# MEDIATION SYSTEMS IN BACTERIAL LIPOPOLYSACCHARIDE-INDUCED HYPOTENSION AND DISSEMINATED INTRAVASCULAR COAGULATION

## I. The Role of Complement*

By RICHARD J. ULEVITCH,‡ CHARLES G. COCHRANE, PETER M. HENSON, DAVID C. MORRISON§, AND WILLIAM F. DOE

*(From the Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)*

A single injection of lipopolysaccharide (LPS)[1] from gram-negative bacteria can produce multiple pathophysiological changes. These include hypotension (1–5), disseminated intravascular coagulation (DIC) (2,3,6–9,10), and changes in numbers of peripheral blood cells (2–4,11). The effector mechanisms responsible for these complex phenomena are not well understood. What is known is that a variety of humoral and cellular mediation systems may be activated by LPS in experimental animals and in in vitro systems. For example, LPS has been shown to activate both the classical and alternative pathways of complement (12,13–17) Hageman factor (18), induce release of vasoactive and coagulation promoting substances from platelets (11,19,20), damage endothelial cells (21–23), and promote the release of effector substances from neutrophils (24), mononuclear cells (25), and macrophages (26).

The following studies were undertaken to establish the role of one of the mediation systems, the complement system, in LPS-induced pathophysiologic changes. We have assessed the role of complement in LPS-induced hypotension and DIC by different means. One approach has involved a comparison of the results of LPS injection into normal rabbits with rabbits either depleted of C3 and terminal complement components by cobra factor or rabbits genetically deficient in the sixth component of complement. In these experiments the role of the anaphylotoxins, as well as complement-mediated cell lysis, could be studied.

A second approach and one unique to this work involved the use of highly purified, structurally defined LPS preparations which differ significantly in

[1] *Abbreviations used in this paper:* CoF, cobra venom factor; DIC, disseminated intravascular coagulation; LPS, lipopolysaccharide.

their ability to reduce the hemolytic complement levels of normal rabbit serum. LPS was purified from *Escherichia coli* 0111:B4 and *Salmonella minnesota* Re595. When tested in vitro 250 $\mu$g of 0111:B4 LPS did not reduce the hemolytic complement activity of normal rabbit serum. In contrast as little as 1 $\mu$g of Re595 LPS was shown to reduce complement levels in normal rabbit serum (D. C. Morrison, personal communication).

A less purified, particulate preparation of LPS from *Serratia marcescens* was also used in some experiments. The use of this LPS preparation allowed direct comparison of our data with those of other workers (3,4,11), as well as determining if soluble and particulate preparations of LPS behave in a similar manner.

Many of the pathophysiologic changes which occur in experimental animals after LPS injection have also been observed in patients with gram-negative bacteremia who develop septic shock (10,27). The studies of Guenter et al. (5) have suggested that the injection of purified LPS preparations into experimental animals represent a useful model for the study of the pathophysiologic changes which occur in patients with gram-negative bacteremias. Thus, a definition of the mechanism of LPS-induced hypotension, DIC, and peripheral blood cell changes may help to define the mediation systems responsible for similar changes in patients with gram-negative sepsis.

## Materials and Methods

*Rabbits.* Male and female 2-2.5 kg New Zealand white rabbits from a closed colony and rabbits genetically deficient in C6 bred into the same colony were obtained from Rancho de Conejo, Vista, Calif. Animals had free access to food an water before the beginning of the experiment.

*LPS.* LPS was prepared from *E. coli* 0111:B4 by phenol extraction and further purified by treatment with ribonuclease, ethanol precipitation, and gel filtration (28). LPS was also purified from *S. minnesota* Re595[2] by the phenol-petroleum ether-chloroform procedure of Galanos et al. (29). Both LPS preparations were free of detectable protein and nucleic acid. LPS 0111:B4 was soluble in aqueous solution and was stored at −70°C. Concentrations of 0111:B4 LPS were determined by measuring the colitose content of the purified material (28). Lyophilized LPS Re595 was solubilized by the addition of 0.1% triethylamine followed by sonication. This material was dialyzed against sterile isotonic saline before use. LPS isolated from *S. marcescens* was obtained from Difco Laboratories, Detroit, Mich. and was used after suspending the lyophilized LPS in sterile, isotonic saline followed by sonication.

*Experimental Procedure.* On the morning of the experiment a cannula (Intramedic Polypropylene Tubing, ID 0.023″) was inserted into the femoral artery approximately to the level of the aortic bifurcation. Surgery was performed after the subcutaneous administration of 1-2 ml of 1% Novocain at the incision site. The animals were strapped in a supine position during surgery and for the remainder of the experiment. The cannula was utilized to remove blood samples from which cell counts and plasma protein concentrations were determined, as well as to measure blood pressure. Blood pressures were measured with a Statham SP 1400 Blood Pressure Monitor (Statham Instruments, Inc., Oxnard, Calif.) and Statham P37B Pressure Transducer. Two pressure measurements as well as 2.5-ml blood samples were taken 30-60 min before LPS injection to establish preinjection base-line values for the various parameters measured during the experiment. Data for all parameters measured is expressed as a percentage of the value determined immediately preceding the LPS injection. Stable base-line values were obtained for all parameters measured. After the injection of LPS, blood samples were removed at 5, 30, 50, 180, and 300 min

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[2] The *S. minnesota* Re595 LPS was prepared while one of us (D. C. M.) was a guest in the laboratory of Professor Otto Lüderitz, Max Planck Instit fur Immunbiologie, Freiburg im Br., Germany.

postinjection, while pressures were monitored at 5, 30, and every 30 min thereafter for the remainder of the experiment. The cannula was routinely flushed every 30 min with no more than 1 ml of a 2 U heparin/ml sterile saline.

In all experiments LPS was injected intravenously in a 1-ml volume during a 30-s time period.

*Quantitation of Peripheral Blood Cells.*    Blood samples were collected into 3.8% sodium citrate (1 vol citrate/9 vol blood) in plastic tubes. Samples of anticoagulated blood were diluted in 2% acetic acid and granulocytes and mononuclear cells counted in a hemocytometer.

Changes in circulating platelets were determined by injection of $^{51}$Cr-platelets 18–24 h before the beginning of the experiment. The rabbit blood was drawn through a 19 gauge needle from the ear artery into plastic tubes with acid citrate dextrose (ACD) (30) (1 vol ACD/6 vol blood). The sample was centrifuged 20 min × 1,500 rpm at 25°C (International PR-2, 12 place rotor), the platelet-"rich" plasma aspirated and recentrifuged for 15 min at 25°C, 3,000 rpm, as above. The platelet "poor" plasma was removed and the pellet was resuspended in 1–2 ml of the platelet poor plasma. After incubation for 60 min at 37°C with $^{51}$Cr (0.23 mCi/tube of whole blood originally collected) the platelets were washed twice with platelet poor plasma, resuspended in platelet poor plasma, and injected into the experimental rabbits. Radioactivity in 0.5-ml aliquots of whole blood was measured in a Packard Automatic gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.) to quantitate changes in circulating platelets. Control experiments in normal, noncannulated rabbits demonstrated that $^{51}$Cr-platelets have a half-life ($t_{1/2}$) of approximately 30 h. When experimental data determined over a 5-h period from normal and cobra venom factor (CoF)-treated rabbits (after cannulation) was compared to a calculated curve for $^{51}$Cr platelet disappearance, good agreement was observed. Thus neither the surgical procedures employed nor CoF treatment have any effect on the survival of $^{51}$Cr platelets.

