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Research Article

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Escherichia coli Bacteremia in the Squirrel Monkey

I. EFFECT OF COBRA VENOM FACTOR TREATMENT

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ABSTRACT Squirrel monkeys were significantly depleted of complement by a nontoxic protein constituent of cobra venom, and the influence of cobra factor (CoF) treatment on the course of *Escherichia coli* bacteremia was studied. Striking neutropenia occurred rapidly in control animals while the rate of occurrence of neutropenia was 20 to 30 times slower in the CoF-treated animals, suggesting that the *E. coli*-induced neutropenia was at least partially a complement-mediated response. In the CoF-treated monkeys, the initial rate of clearance of the *E. coli* from the circulation tended to be slower and the resultant levels of bacteremia were higher than in control animals. These observations are consistent with a hypothesis that complement-mediated neutrophilic leucocyte function is an important host defense mechanism in gram-ngeative bacillary bacteremia.

INTRODUCTION

The basic pathogenetic mechanisms that underlie the physiological changes which occur during bacteremia with gram-negative bacilli remain elusive. There is a body of evidence which suggests interactions between gram-negative bacteria and the complement system. Electron micrographs of gram-negative bacteria after incubation with antibody and complement demonstrate the development of circumscribed defects in the bacterial cell walls (1, 2). The in vitro incubation of endotoxin with a variety of animal sera results in the inactivation of selected complement components with the generation of complement-derived chemotactic and anaphylatoxic factors (3–12). Further, in vivo studies both in rabbits and in dogs demonstrate a fall in serum complement titers after the intravenous administration of endotoxin (13–16).

A nontoxic protein of cobra venom combines with a noncomplement plasma protein, recently designated C3-proactivator, with the resultant inactivation of C3 (17–21). Utilization of this cobra factor (CoF)[1] made feasible in vivo studies of the influence of complement depletion on experimental gram-negative bacillary bacteremia. A subhuman primate was utilized to approximate human physiologic responses (22–30).

Because of the indirect evidence that endotoxin and complement interact, the following experiments were designed to determine the influence of in vivo complement depletion upon the clearance of bacteria and upon the concomitant host responses to bacteremia.

METHODS

Preparation of the anticomplementary factor (CoF) from cobra venom. Lyophilized venom of the Asian cobra *Naja naja*[2] was fractionated and quantitated for anticomplementary activity as described by Cochrane, Müller-Eberhard, and Akin except that the final Sephadex gel filtration step was not performed (18). Sprague-Dawley derivative rats which were injected with 300 U of CoF per kg in four equally divided intraperitoneal doses over 24 h showed no evidence of neurotoxicity.

Experimental animals and procedures. 1–4 yr old male Brazilian squirrel monkeys, *Saimiri sciureus*,[3] weighing 500–800 g, were utilized. A total dose of CoF of 300 U/kg was given i.p. as four equally divided doses over 24 h. Control animals were given no CoF. Bacteremia was induced in test animals 24–48 h after the last CoF injection. Animals were allowed food and water ad libitum prior to study.

Animals were restrained supine, and the inguinal areas were prepared by shaving, scrubbing with 3% hexachlorophene followed by 70% alcohol, and analgesia was accomplished with approximately 1.0 ml of 0.01% lidocaine hy-

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[1] *Abbreviations used in this paper:* CoF, cobra factor; RES, reticuloendothelial system; t/2, 50% clearance time.

[2] Ross Allen Reptile Institute, Silver Springs, Fla.

[3] Tarpon Zoo, Tarpon Springs, Fla.

drochloride administered subcutaneously. No additional sedatives or analgesics were given. A femoral vein and artery were cannulated (20 gauge long-dwell catheter needle[4]). After insertion, the catheters were filled with heparinized saline (10 U heparin U.S.P. per ml of normal saline). The monkeys were then turned and restrained in a prone position with extremities dependent, a rectal thermal probe was inserted, and the temperature allowed to stabilize for 30–60 min.

