CoF) N = 8	3437 ± 2156	1–3	0	0	2786 ± 2125	1 ± 1.02	80	$P < .001$
B (250 μ/kg CoF) N = 8	2600 ± 780	3–4.6	—	—	10937 ± 2556	—	20	$P > .05$
C (control) N = 8	1688 ± 732	100	135	2–4+	13700 ± 3900	4.7 ± 1.8	—	—
D (600 μ/kg CoF) N = 8	3715 ± 1432	0–3.0	0	0	5490 ± 3680	1.64 ± 1.16	60	$P < .001$

* In each experimental group, three sponges were removed from each of 8 animals at 24 hours, the fluid was expressed, and counts were made in triplicate. Each value of the inflammatory neutrophil counts in this table represents the mean ± SD of 24 different sponge fluids. There was no significant difference between the mean counts performed by hemocytometer and those by Coulter Counter.

† Reverse Arthus reactions were graded according to degree of edema, hemorrhage and necrosis on a semiquantitative scale (0 to 4+).

‡ Differences in inflammatory fluid neutrophil counts and total cell counts between CoF-treated animals and controls.

Table 2—Determinations of CoF Activity Using Fresh Human Serum from Different Donors

Donor	CoF titer (units/ml)
CS	990
SW	300
CM	480
MU	320

half that amount. Freshly drawn serum was obtained from four human volunteers and was used to assay the same aliquot of purified CoF according to Ballow.[6] The results indicate that use of different human serums can lead to as much as a threefold difference in the number of units of CoF. Because of this we now use frozen serum from a single individual to assay different preparations of CoF.

Table 3 indicates the effect of relatively high local concentrations of CoF on chemotaxis at 24 hours. The total number of cells present at 24 hours is not significantly different from the control group. There is, however, a significant increase in the amount of fluid present in the sponges containing CoF. This dilutes the cells and results in lower neutrophil concentrations in the CoF-containing sponges. The increased fluid phase may be due to the generation of permeability increasing factors locally by the interaction of CoF and complement components. There is excellent absorption of the amounts of CoF implanted in each animal with C3 levels depleted to between 4 to 12% of normal. This was assayed by immunodiffusion.

One animal in this experimental group was found to have all four sponges infected with *Streptococcus mitis* at 24 hours. It was not included in the data in Table 3. The counts in the infected sponges were markedly elevated on both the saline and CoF sides. The cell counts were 37 and 50×10^3 on the saline side and 29 and 55×10^3 on the CoF side. This demonstrates that the presence of a high concentration of CoF in a local area does not impair the microvasculature and prevent bacterially stimu-

Table 3—The Local Effect of Cobra Venom Factor on Chemotaxis

Sponges containing	PMN/cu mm $\times 10^3$	Sig	dF	PMN/ sponge $\times 10^6$	Sig	dF	Sponge fluid volume (μl)	Sig	dF
.15 M saline	12.5 ± 5	.001	18	3.99 ± 1.96	NS	18	313 ± 69	.01	18
.15 M saline + 5 units CoF	6.11 ± 2.63	.001	18	2.61 ± 1.37	NS	18	406 ± 53	.01	18

PMN = polymorphonuclear leukocytes

lated chemotaxis. The bacteria caused a three- to fourfold increase in chemotaxis.

Table 4 indicates the concentration of leukocytes still present in sponges after 4 hours *in vivo*. The leukocytes in these sponges were obtained from other rats as described above. No differences in cell concentrations or in the trypan blue counts were present between saline- and CoF-containing sponges. No change in cell count occurred after a 2-hour exposure to CoF *in vitro*, and no difference in the percent of cells staining with trypan blue was found between saline- and CoF-treated cells.

Discussion

Nonimmune chemotaxis and the Arthus reaction were both inhibited by CoF-mediated depletion of serum complement in groups A and D. The effects were associated with C3 depletion to less than 3% and with total absence of hemolytic complement activity in the serum. Failure to deplete animals to a C3 level below 3% allowed a normal chemotactic response (group B). The need to deplete complement to low levels to inhibit the Arthus reaction has been observed by Ward.[12] The degree of inhibition of chemotaxis into sponge fluid was 60 to 80%.

The simple sponge system described provides a quantitative estimate of the role that serum complement plays in nonimmune sterile neutrophil chemotaxis *in vivo*. The discrepancy between the dose of CoF given and the degree of C3 depletion and chemotaxis inhibition found in groups A and D is of interest. This can best be accounted for by the variable number of units that may be found in the same CoF sample when assayed against fresh sera (Table 2) from different individuals. This was the case in these studies. The different activity may be due to larger amounts of C3 proactivator present in sera, which would yield larger assay values for activity units of CoF present or to variations of the amount of C3 present in those sera.