*Complement Measurements.*    Plasma samples were analyzed for C3 by radial immunodiffusion (31). The anti-C3 antiserum was obtained by immunizing guinea pigs with zymosan that had been treated with normal rabbit serum and extensively washed. The area of the precipitin ring was calculated, and changes in C3 concentration were determined by comparing the C3 concentration after injection to the C3 concentration determined prior to LBS injection. The C3 levels determined from the two preinjection plasma samples differed by less than 10%. In some experiments serum was collected, and $CH_{50}$ values were determined according to Kniker and Cochrane (32).

The concentration of C6 in the serum of offspring of a C6-deficient doe and a heterozygous buck was determined hemolytically. Aliquots of the test serum were added to C6-deficient serum and assayed in the hemolytic complement assay (32). Pooled normal rabbit serum was used as a standard and considered to be 100%. The heterozygous rabbits tested had between 35–45% of normal C6 levels.

*Depletion of C3.*    CoF was purified by DEAE Sephadex A-50 chromatography according to Cochrane et al. (33). Decomplementation of normal 2–2.5 kg rabbits was accomplished with 600–750 U of CoF administered by four or five intraperitoneal injections of 150 U of CoF during a 36-h period ending 24 h before the start of the experiment. In all experiments the plasma C3 concentration, as determined by quantitative immunodiffusion techniques, was always less than 5% of the concentration before CoF injection.

*Gross and Microscopic Observations.*    Rabbits were sacrificed for autopsy examination 24 h after injection of LPS or, in cases in which rabbits failed to survive, were autopsied as close to the time of death as possible. After examination of the organs macroscopically, sections were taken of the lung, liver, spleen, and kidney routinely along with sections from any tissue of other organs appearing abnormal. In initial experiments, microscopic sections were prepared from all organs of the body. Sections were fixed in 10% neutral formalin for embedding in paraffin and were quickly frozen at −70°C for fluorescent microscopic observations. Paraffin-embedded tissues were stained with hematoxylin and eosin, phosphotungstic acid hematoxylin, and periodic acid Schiff reagent. Frozen sections were treated with fluorescent antibodies directed to rabbit fibrinogen, gamma globulin, C3, and albumin. As control, specific antibodies were absorbed from the antibody solution by addition of purified antigen before treatment of the frozen sections.

## Results

*Hypotension Induced in Rabbits by Three Preparations of Bacterial LPS.*    To compare the various preparations of LPS used in these studies the relationship
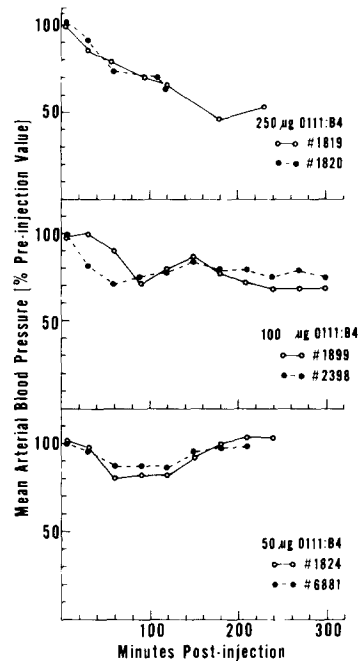
FIG. 1.   The dependence of blood pressure changes on the concentration of *E. coli* 0111:B4 LPS. The data are expressed as a percentage of the preinjection mean arterial blood pressure. Results for two individual animals are shown in each panel.

between dose of LPS and blood pressure fall during a 5-h period after injection was determined for the soluble LPS preparations from *E. coli* 0111:B4 and *S. minnesota* Re595 and the particulate LPS from *S. marcescens*.

Representative data from several individual animals injected with 50, 100, or 250 μg of LPS 0111:B4 is shown in Fig. 1. Dose response experiments for both Re595 and *S. marcescens* LPS were also performed to determine the minimal concentration of these molecules which would initiate and maintain a 30% decrease in blood pressure during a 5-h period. In most subsequent experiments concentrations of LPS were used which produced and sustained at least a 30% fall in mean arterial blood pressure over 5 h, namely 100 μg for LPS 0111:B4 and *S. marcescens* and 50 μg for LPS Re595. Although the minimal dose usually did not result in death during a 24-h period all animals injected with LPS were characterized as visibly sick. For example, in one series of experiments 2/12, 1/9, and 1/6 rabbits injected with the minimal hypotensive dose of Re595, *S. marcescens*, and 0111:B4, respectively, were dead at 24 h postinjection. However, all of the survivors in each group were characterized as sick, and as to be described in a subsequent section, presented evidence of DIC. Animals injected with saline demonstrated less than 10% change in mean arterial pressure during the 5 h of the experiment (shaded areas, Figs. 2-4).

During the experiment 2.5-ml blood samples were removed. To eliminate the possibility that the blood sampling potentiated blood pressure decreases after LPS injection, a group of animals that were bled twice before injection and then at 5, 30, 90, 180, and 300 min postinjection (17.5 ml blood removed) were
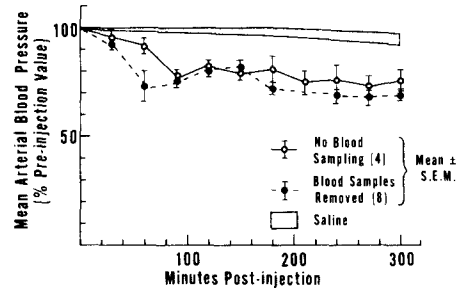
FIG. 2. The effect of removal of blood samples on LPS-induced hypotensive changes. Two groups of rabbits were injected with 100 μg of 0111:B4 LPS. One group had no blood samples removed, while the other had 2.5-ml samples removed twice before LPS injection and at 5, 30, 90, 180, and 300 min postinjection. The numbers of animals in each group is given in parentheses. Blood pressure changes in animals injected with saline are shown in the shaded area.
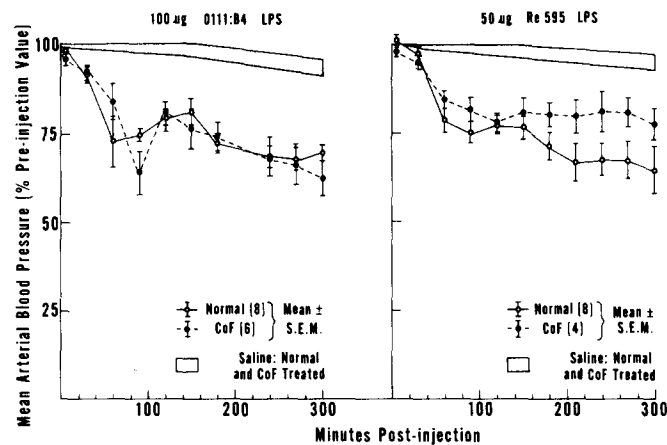