From each animal, a base-line blood sample was obtained for bacterial culture, serum complement, antibody titer, hematocrit, and total and differential leukocyte count. Following administration of the bacterial inoculum through a femoral vein catheter, subsequent blood samples were drawn from the femoral artery catheter. To avoid significant blood volume depletion, sample volumes were replaced with identical volumes of normal saline solution. Furthermore, the total blood sample volume obtained prior to sacrifice was always less than 3.0 ml. Terminally, blood for quantitative blood culture and leucocyte count was obtained from the inferior vena cava and the right and left ventricles to detect spurious elevations in bacterial counts which might be due to residual bacteria in the catheters. All blood samples were placed directly into polyethylene tubes (12 × 75 mm)[5] containing approximately 1 U of dried heparin. Plasma specimens not studied immediately were stored at −70°C.

Complement determinations. Two parameters of complement activity were measured. Total hemolytic complement titers were performed in duplicate for each sample by the method of Mayer, modified by the reduction of all reagent volumes by 60% (31). Plasma samples from control animals were diluted 1:225 and plasma samples from CoF-treated animals were diluted 1:80 in isotonic veronal buffer, pH 7.3. As a control, a normal human serum with a known hemolytic titer was included in each set of determinations. The concentration of C3 was estimated by the radial immunodiffusion technique utilizing agar plates containing anti-human C3[6] (32).

Escherichia coli preparation and quantitative blood cultures. E. coli 075 was incubated overnight at 37°C in trypticase soy broth.[7] The bacteria were sedimented and washed three times in sterile water. The final concentration was adjusted to an estimated 1.0×10^9 per ml using a photocolorimeter[8] and confirmed by plating 10-fold serial dilutions on MacConkey agar.[7] A dose of 4.0×10^9 *E. coli* per kg, assumed to represent a lethal dose, was administered (33, 34).

Bacteremia was quantitated by utilization of 0.1 ml of blood in serial 10-fold dilutions of MacConkey agar. Due to the small quantity of blood employed, bacterial counts of fewer than 100 colony-forming units per ml could not be detected.

^{32}P-labeled E. coli and scintillation counting. E. coli 075 and 1 mCi of carrier-free ^{32}P (footnote 9) were added to a low phosphate medium consisting of 1% proteose peptone no. 3,[10] 0.5% casamino acids,[10] and 0.05% dextrose (35, 36). After 16 h of incubation at 37°C, the organisms were collected by centrifugation, washed three times in sterile water, and the number of viable *E. coli* determined in triplicate by colony plate counts on MacConkey agar. The bacteria were then heat-killed by incubation at 60°C for 1 h, recultured to verify loss of viability, and stored at 4°C. On the day of experimental use, the ^{32}P-labeled *E. coli* were washed an additional four times in the original volume of sterile water. A dose of 3.0×10^9 *E. coli* per kg was administered.

The method of liquid scintillation counting was the same for the ^{32}P-labeled *E. coli* cultures and the blood samples. To 0.1 ml of either sample was added 1.0 ml of 0.75 N sodium hydroxide, 0.5 ml of 30% hydrogen peroxide, and 15.8 ml of a triton-toluene scintillation mixture (2,5-diphenyloxazole 14 g, 1,4-bis[2-(5-phenyloxazolyl)]benzene 0.2 g, toluene 2,000 ml, triton X[11] 1,000 ml, and 2.5 M hydrochloric acid 160 ml). The *E. coli* samples were diluted 1:10 and 1:100 and counted in triplicate. All counts were corrected by internal standardization. To determine the amount of unbound ^{32}P in either the *E. coli* suspension or plasma, samples were diluted 1:10 in normal saline, passed through a 0.45 μm Millipore filter,[12] and 0.1 ml portions counted as described. Counts were corrected for background radiation, unbound ^{32}P, and decay of the isotope. The ratio of unlabeled to labeled bacteria was obtained by dividing the number of colony-forming units per milliliter by the calculated number of counts per minute per milliliter of the ^{32}P-*E. coli* suspension.

Bacterial clearance rates. Animals were given an i.v. injection of either 5.0×10^7 viable *E. coli* per kg or 3.0×10^9 ^{32}P-labeled heat-killed *E. coli* per kg. Timed arterial blood samples were obtained and clearance curves were constructed using either the number of *E. coli* colony-forming units per ml or the corrected ^{32}P cpm per ml of blood (37).