Table 4—The Effect of Cobra Venom Factor on Neutrophil Survival In Vivo After 4 hours

Group	Sponges containing	PMN/cu mm $\times 10^3$	Sig	dF	PMN/sponge $\times 10^6$	Sig	dF	Trypan blue positive (%)
A	Saline, inflammatory fluid & neutrophils	5.4 ± 3	NS	22	2 ± 1.2	NS	22	3
B	CoF, inflammatory fluid & neutrophils	4.1 ± 1.82	NS	22	1.7 ± .9	NS	22	3

Sponge capsular tissue biopsies from CoF-treated and control rats revealed tissue infiltration by neutrophils; it was not possible to reliably quantitate the amount and degree by such limited histologic sampling. The sponge fluid serves as a sink in which the neutrophils leaving the circulation and migrating through the tissue accumulate.

The path from the connective tissue blood vessel lumen to the sponge involves leukocyte margination, movement through the vessel wall between endothelial cells and directed migration through the tissue extracellular space. The degree of this reaction is also dependent on the circulating neutrophil pool.

The experiments presented in Tables 3 and 4 were designed to consider the possibility that CoF may have other direct effects on chemotaxis independent of complement depletion. The areas of concern were the neutrophil, the microcirculation and the connective tissue matrix.

The local effect of a relatively large amount of CoF (5 units) was to increase the amount of fluid phase without affecting the total number of neutrophils migrating into the sponges (Table 3). CoF was absorbed from the sponges into the circulation and depleted the serum level of C3 to less than 12%. This provided lower concentrations of complement for diffusion into the sponge site. CoF also interacted locally with the complement system proteins that diffused into the sponges from the surrounding blood vessels during the first 4 hours after implantation. This interaction generated chemotactic and vascular permeability factors in the sponge fluid. These CoF effects on the serum pool of C3 and the local pool of C3 in the sponge are complex and vary with time. The net effect of these two processes in this experiment was to allow a normal number of neutrophils to accumulate in the sponges.

The major purpose of this experiment was to show that CoF did not work primarily by damaging the microcirculation locally and therefore preventing perfusion and leukocyte access to the sponge space. The fact that normal numbers of cells were able to accumulate despite the presence of high concentrations of CoF makes damage to the circulation or the extravascular ground substance less likely. The animal with the infected sponges provided dramatic proof of the excellent chemotaxis possible in an area containing high concentrations of CoF. Histologic studies of the capsular tissue showed no obvious damage to the capillaries or ground substance after exposure to high concentrations of CoF. The role of CoF-generated complement components on neutrophil mobility, level of chemotactic factor inactivator or ability to respond to another chemotactic stimulus was not measured in this experiment.

Table 4 provides evidence against any adverse effect of CoF on

neutrophils. Neutrophil survival *in vivo* or *in vitro* as measured by cell counts and trypan blue studies showed no differences between CoF- and saline-treated cells. Cochrane has shown that neutrophils from CoF-treated rabbits behave normally in Boyden chambers when exposed to a chemotactic stimulus.[9] Peripheral neutrophil counts are increased twofold in CoF-treated rats. This agrees with Cochrane's observations in the rabbit.[9] These studies indicate a lack of effect of CoF directly on the microcirculation, connective tissue or neutrophils. The effect appears to best be explained by loss of complement-derived chemotactic substances. The *in vivo* action of CoF is to deplete C3, C5 and C6, as well as terminal components. It is likely that loss of some combination of these led to impaired chemotaxis *in vivo*.

These findings on the effects of complement depletion on acute inflammation in connective tissue are in agreement with work of Hill and Ward,[2] who produced acute myocardial infarction in CoF-treated rats by coronary ligation. These authors were unable to quantitate the degree of chemotactic inhibition produced.

As described in a recent abstract,[11] ^{51}Cr-labeled peritoneal exudate cells were injected intravenously into CoF-treated rats. These rats were also given a subplantar injection to carrageenin, and the normal and injected paws were amputated. Radioactivity in circulating cells, plasma and paws was determined at 5 and 24 hours. In these studies only 5% of the injected cells were still circulating at 1 hour. There was no evidence that the circulating labeled pool could be maintained at constant specific activity so as to give meaning to the counts found in the injected limb. Other problems with this model include a) the method cannot differentiate between leukocytes pooled in local vessels and those migrating into tissue and b) the heterogeneity of the injected cell population, which also includes mononuclear cells. This system for the quantitative study of chemotaxis *in vivo* is not clearly defined. The Rebuck skin window has also been used in the past, but this gives only a semiquantitative measure of chemotaxis. Peritoneal exudates may be studied, but these are more difficult to work with and quantify than the sponge system.

The model presented does not require leukocyte isolation, labeling and reinjection with the possibility of subtle damage to the cells or the need for controls to correct for the vascular content of ^{51}Cr. The use of locally toxic materials is avoided. The sponge model should be of use in examining *in vivo* chemotaxis stimulated by necrotic tissue, bacteria and immune complexes as well as providing an assay system for pharmacologic agents affecting chemotaxis.

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