FIG. 3. The effect on mean arterial blood pressure of injection of 0111:B4 LPS (left panel) and Re595 (right panel) in normal and CoF-treated rabbits. Normal rabbits were depleted of C3 by multiple intraperitoneal injections of 150 U/injection of CoF. CoF-treated animals had less than 5% of the pretreatment C3 levels at the time of LPS injection and during the experiment. The mean arterial blood pressure in normal and CoF-treated rabbits injected with saline is shown in the shaded area. The numbers of rabbits in each group is given in parentheses.

compared with a group of animals that had no blood removed. The results of experiments with 0111:B4 LPS are shown in Fig. 2. These data indicate that only at 30 min postinjection is there a difference between the two groups of animals. Even when up to 35 ml of blood were removed (data not shown) the pressure changes observed were of the same magnitude as the two groups of experimental animals shown in Fig. 2. Other experiments (data not shown) indicated that blood sampling had no effect on animals injected with Re595 or *S. marcescens* LPS.

*Blood Pressure Changes in CoF-Treated Rabbits.* To assess the role of C3 in LPS-induced hypotension normal rabbits were depleted of C3 and terminal complement components by multiple intraperitoneal injections of CoF. At the
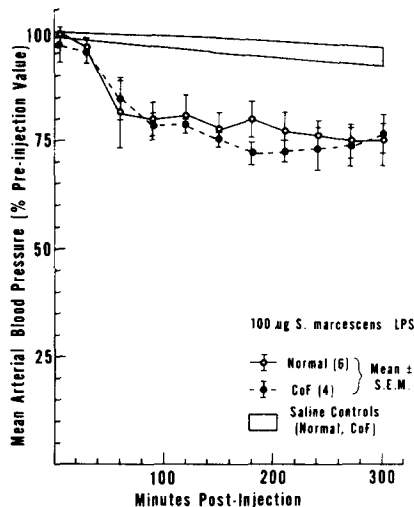
FIG. 4.   The effect on mean arterial blood pressure of injection of 100 µg of *S. marcescens* LPS into normal and CoF-treated rabbits. CoF-treated animals had less than 5% of their circulating C3 at the time of injection and during the experiment. The numbers of rabbits in each group is given in parentheses.

start of the experiment and during the 5 h of measurement the plasma C3 levels were less than 5% of the pre-CoF treatment value.

Results obtained after the injection of 100 µg of 0111:B4 LPS or 50 µg of Re595 LPS are given in Fig. 3. Normal animals show a blood pressure fall characterized by an initial decrease of 25–30% within 30–60 min postinjection, no change or a partial return in the pressure 60–120 min postinjection, and a secondary small drop occurring beyond 120 min postinjection. When 100 µg of 0111:B4 LPS was injected into CoF-treated rabbits both the rate and extent of the pressure drop was identical to that observed in normal rabbits. (Fig. 3, left panel) Injection of 50 µg of Re595 into CoF-treated rabbits produced an initial decrease in blood pressure (60 min postinjection) paralleling that in normal rabbits, but the secondary drop in pressure was somewhat reduced when compared to that of normal animals (Fig. 3, right panel).

Experiments were also performed with 100 µg of *S. marcescens*. These results are shown in Fig. 4, and it is apparent that the blood pressure decreases observed in CoF-treated rabbits parallel those seen in normal animals. In additional experiments, 12.5 mg of *S. marcescens* was injected into normal and CoF-treated rabbits (six rabbits in each group). The hypotension that resulted was identical in the two groups and similar in intensity and time-course to that shown in Fig. 4 with 100 µg of the *S. marcescens* LPS.

*Blood Pressure Changes in C6-Deficient Rabbits.*   Although the blood pressure changes in CoF-treated rabbits parallel those observed in normal rabbits a role for the terminal components C5–C9 cannot be completely excluded. The low level of C3 (5%) in CoF-treated rabbits could be sufficient to permit lysis of a critical target cell by C5–C9. Thus participation of the terminal sequence of complement components (C5–C9) in LPS-induced hypotension was assessed using rabbits genetically deficient in C6. Rabbits deficient in C6 were injected
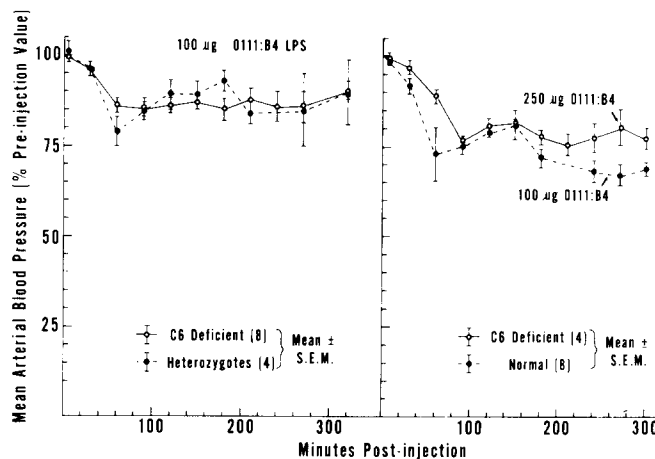
FIG. 5.   (Left panel) The effect of injection of 100 μg of 0111:B4 LPS on mean arterial blood pressure in C6-deficient rabbits and rabbits heterozygous for the C6 deficiency. The heterozygotes have 35–45% of normal C6 levels. (Right panel) The effect of injection of 250 μg of 0111:B4 LPS into C6-deficient rabbits compared with the effect of injection of 100 μg of 0111:B4 LPS into normal rabbits. The numbers of animals in each group is given in parentheses.

with 100 μg of 0111:B4 LPS, and the decrease in blood pressure was compared with that observed in normal rabbits. These results are shown in Fig. 5 (left panel). Injection of 100 μg of 0111:B4 LPS into C6-deficient rabbits induced a decrease in blood pressure in C6-deficient rabbits over the 5-h period. Between 3 and 5 h, the hypotension was less than that observed in normal animals. This decreased response could occur as a result of strain differences between the C6-deficient rabbits and the normal rabbits or a participation of C5–C9 in mediating LPS-induced hypotension. To distinguish this, rabbits heterozygous for the C6 deficiency trait (35–45% normal C6 levels) were injected with 100 μg of 0111:B4 LPS. The fall in blood pressure observed paralleled that seen with the C6-deficient rabbits. These results suggest that a strain difference could account for the diminished response to 0111:B4 in both C6-deficient and rabbits with a partial deficiency of C6 as compared to normal rabbits. This difference would be reflected by a different dose response behavior with respect to 0111:B4 LPS. To test this a group of C6-deficient rabbits were injected with 250 μg of 0111:B4. These data are shown in Fig. 5 (right panel). The C6-deficient rabbits showed an early fall in mean arterial blood pressure which approached that seen in normal rabbits, although the maximum decrease occurred 20–30 min later. Some difference was observed in the second phase of the hypotension in which the C6-deficient animals did not demonstrate as large a fall.