Plasma bactericidal activity. Plasma specimens from animals treated with CoF and from normal animals were tested for in vitro bactericidal activity against *E. coli* 075. Human serum from a single donor (D. G.) was studied as a control. Plasma specimens to be tested were passed through a Millipore filter, diluted with cold isotonic veronal buffer, pH 7.3, to a final plasma concentration of 10%, and 0.9 ml placed in each of four test tubes. 0.1 ml of *E. coli* suspension containing 1×10^7 organisms per ml was added to each tube. Viable bacterial counts were performed at zero time on one tube. The remaining three tubes were incubated at 37°C for 120 min, following which viable bacterial counts were determined and the percentage of surviving bacteria calculated (38, 39).

E. coli 075 agglutination titers. The antibody titer against *E. coli* 075 was determined on all base-line plasma specimens by a microtiter technique utilizing heat-killed formalinized antigen (40). Each plasma specimen was tested both with and without 0.05 M 2-mercaptoethanol. The antibody titer was interpreted as the highest plasma dilution showing complete dispersion of antigen.

Hematologic studies. Microhematocrits were determined at the start and end of each experiment. Leukocyte counts were determined on heparinized blood within 30 min of obtaining the sample utilizing a hemocytometer.

RESULTS

Complement depletion by CoF. The degree of complement depletion was determined with plasma ob-

[4] Becton, Dickinson & Co., Rutherford, N. J.
[5] Falcon Plastics, Los Angeles, Calif.
[6] Immunoplate, Hyland Labs, Costa Mesa, Calif.
[7] BBL Division of Becton, Dickinson & Co., Cockeysville, Md.
[8] Bausch & Lomb, Rochester, N. Y.
[9] New England Nuclear Corp., Boston, Mass.
[10] Difco Laboratories, Detroit, Mich.
[11] Beckman Instruments, Fullerton, Calif.
[12] Millipore Corp., Bedford, Mass.

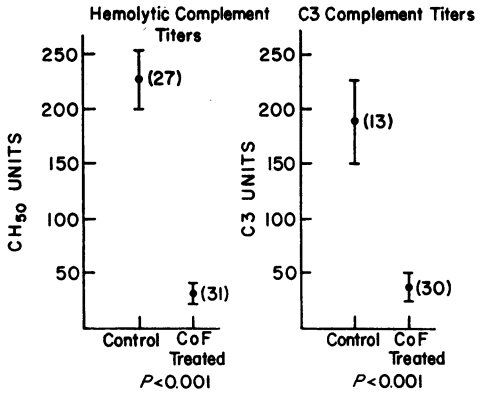

FIGURE 1 Hemolytic and C3 complement levels in control and CoF-treated monkeys. Hemolytic complement titers expressed as 50% hemolytic U (CH_{50}), C3 titers as units of human C3. Values represent means ±2 standard errors of the mean. Numbers in parentheses indicate number of observations. The significance of differences was assessed using Student's *t* test.

tained just prior to the induction of experimental bacteremia. The mean hemolytic complement titer was 226 U in control animals compared with 31 U in CoF-treated monkeys (Fig. 1). The mean C3 value as determined by radial immunodiffusion was 187 U in control animals and 36 U in complement-depleted animals.

Effect of E. coli bacteremia on complement values. In the early experiments, the initial blood samples were obtained 30–60 min after the injection of *E. coli*. Hemolytic complement activity decreased an average of 32% at 60 min (Fig. 2). In two subsequent experiments, CH_{50} determinations were performed sequentially over the first 5 min. In both animals, CH_{50} values decreased within 30 s: 312–223 CH_{50} U (28% decrease) and 283–135 CH_{50} U (52% decrease).

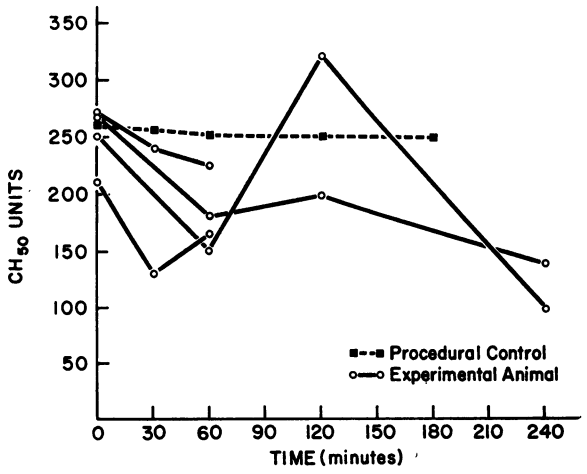

FIGURE 2 Effect of *E. coli* bacteremia on hemolytic complement activity. Time following intravenous challenge with viable *E. coli* is plotted on the abscissa.

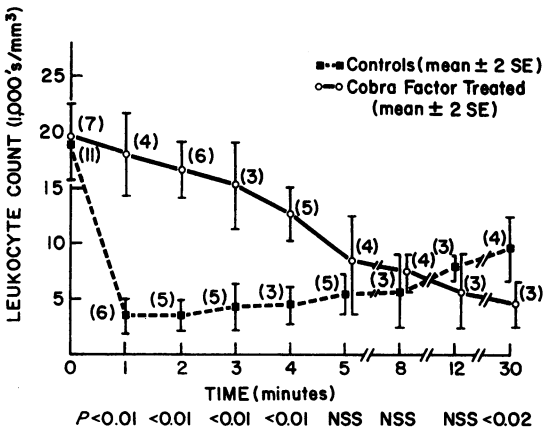

FIGURE 3 Effect of *E. coli* bacteremia on leukocyte counts of CoF-treated and control monkeys. Total leukocyte counts are plotted on the ordinate and interval in minutes following i.v. challenge with viable *E. coli* is plotted on the abscissa. The significance of differences was assessed using Student's *t* test. NSS, not statistically significant.

Effect of E. coli bacteremia on leukocyte counts. In control monkeys a dramatic decrease in leukocyte count was demonstrated after the i.v. administration of *E. coli* (Fig. 3). Maximal depression of the leukocyte count occurred within 1–2 min and occasionally was demonstrated as soon as 45 s after injection. Both viable and heat-killed ^{32}P-labeled *E. coli* were used in these experiments, and no differences were noted between their effects on the leukocyte count. In complement-depleted monkeys, leukocyte counts also decreased after the i.v. administration of *E. coli;* however, the decrease occurred at a much slower rate (Fig. 3). In CoF-treated animals, the maximal decrease in leukocyte count usually occurred between 12 and 30 min after injection, but two animals required 2 h to reach minimum values. Differential leukocyte counts demonstrated that the leukopenia was the result of neutropenia. Neutrophiles reappeared in normal animals earlier than in CoF-treated animals. In both groups during the phase of rebound many immature granulocytic forms were demonstrable in peripheral blood smears.

A control and a CoF-treated animal were sacrificed at 2, 5, or 10 min after the injection of *E. coli* and the difference in total granulocyte counts between blood obtained from the right and left ventricles was determined. In control animals at 2 min the granulocyte counts in left ventricular blood were 586 per mm^3 less than right ventricular values and at 5 min, 1,380 per mm^3 less. By 10 min, granulocyte counts in left ventricular blood exceeded the right ventricular values by 1,608 per mm^3. In contrast, in CoF-treated animals the granulocyte counts in left ventricular blood exceeded the right ventricular values at 2 and 5 min and a net decrease in granulocyte counts across the pulmonary circulation was not

TABLE I

Clearance Rates of E. coli

Experimental group	No. of animals	Clearance rate* Fast component	Clearance rate* Slow component
Viable *E. coli*			
Control	3	1.5, 2.0, 2.0	4.0, 5.0, 8.0
Complement-depleted	3	2.0, 3.0, 3.0	6.5, 6.5, 7.0
Killed ^{32}P-labeled *E. coli*			
Control	6	0.6, 0.6, 0.6, 0.75, 0.75, 0.75	
Complement-depleted	6	0.6, 0.8, 0.8, 1.0, 1.2, 1.5	

* t/2 in minutes.

evident before 10 min. At 10 min, granulocyte counts in left ventricular blood from the CoF-treated monkeys were 2,286 per mm^3 less than controls.