*Peripheral Blood Cell Changes.*   The role of complement in alterations in numbers of circulating platelets, neutrophils, and mononuclear cells was investigated using normal, CoF-treated, and C6-deficient rabbits. These experiments were performed for several reasons: (a) To define what cells are affected by LPS injection and the kinetics of these changes, (b) to determine the role of complement in changes in peripheral blood cell numbers, and (c) to determine which, if any, cell changes correlate with blood pressure decreases. Measurements were
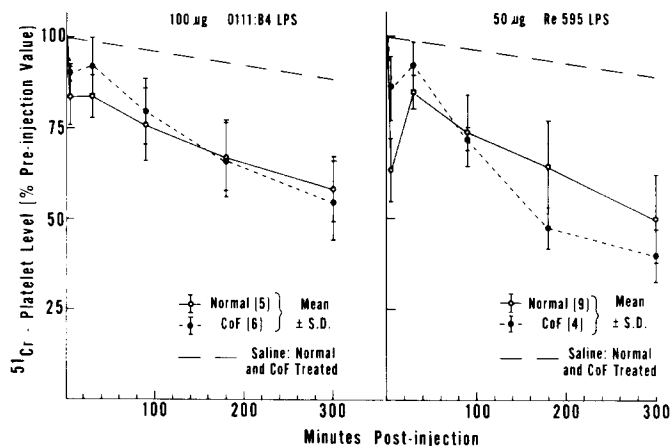
FIG. 6.   The effect of LPS injection on circulating ^{51}Cr-platelets. (Left panel) The effect on platelets of the injection of 100 μg of 0111:B4 LPS into normal and CoF-treated animals. (Right panel) The effect on platelets of the injection of 50 μg of Re595 LPS on circulating platelets in normal and CoF-treated rabbits. The numbers of animals in each group are given in parentheses. The dashed line represents the theoretical curve for platelet turnover based upon a $t_{1/2}$ of 30 h (see Materials and Methods).

obtained in the same experiments that monitored blood pressure changes after LPS injection. The various cells were quantitated as described in Materials and Methods.

*Platelet Changes.*   Control experiments described in Materials and Methods indicated that ^{51}Cr-platelets behaved in a similar manner when normal noncannulated rabbits were compared with either normal or CoF-treated experimental control animals. A $t_{1/2}$ for platelet survival of approximately 30 h was determined. Preliminary experiments demonstrated a correlation between ^{51}Cr-platelet changes and changes in number of platelets determined by visual quantitation of platelets in a hemocytometer.

After the injection of the different LPS preparations, two distinct patterns of ^{51}Cr platelet disappearance were observed in normal animals. Injection of 100 μg of 0111:B4 LPS into normal rabbits produced a small decrease (<10%) in circulating platelets 5 min after injection. The remaining platelets disappeared at a rate that appeared to be only slightly more rapid than that observed in control animals (Fig. 6, left panel). When 100 μg of 0111:B4 was injected into CoF-treated rabbits and the results compared to normal rabbits, only a slight reduction of the small drop observed at 5 min was noted (Fig. 6, left panel). When experiments were performed in C6-deficient rabbits injected with 100 μg of 0111:B4 LPS platelet changes identical to those observed in normal rabbits were seen.

In contrast to results obtained with 0111:B4 LPS, injection of 50 μg of Re595 LPS into normal rabbits produced a rapid and substantial decrease in circulating platelets (Fig. 6, right panel) at 5 min. The decrease was followed by a return to about 80% of the preinjection value and then a secondary disappearance phase. This secondary disappearance phase proceeded at an increased rate when compared to that observed in control animals. CoF treatment greatly reduced
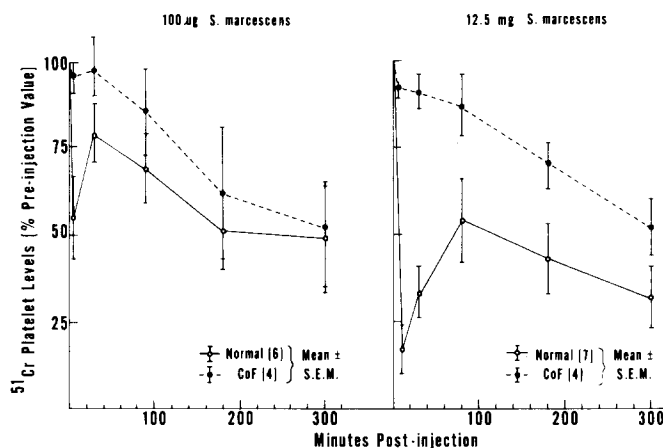
Fig. 7.    The effect of LPS injection on circulating $^{51}$Cr-platelets. (Left panel) The effect of injection of 100 μg of S. marcescens LPS on circulating platelets in normal and CoF-treated rabbits. (Right panel) The effect of injection of 12.5 mg S. marcescens LPS on circulating platelets in normal and CoF-treated rabbits. The number of animals in each group are given in parentheses.

the initial decrease in circulating platelets observed after Re595 injection, while having little effect on the rate of the secondary disappearance of the remaining platelets.

Similarly when either 100 μg or 12.5 mg of S. marcescens was injected, a biphasic platelet change was observed with the rapid and substantial drop in platelets being markedly reduced by CoF treatment (Fig. 7). At either concentration the secondary disappearance of platelets proceeded at a greater rate than that observed in control animals. Experiments in which S. marcescens was injected into C6-deficient rabbits also demonstrated a biphasic platelet response (data not shown).

Two distinct patterns of platelet behavior have been noted. The injection of 0111:B4 LPS was characterized by a change in circulating platelets that occurred despite treatment with CoF, while in marked contrast a biphasic platelet change is observed after injection of Re595 or S. marcescens LPS. The initial rapid drop observed after the injection of these LPS molecules is largely reduced by prior administration of CoF to eliminate C3, while the secondary disappearance of platelets remained unaffected.

*Neutrophils and Mononuclear Cells.* Injection of all three preparations of LPS into normal, CoF-treated, or C6-deficient rabbits produced a rapid and virtually complete disappearance of neutrophils followed by a gradual return beginning about 180 min postinjection. The effects of injection of LPS 0111:B4 or Re595 on neutrophils is shown in Fig. 8. No differences between normal and CoF-treated rabbits were noted. A rapid fall in mononuclear cells to 10–20% of preinjection levels was also observed after the injection of the different LPS molecules into normal, CoF-treated, and C6-deficient rabbits. Changes in circulating neutrophils and monocytes have been observed with as little as 0.1 μg of 0111:B4 LPS.