Bacterial clearance and tissue distribution of E. coli. In both control and CoF-treated animals, biphasic vascular clearance curves were obtained after the administration of either viable or heat-killed ^{32}P-labeled *E. coli*. 50% clearance times (t/2) were determined for the fast initial and slower secondary phases (Table I). The t/2 values for both the fast and slow phases were slightly greater in complement-depleted animals, but because of the small numbers of animals, the values did not achieve statistical significance.

Quantitation of bacteremia. Quantitation of *E. coli* in blood at 1, 2, 3, and 4 h demonstrated consistently higher levels in complement-depleted animals than in controls (Fig. 4). The differences between control and CoF-treated animals were statistically significant at all time intervals studied beyond 30 min. Simultaneous blood samples were obtained at the time of sacrifice from the catheter, inferior vena cava, and both ventricles; in no instance was there a significant difference between bacterial counts on blood obtained from the several sources in a given animal.

Experiments were performed in CoF-treated animals in which reconstitution of the complement system was attempted. Two animals depleted of complement with CoF subsequently were given 800 and 2,400 hemolytic units of human C3, respectively. The CH_{50} and C3 titers did not increase from baseline values. Following i.v. challenge with 4×10^9 viable *E. coli* per kg, the number of circulating *E. coli* remained in the range observed for complement-depleted animals. In contrast to these observations, the administration of 3.5 ml of whole fresh monkey serum to a complement-depleted animal prior to i.v. *E. coli* resulted in a level of bacteremia which was in the range for control animals (Fig. 5). In this latter

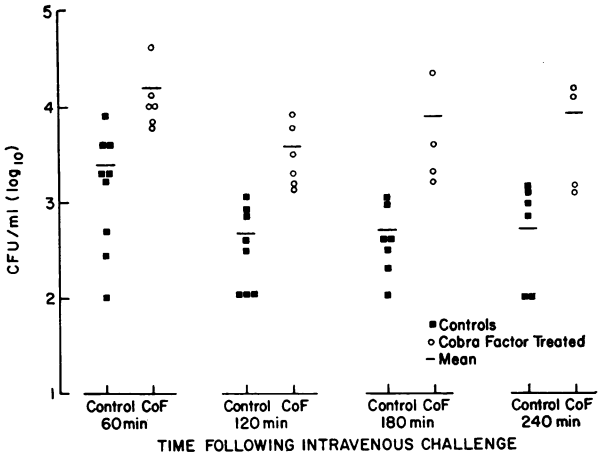

FIGURE 4 Number of colony forming units (CFU) of *E. coli* cultured from blood at intervals following i.v. challenge in CoF-treated and control monkeys.

animal, the hemolytic complement titer rose from 40 to 105 U and the C3 level from 34 to 60 U, both of which values remain below normal.

Effect of CoF treatment on in vitro serum bactericidal activity. Fresh normal monkey serum was bactericidal for the strain of *E. coli* 075 utilized in these studies, as demonstrated by 96–99% reduction in viable bacterial counts when 1×10^6 *E. coli* 075 were incubated for 120 min in 10% fresh monkey serum (Fig. 6). Monkey serum that was heat-inactivated (56°C for 30 min), or fresh serum from monkeys treated with CoF, had no bactericidal activity and often allowed some bacterial

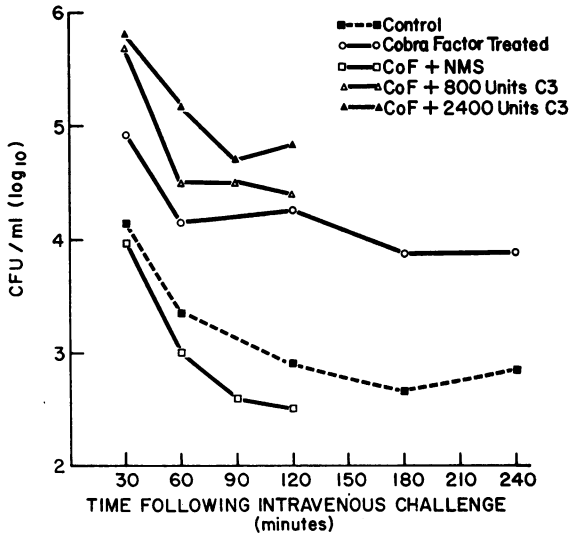

FIGURE 5 Effect of "repletion" with normal monkey serum or human C3 in CoF-treated monkeys. Number of colony forming units of *E. coli* cultured from blood at intervals following i.v. challenge. NMS, normal monkey serum.