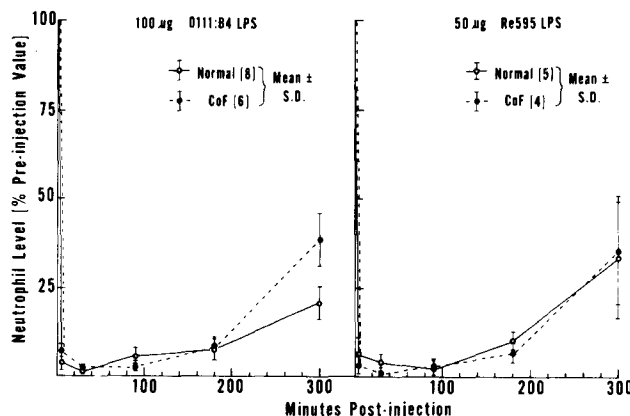*Gross and Histologic Evidence of DIC.*    Gross and microscopic observations

FIG. 8.   The effect of LPS injection on levels of circulating neutrophils. (Left panel) The
effect of injection of 100 μg of 0111:B4 LPS in normal and CoF-treated rabbits on levels of
neutrophils. (Right panel) The effect of injection of 50 μg of Re595 LPS into normal and CoF-
treated rabbits on levels of neutrophils. The numbers of animals in each group are given in
parentheses.

were performed on normal rabbits, rabbits depleted of C3, and C6-deficient
rabbits injected with the three types of LPS described in the experiments above.
Qualitatively similar changes occurred in rabbits injected with each type of LPS
although the quantity and severity of lesions (described below) were greater
when 50 μg Re595 LPS or 100 μg S. marcescens LPS, rather than 100 μg 0111:B4
LPS, were injected. In the normal rabbits, at these dosages of LPS, the lungs
were reddened and occasionally hemorrhages were noted in both the lungs and
along the serosal surface of the large and small bowel. In some rabbits, yellow-
ish flecks of 1–2 mm, representing zones of coagulative necrosis, were observed
on the surface of the liver with penetration occurring several millimeters into
the cut surface. The kidneys in less than 10% of normal rabbits showed early
evidence of coagulative necrosis, but otherwise appeared normal. In rabbits
pretreated with CoF and injected with 50 μg Re595 or 100 μg S. marcescens,
greater severity of the DIC was observed grossly than in rabbits with the LPS
alone. Four control rabbits with CoF pretreatment only, did not show evidence
of DIC grossly. No differences were noted when normal and C6-deficient rabbits
injected with 0111:B4 LPS compared.

Microscopically, fibrin thrombi were seen in a great majority of rabbits
injected with each LPS. The distribution and extent of thrombi in the case of
LPS Re595 is shown in Table I, and the appearance of fibrin-rich thrombi are
shown in Fig. 9. No thrombi were observed in saline controls. In the case of
normal rabbits injected with 50 μg Re595, 8/10 showed thrombi microscopically
24 h after injection of LPS, while 15/19 rabbits injected with LPS of E. coli
0111:B4 and all rabbits injected with 100 μg S. marcescens demonstrated mi-
crothrombi of a moderate to mild degree. Increasing the dose of each LPS to over
3 mg resulted in greater severity in all cases with thrombi appearing in each
section of each organ. The thrombi stained blue with phosphotungstic acid
hematoxylin and reacted with fluorescent antifibrin but not antialbumin. IgG
and C3 were present in only small amounts in the thrombi.

TABLE I

*Microscopic Evaluation of Intravascular Coagulation S. minnesota Re595 (50 µg)*

| | Normal rabbits | | | CoF rabbits | | | |
|---|---|---|---|---|---|---|---|
| | Moder-ate* | Mild | Neg | Severe | Mod | Mild | Neg |
| Liver | 3 | 5 | 3 | 1 | 2 | 1 | — |
| Lung | 2 | 6 | 3 | 1 | 1 | 2 | — |
| Kidney | 1 | 4 | 6 | 1 | 2 | 1 | — |
| Spleen | 4 | 5 | 2 | 1 | 1 | 2 | — |

* The numbers represent individual rabbits bearing lesions of the stated severity 24 h after injection of LPS. Severe: marked numbers of thrombi in each section of tissue with evidence of renal cortical necrosis. Moderate: occasional thrombi in each section of tissue. Mild: rare thrombi observed in occasional sections.

Rabbits depleted of C3 and terminal components with CoF before injection with LPS demonstrated qualitatively similar changes as normal rabbits, but the lesions were more numerous and of greater severity (Table I). Frequently with *S. marcescens* (4/4) and occasionally with *E. coli* 0111:B4 (3/11), rabbits pretreated with CoF developed severe thrombotic lesions. Of four rabbits injected with CoF alone intraperitoneally (without a second injection of LPS), one showed a single microthrombus in the lung. In five C6-deficient rabbits, injections of 100 µg *E. coli* 0111:B4 produced microthrombi to an extent similar to that in normal rabbits.

*In Vivo Complement Measurements.* To determine whether the LPS preparations used do activate complement in vivo, plasma was analyzed for C3 by radial immunodiffusion in all experiments in which blood pressure and peripheral blood cell changes were studied. In addition, separate experiments were performed in rabbits that were injected with up to 30 times the minimal hypotensive dose, and serum samples were collected for $CH_{50}$ determination.

Using the minimal concentration of each LPS that produced hypotension and DIC, no changes in plasma C3 concentration were detected. These data are shown for individual animals in Fig. 10 (solid line), but are representative of the 22 animals tested. Only when doses 30–100 times the minimal hypotensive dose were tested slight decreases in C3 were observed and are most apparent in animals injected with Re595. Simultaneous measurement of RSA or rabbit IgG by radial immunodiffusion revealed no changes in these proteins during the time when the modest decrease in plasma C3 concentration was observed.

Changes in $CH_{50}$ measured at various times after injection of 3 mg 0111:B4 or Re595 indicated no consistent pattern of decrease. (Fig. 11) Even when decreases were detected the changes were only 20–30%. Again these experiments were performed with concentrations of LPS much greater than that required to produce hypotension or DIC.

*Hypotensive Changes Associated with Complement Activation.* The previous data suggest that complement is not required for the production of LPS-induced hypotension. The following experiment was performed to directly examine the effect of intravascular complement activation on blood pressure changes.

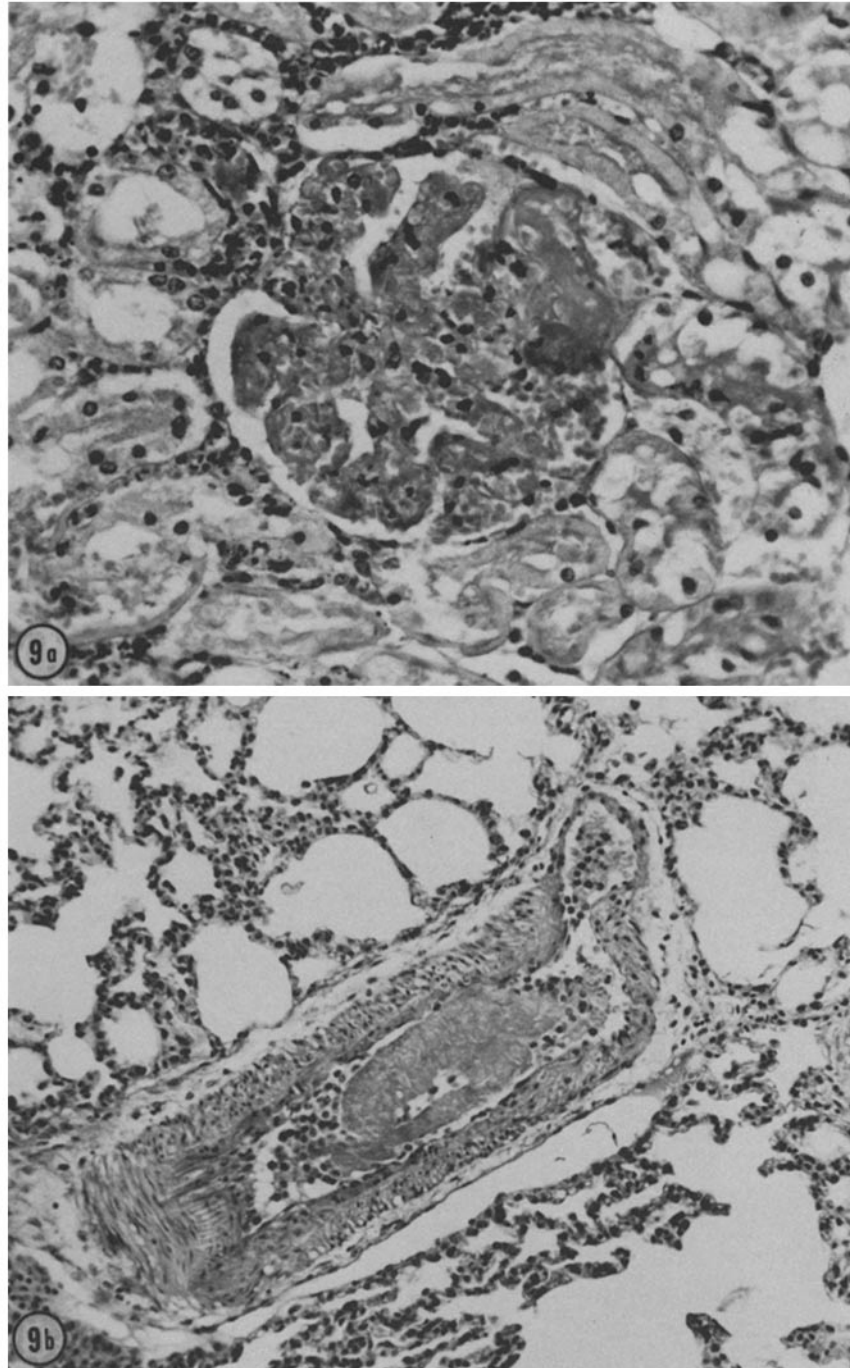FIG. 9.   (a) Photomicrograph of a glomerulus of a rabbit injected 24 h previously with 50 μg of Re595 LPS. Abundant fibrin deposits are noted in the capillary lumens of the glomerular tufts. Advanced necrosis of the renal parenchyma has occurred in this severe reaction. PAS stain × 320. (b) Photomicrograph of a fibrin deposit in a pulmonary vessel. PAS stain × 100.
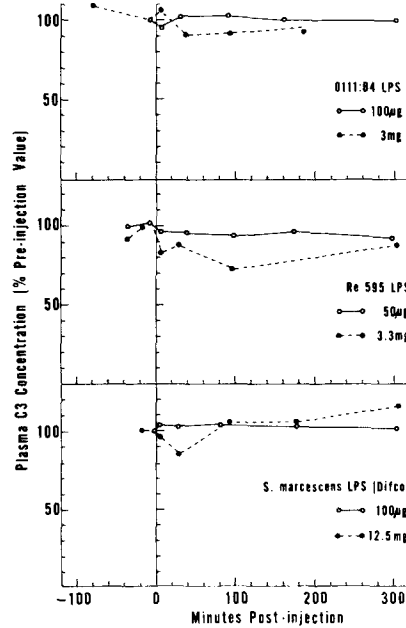
FIG. 10.    The effect of LPS injection on plasma C3 levels determined by radial immunodiffusion measurements. Each panel shows results obtained with individual animals injected with 0111:B4, Re595, or *S. marcescens* LPS.
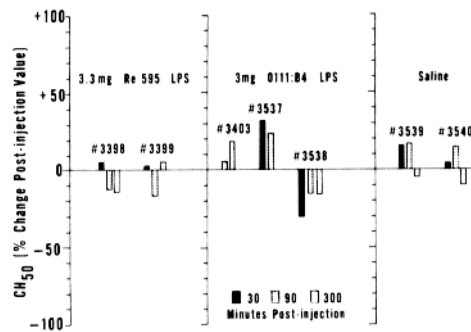


FIG. 11.    The effect of LPS injection on in vivo $CH_{50}$ values determined after injection of LPS. The data is expressed as a percentage change from the preinjection value. Results for individual animals are given in each panel.

To activate the complement system, intravenous or intraperitoneal injections of 300 U of CoF were administered to normal rabbits, and the effect of the injections on blood pressure followed. The experimental procedure utilized in this experiment was identical to that described for the LPS experiment except that only CoF was administered. Blood samples were removed at various times before and after CoF injection. Changes in plasma C3 levels were measured by radial immunodiffusion and used to assess complement activation. The effects of CoF injection on mean arterial blood pressure and C3 concentrations are shown in Fig. 12. As noted a fall in blood pressure occurred immediately after the
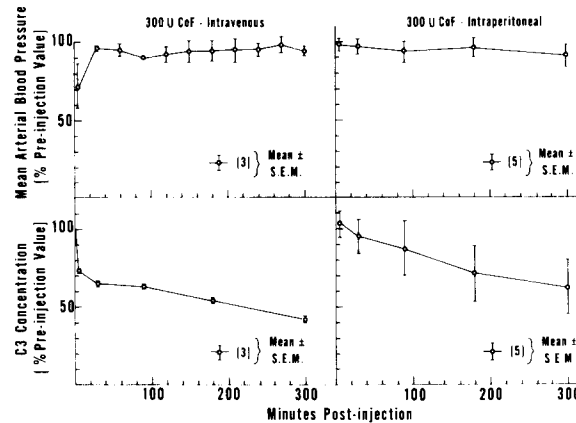
FIG. 12.   A comparison of blood pressure changes and changes in plasma C3 levels after the intravenous and intraperitoneal injection of 300 U of CoF. The numbers of animals in each group is given in parentheses.

injection of CoF intravenously, but not after its injection intraperitoneally. The drop was short lived, with normal blood pressure levels resumed within 30 min. The fall was accompanied by a rapid fall of C3 levels, with a decrease of 30–40% occurring in the first few minutes after injection. The amount of C3 activated could have been even greater, since clearance of the activated C3 from the circulation was required to detect decreases by the immunologic technique employed. In rabbits injected with 300 U of CoF intraperitoneally the levels of C3 fall to approximately the same extent, but the rate of fall was much slower. In these rabbits hypotension was not observed.

## Discussion

*Hypotension Induced by Bacterial LPS. Its Relationship to Complement Activation.*   The results suggest that C3 and terminal complement components are of minimal importance in the initiation of LPS-induced hypotension and DIC. This conclusion is based upon experiments that compared the effect of injection of three different LPS preparations into normal, CoF-treated, and C6-deficient rabbits and upon experiments designed to examine the hypotension associated with activation of complement. Depletion of C3 with CoF before injection of LPS failed to prevent the hypotension and DIC. In addition, hypotension and DIC were observed in C6-deficient rabbits injected with LPS.