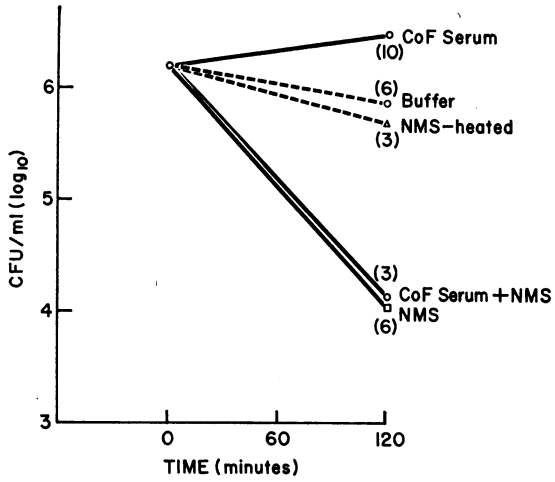

FIGURE 6 Effect of CoF treatment on in vitro serum bactericidal activity against *E. coli*. NMS, normal monkey serum. Numbers in parentheses indicate number of experiments.

proliferation. If fresh monkey serum (10% final concentration) was added to serum from CoF-treated monkeys, the serum bactericidal activity was restored

E. coli 075 antibody titers. The antibody titers were all very low (maximum titer 1:8) with no significant differences between control and experimental animals. In no instance was a titer detectable ($\geq$ 1:4 dilution) after serum incubation with 2-mercaptoethanol. No correlation was demonstrable between antibody titer and level of bacteremia or clearance rate.

DISCUSSION

It is now known that complement activation can occur in one of two ways: the classical pathway requiring antibody and C1, or an alternate pathway which bypasses C1, C4, and C2 and activates C3 via at least two plasma protein components, factor A and factor B of the properdin system (20, 21, 41). Factor B is considered identical to C3-proactivator (C3PA) (42). CoF is known to combine with C3PA to form an enzymatically active complex which cleaves and inactivates C3 (19).

Current knowledge of the noncomplement physiologic effects of CoF administration is limited. CoF administered in a single large injection to a variety of experimental animals produced considerable hemolysis (18). Subsequently, a low molecular weight component of CoF was shown to have in vitro hemolytic activity, and CoF administration regimens in vivo were altered to avoid clinically significant hemolysis (18, 43). Recently, platelets, from guinea pigs, rabbits, dogs, and humans were shown to aggregate and release their intracellular constituents after in vitro exposure to CoF (44). Cochrane noted an increase in blood leukocyte counts in nonprimate experimental animals given CoF (18).

A fall in hemolytic complement titers has been observed in rabbits and dogs after intravenously administered endotoxin (13–16). The present studies demonstrated the same phenomenon in monkeys, but the rapidity of the decrease was striking. In all control animals, a significant decrease ocurred within 30 s after the administration of *E. coli*. The rapidity with which the plasma complement concentration falls suggests that complement has a pivotal role. In addition, it is notable that the rapid fall in complement paralleled the rapid fall in circulating neutrophils.

Granulocytopenia after endotoxin has been well documented in man and other mammalian species (45–47). Through *in vivo* microscopic studies in animals having received bacteria intravenously, it has been shown that neutrophils rapidly adhere to capillary endothelium and engage in phagocytosis (48–50). Similar rapid adherence of circulating neutrophils to vascular endothelium ("margination") was recently shown in volunteers given small intravenous doses of endotoxin (51, 52). This margination phenomenon may represent a complement-mediated immune adherence reaction (48, 49, 53). Recently granulocyte receptors for the immune adherence reaction have been demonstrated (54).