The studies were conducted with three different preparations of LPS to maximize observation of potentially different effects on hypotension, DIC, and changes in blood cells and plasma protein systems produced by LPS. The three different LPS preparations were chosen for the following reasons: (a) LPS 0111:B4 , as prepared, is soluble in aqueous solution, and in vitro was not an efficient activator of hemolytic complement, (b) LPS Re595, although insoluble as purified, was chemically solubilized. This molecule, in contrast to 0111:B4 LPS was a potent activator of serum hemolytic complement, and (c) LPS from *S. marcescens* used in these studies was only partially soluble in aqueous solution and was not a highly purified preparation. LPS from *S. marcescens* has been

utilized by other investigators and therefore allows our studies to be compared with previous reports.

Dose-response studies were performed to determine a minimal concentration of LPS required to produce a 30% decrease in mean arterial blood pressure during the 5-h period of measurement. While the 30% drop was chosen arbitrarily, the use of a threshold dose permitted the study of hypotension at a dose of LPS that was not lethal and that would be most sensitive for experimental manipulation of complement. No significant differences were observed in the three LPS preparations in their ability to induce a hypotensive change in normal rabbits. Furthermore, we established that the removal of blood samples during the experiment had little effect on the hypotensive changes observed.

Changes in blood pressure observed after injection of any one of the three preparations used could be divided into two phases. The first drop occurred within 30–80 min of injection, and then the blood pressure was either maintained at this level or partially returned to normal during the subsequent hour. A secondary small decrease occurred between 120 min postinjection and the final measurement at 300 min.

The minimal importance of C3 in the establishment of the first phase of the pressure fall is readily apparent when CoF-treated rabbits injected with 0111:B4 or *S. marcescens* LPS are compared with normal animals (Fig. 3 and 4). No differences are observed in these groups. Furthermore, the second phase of the pressure drop was similar when normal and CoF-treated rabbits injected with either 0111:B4 or *S. marcescens* are compared. Only in the group injected with Re595 does CoF treatment produce any difference in blood pressure changes. In this group the secondary fall in blood pressure does not occur, but the mean arterial blood pressure is maintained at levels between 75–85% of the preinjection blood pressure. Thus with this type of LPS it appears that C3 and terminal complement components may play a role in the potentiation of the LPS-induced blood pressure fall. Experiments in which 12.5 mg of *S. marcescens* were injected into normal and CoF-treated rabbits also demonstrated no difference in the LPS-induced hypotension when these two groups were compared.

Experiments with C6-deficient rabbits also provide evidence that the complete sequence of terminal components C5–C9 is not required for LPS-induced hypotension. These data indicate a minimal role for either "innocent bystander" lysis (37) of various effector cells or direct lysis of target cells which might have LPS bound to their surface. When 100 $\mu$g of 0111:B4 were injected into C6-deficient rabbits the mean arterial blood pressure decreased about 15% in contrast to the 30% decrease observed in normal rabbits. When the same concentration of LPS was injected into rabbits that were heterozygous for C6-deficiency (35–45% of normal C6 levels) a 15% decrease in pressure was also observed. These data suggest that there is a strain difference between our normal New Zealand white rabbits and the C6-deficient animals. Inasmuch as we used a threshold dose of LPS determined from a dose response in normal animals this difference might be accentuated. This argument is supported by the result obtained when 250 $\mu$g of 0111:B4 were injected into C6-deficient rabbits. At this concentration the change in blood pressure after LPS injection parallels that seen in normal animals injected with 100 $\mu$g of 0111:B4.

These data therefore provide evidence for a minimal role of C3 and terminal complement components in LPS-induced hypotension. At the present time there are still several other possible roles of complement components in LPS-induced hypotension which cannot be excluded. First the involvement of proteolytic enzymes from neutrophils or other cells (34) which could cleave C5 to produce the biologically active fragment C5a cannnot be completely excluded from experiments. However, preliminary experiments have indicated that neutrophil depletion does not block LPS-induced hypotensive changes.[3] It is also possible that a component or components reacting with the LPS before C3PA or in the early components of the classical pathway could be involved in LPS-induced hypotension. However, it is clear from our measurements of complement activation in vivo that little if any complement is activated by the LPS preparations tested even when concentrations of LPS are used which are far in excess of that required to produce hypotension.

*Hypotension Associated with Activation of Complement.* The studies on hypotension associated with the activation of complement revealed significant differences between complement and LPS-induced hypotension. Considerable activation of C3 (greater than 30% of that in the circulation) was required to produce hypotension after injection of CoF. In addition, the consumption of C3 had to take place rapidly, i.e., within a 5-min period. Slower consumption of C3, as occurred after an intraperitoneal injection of CoF, failed to induce hypotension. Furthermore, the fall in blood pressure was ephemeral, with levels returning to normal within 30 min in marked contrast to LPS-induced hypotensive changes. Studies examining the causal relationship between activation of C3 and the development of hypotension will be the subject of a later publication.

Other investigators have also studied the role of complement in LPS-induced hypotension. One of the first studies of the role of the complement system in LPS-induced hypotensive changes was performed by From et al. in 1970 (4). Using CoF, these investigators depleted C3 in dogs and compared the hypotensive changes observed after LPS injection into normal and CoF-treated dogs. LPS injection into normal dogs produced an immediate fall in blood pressure (within 2 min postinjection), a gradual return to about 60–80% of normal within 30–60 min, followed by a decrease in pressure measured over 3 h. CoF treatment abrogated the initial rapid drop but had no effect on the secondary drop in blood pressure. This early drop in blood pressure seen in normal, but not CoF-treated dogs, may be analogous to the decrease in mean arterial blood pressure we observed after intravenous CoF injection. That is, the drop is associated with activation of complement and is easily reversible, possibly due to the generation of short lived mediators such as the anaphylotoxins. The role of complement in LPS-induced hypotension in felines has also been studied by Kitzmiller et al. (3). CoF treatment of felines had little effect on the hypotensive change observed after the injection of LPS.

Recently, however, another study in dogs by Garner et al. (2) indicated that CoF-treated dogs did not demonstrate any fall in mean arterial blood pressure after LPS injection. Although similarly to the report of From et al. (4), these

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[3] Ulevitch, R. J., C. G. Cochrane, P. M. Henson, and D. C. Morrison. Unpublished results.

authors abrogated the initial fall in blood pressure observed after LPS injection into dogs depleted of C3 by CoF, they also indicated that CoF treatment abrogated any secondary fall in mean arterial pressure up to 3 h after LPS injection. An explanation of the differences between this study and the results of From et al. (4) is not clear at this time.

It is of interest that Johnson and Ward (35) observed a protection in normal rabbits to the lethal effects of 200 $\mu$g of LPS that was not shared by C6-deficient rabbits. Lethality was measured after 48 h. He postulated that the terminal components of complement could play a role in detoxifying LPS. The present data indicate that the acute effects of LPS (24 h) are similar in C6-deficient and normal rabbits, although of slightly less intensity in the former, and do not bear on the longer term (48 h) effects. No difference in the lethal effects of the three LPS preparations was observed at 24 h in the present studies, as sublethal doses were employed.

*Coagulation Associated with LPS in Normal, CoF-Treated, and C6-Deficient Rabbits.*    The present studies indicate that the coagulative effects of LPS can occur despite prior depletion of C3 and in the absence of C6. This suggests that the active fragments C3a and C5a or the complement-mediated lysis of cells are not essential for initiation of clotting by LPS.

It was of interest that prior treatment of the rabbits with CoF to deplete C3 if anything enhanced the coagulative effects of LPS. This observation is being pursued in current studies. Muller-Berghaus and Lohmann (9) have recently demonstrated hematological changes and renal glomerular microthrombi to occur in C6-deficient rabbits infused with LPS which were indistinguishable from those changes observed in normal rabbits treated with LPS. These results also suggest that the terminal complement components are not required for the consumptive coagulopathy induced by LPS.

*The Effect of LPS on Blood Cells.*    Injection of all three preparations of LPS into normal, CoF-treated, and C6-deficient rabbits produces changes in circulating neutrophils, mononuclear cells, and platelets.

The behavior of neutrophils in normal rabbits injected with the minimal hypotensive dose of any of the three LPS molecules used in this study is characterized by a rapid, within 5-min postinjection, and almost complete disappearance of cells from the circulation. The neutrophil levels remain between 0–10% of the starting value for approximately 2 h and then gradually return to the circulation reaching values of 25–50% of the initial preinjection level by 5 h postinjection. This pattern of neutrophil disappearance and return is identical in CoF-treated and C6-deficient rabbits. The pattern of the disappearance and return of the mononuclear cells in normal rabbits is essentially the same as that of the neutrophils. The principal difference is that the mononuclear cells only fall to 10–20% of the preinjection level. This same pattern of mononuclear cell disappearance was observed in rabbits treated with CoF and in C6-deficient rabbits. Therefore the changes in circulating neutrophils and mononuclear cells appears not to require the presence of C3 or terminal complement components. At the present time the mediation system responsible for the disappearance of the neutrophils and mononuclear cells has not been defined. This phenomenon appears to be different from the neutropenia followed by neutrophilia occurring after CoF injection as described recently by McCall et al.

(36). These investigators postulated that the neutrophil changes observed after CoF injection were mediated by some product of complement activation.

Two distinct patterns of $^{51}$Cr-platelet behavior have been observed after LPS injection. Injection of 100 $\mu$g 0111:B4 LPS into normal and CoF-treated rabbits produced parallel changes in circulating platelets, characterized by a gradual decrease in platelets over a 5-h period which appeared to be slightly increased from that observed in control animals. The behavior of platelets in C6-deficient rabbits injected with 100 $\mu$g of 0111:B4 was indistinguishable from normal animals. Thus it appears that neither C3 nor the terminal complement components influence the behavior of $^{51}$Cr-platelets in rabbits injected with 0111:B4 LPS.

In contrast, however, are the changes in circulating platelets observed after the injection of 50 $\mu$g of Re595 and 100 $\mu$g or 12.5 mg of S. marcescens LPS. Injection of either 50 $\mu$g of Re595 or 100 $\mu$g of S. marcescens induced a biphasic change in circulating platelets. This change is characterized by a 30–60% decrease within 5 min, a return to 70–90% of the preinjection value by 30 min, and a secondary disappearance of platelets occurring at a rate greater than that observed in control animals. Injection of 12.5 mg of S. marcescens differed primarily in the magnitude of the disappearance and the extent of the return to the circulation. That is, platelet levels fall rapidly to less than 25% of initial values and only returned to 40–65% of starting levels. When Re595 or S. marcescens was injected into CoF-treated rabbits the extent of the initial rapid disappearance of platelets was greatly reduced, while little if any effect was observed in the subsequent disappearance of platelets. Preliminary experiments, in which C6-deficient rabbits were injected with S. marcescens demonstrate the same biphasic platelet change observed in normal rabbits injected with S. marcescens LPS. The demonstration of a C3-dependent platelet disappearance after the injection of either Re595 or S. marcescens is in agreement with published observation of Brown and Lachmann (11). These investigators also observed a biphasic change in $^{51}$Cr-platelets after injection with 1–5 mg of S. marcescens into normal and C6-deficient rabbits, but markedly reduced the initial disappearance phase by depletion of C3 with CoF.

During the initial decrease and return phase of the platelets a portion of the platelets are either irreversibly sequestered or are lysed, since the level of platelets never returns to the 100% value. Whether one or both of these phenomena are operative is unknown at this time. However what can be concluded is that the presence of C3 is required for this change to occur. The exact mechanism of these platelet changes is also unknown but at least several possibilities exist. They are: (a) an "immune adherence" reaction involving the interaction of platelets with C3b bound to their surface (37), (b) a lytic reaction in which C$\overline{567}$, C8, C9 lyse the platelet membrane (38), (c) C3a mediated release of other factors that could affect platelet behavior, i.e., PAF from basophils (39), and (d) LPS-mediated release of ADP from cells by direct or indirect mechanisms (39).

## Summary

We have studied the role of complement in lipopolysaccharide (LPS)-induced hypotension and disseminated intravascular coagulation (DIC) by comparing the effects of injection of three preparations of LPS from E. coli 0111:B4, S.

*minnesota* Re595, and *S. marcescens*. Injections of nonlethal doses of these LPS preparations into normal rabbits produced decreases in mean arterial blood pressure during a 5-h period. When rabbits treated with cobra venom factor (CoF) to deplete C3 were injected with the various LPS preparations, mean arterial pressures fell at a rate and extent essentially identical to that observed in normal rabbits. Rabbits genetically deficient in C6 also demonstrated LPS-induced hypotensive changes. Only minimal, or no changes in plasma C3 levels or serum $CH_{50}$ values were detected in normal rabbits after LPS injection.

Hypotensive changes were also induced in rabbits when complement was rapidly activated by intravenous injection of CoF. In contrast to the hypotension induced by LPS, the fall in arterial pressure associated with the consumption of complement was short lived and required the rapid consumption of considerable amounts of C3.

The occurrence of DIC noted in normal rabbits injected with each preparation of LPS was not inhibited in either rabbits treated with cobra factor or in C6-deficient rabbits. The DIC was most pronounced after injection of Re595 and *S. marcescens* LPS.

Injection of the various LPS preparations produced a rapid disappearance of circulating neutrophils and mononuclear cells, which occurred with the same kinetics and to the same extent in normal, CoF-treated, and C6-deficient rabbits. Injection of either Re595 LPS or *S. marcescens* LPS produced a biphasic disappearance of circulating $^{51}$Cr-platelets. In contrast, injection of 0111:B4 LPS affected only slightly the rate of disappearance of $^{51}$Cr-platelets. Depletion of C3 by cobra factor treatment had no effect on the disappearance of platelets in animals injected with 0111:B4. In marked contrast cobra factor treatment greatly reduced the initial rapid disappearance of platelets in rabbits injected with either Re595 or *S. marcescens* LPS, but had no effect in the secondary disappearance phase.

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