Little is known of the neutrophil response to endotoxin in vivo when the complement system is inhibited. CoF treatment had no influence on the endotoxin-induced neutropenia of the Shwartzman reaction in rabbits but events during the first 4 h postinjection were not reported (16). The accumulation of neutrophils in inflammatory exudates following i.p endotoxin administration was markedly retarded in C5-deficient mice (55). In the studies reported here, CoF-treated monkeys required 20–30 times longer to develop maximal neutropenia after bacterial challenge than did control animals. This blunted response was paralleled by the observation that the sequestration of neutrophils in the pulmonary circulation during the first few minutes following *E. coli* injection into the CoF-treated animals was decreased. Thus, the development of neutropenia during *E. coli* bacteremia may be at least partially complement-mediated. The assumption that complete inhibition of the complement system would totally preclude the development of neutropenia cannot be reached from these studies since CoF-treated animals retained approximately 14% of their plasma complement activity.

The bloodstream clearance of *E. coli* has been divided into three phases (56). The initial phase is one of rapid clearance with the disappearance of 90–99.9% of the injected organisms within 30 min or less. During the second phase, the *E. coli* persist in the circulation at low concentrations while the third phase is characterized either by increasing bacteremia or sterilization of the bloodstream. Multiple factors are involved in the initial

clearance of *E. coli* from the bloodstream, e.g., fixed phagocytic cells of the reticuloendothelial system (RES), polymorphonuclear neutrophils, complement, and antibody (56). The RES is generally considered to perform a major role in the initial clearance phase (37). The relation of the complement system to RES function remains controversial. Some investigators have found the presence of complement to be necessary for clearance while others have found that the presence of complement has no influence on RES clearance of endotoxin or gram-negative bacteria (57, 58). Spielgelberg, Miescher, and Benacerraf demonstrated an impaired clearance of *E. coli* in mice which were depleted of complement by the injection of heat-aggregated human gamma globulin (59). Circulating neutrophils have not been considered to have a significant role in vascular clearance during the initial phases of bacteremia (37). However, the demonstration that prompt neutropenia following bacteremia is blunted by CoF treatment and the observed tendency toward impaired initial bacterial clearance are consistent with a hypothesis that marginated neutrophils may have an important role. Thus, the tendency toward the impaired initial bacterial clearance in the present studies may have been the consequence of a delay in the immediate sequestration of large numbers of neutrophils from the circulation.

In the second phase of clearance of *E. coli,* CoF-treated animals demonstrated significantly higher levels of bacteremia than did controls. This phenomenon may be complement-dependent since it was corrected by the administration of fresh monkey serum to one experimental animal. The complement-dependent serum bactericidal reaction also has been implicated as a determinant of the ultimate level of bacteremia (38, 39). These conclusions were supported by the present studies and those of other workers which demonstrated that CoF treatment of monkeys interfered with in vitro plasma bactericidal activity (20). As a consequence of the impairment in initial clearance, more bacteria would persist in the circulation. With a concomitant impairment of complement-dependent plasma bactericidal activity, the prolonged persistence of higher levels of bacteremia would be expected.

Impaired phagocytosis by neutrophils may be another contributing factor to the higher bacteremia levels in the CoF-treated monkeys. Other investigators have shown in vitro a loss of serum opsonic activity for the phagocytosis of *E. coli* by neutrophils (60). Additional in vitro studies with human sera deficient in Clq and C2 and guinea pig serum deficient in C4 showed no impairment of neutrophil phagocytosis (61, 62). These results strengthen the role of the CoF-inactivated alternate pathway in assisting neutrophil phagocytosis.

The present experiments provide data which implicate the complement system in the pathophysiology of *E. coli* bacteremia. Using a subhuman primate, it was demonstrated that CoF treatment significantly decreased the animals' plasma concentration of C3 as well as the total hemolytic complement titer. Using this model, i.e., the CoF treated monkey in which the plasma concentration of C3 was significantly decreased, the induction of experimental *E. coli* bacteremia was followed by a blunted granulocytopenic response and a higher level of bacteremia than was observed following the induction of *E. coli* bacteremia in control monkeys. Based upon our current knowledge of the biologic activities of the complement system, it is proposed that CoF treatment produced the above results by impairing the alternate complement activation pathway which is involved in the bactericidal reaction and the normal neutrophil margination response to gram-negative bacillary bacteremia. Furthermore, these observations are consistent with the hypothesis that phagocytosis of intravascular microorganisms is more efficiently performed by marginated neutrophils.

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