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### Research Article

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# Interactions of the Classical and Alternate Complement Pathway with Endotoxin Lipopolysaccharide

## EFFECT ON PLATELETS AND BLOOD COAGULATION

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**ABSTRACT** The contributions of the classical and alternate pathways of complement activation to the biological effects of endotoxin have been examined in the guinea pig, with particular reference to thrombocytopenia, leukopenia, and the development of the hypercoagulable state. Injection of endotoxin into normal guinea pigs led to a 95% fall in the level of circulating platelets within 15 min as well as a fall in circulating granulocytes. C4-deficient guinea pigs, known to have a complete block in the activity of the classical complement pathway, but with the alternate pathway intact, sustained no fall in platelets. The development of granulocytopenia proceeded normally. Endotoxin did activate the alternate complement pathway in C4D guinea pigs, as evidenced by the fall in C3-9 titers. With restoration of serum C4 levels, endotoxin-induced thrombocytopenia was observed in C4D animals. Thus, function of the classical complement pathway was an absolute requirement for the development of thrombocytopenia. Experiments performed in cobra venom factor (CVF)-treated normal guinea pigs, with normal levels of C1, C4, and C2, but with less than 1% of serum C3-9 demonstrated the importance of the late components in the development of thrombocytopenia but not leukopenia.

C4-deficient guinea pigs had normal clotting times demonstrating that C4 was not required for normal clotting. In addition, development of the hypercoagulable state, evidenced by a marked shortening of the clotting time, was not observed on injection of endotoxin into C4D animals. Therefore, development of the hypercoagulable state paralleled the development of thrombocytopenia and required function of the classical complement

pathway. Again, the importance of the late components of complement was emphasized by the failure of CVF-treated normal animals to develop hypercoagulability. These results demonstrate that endotoxin is capable of activating both the classical and alternate complement pathways in guinea pigs but that function of the classical pathway is an absolute requirement for the development of thrombocytopenia and the hypercoagulable state.

### INTRODUCTION

For more than 20 years it has been known that thrombocytopenia, leukopenia, and hypercoagulability of blood occur in many species after the administration of bacterial endotoxins (1). The pathophysiologic basis for the thrombocytopenia has been a matter of controversy. Experiments *in vitro* have demonstrated a requirement for divalent cations and heat labile serum factors for endotoxin-mediated platelet damage (2, 3). Other findings have suggested that complement is not involved: the metal requirements for the endotoxin effects *in vitro* are not those which have been shown to maximize complement-fixation reactions or complement-mediated cytotoxic reactions, and no consumption of whole complement has been demonstrated in the *in vitro* reaction (4). Nevertheless, the resemblance between the thrombocytopenia induced *in vivo* and the complement-mediated immune adherence reaction has been noted by several authors (5, 6). Development of the hypercoagulable state has been thought to be a consequence of platelet damage (7).

In this report the role of complement in these reactions has been evaluated with particular reference to the contribution of the two recently described pathways of complement activation. The classical complement pathway

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proceeds through activation of the early complement components, C1, C4, and C2, to activation and cleavage of C3 and the complement proteins which act later in the sequence. The alternate or properdin pathway acts through another series of proteins to enter the complement sequence at the C3 step (8-11). In defining the role of these two pathways, this study utilized the recently described strain of guinea pigs with a genetically controlled deficiency in C4 (C4D)<sup>1</sup> (12). These animals have an intact alternate pathway but absence of function of the classical pathway due to the deficiency in C4. Bacterial endotoxins are known to be potent activators of the alternate pathway (13, 14) and their effects are thought to be mediated by the classical pathway only in rare circumstances (15). By studying the biological consequences of endotoxin administration in C4D animals it has been possible to establish the importance of the classical complement pathway in mediating these phenomena. By extending these studies to cobra venom factor (CVF)-treated normal animals it has also been possible to show that activation of the late complement components is required.

## METHODS

*Endotoxin lipopolysaccharide (LPS).* The LPS used for all the experiments was a phenol-water extract of *Escherichia coli*. (0127:B8) obtained from Difco Laboratories (lot 562875), Detroit, Mich. The mean lethal dose (LD<sub>50</sub>) of the *E. coli* endotoxin for NIH multipurpose guinea pigs was 1.0 mg/kg and this dose of endotoxin was administered in all studies reported here.

*Experimental animals.* "NIH multipurpose" guinea pigs, the strain from which the C4D guinea pigs were derived, were obtained from the NIH animal production section, and used as the normal guinea pigs. C4D guinea pigs were also obtained from the NIH animal production service, and screened by a hemolytic assay for the presence or absence of C4. Sera from 20 representative C4D and NIH animals were examined for anti-endotoxin antibody by a flocculation test utilizing *E. coli*-LPS-coated bentonite particles (16). Utilizing this technique, no antibody could be detected at the lowest serum dilution tested (1/3).

*Preparation of C3-9-depleted animals with CVF.* Vials of 500 U lyophilized purified CVF (lots 40021 and 10012) were obtained from Cordis Corp., Miami, Fla., and stored at -20°C. Just before use, the lyophilized material was dissolved in 5 ml ice-cold distilled water. Injections into NIH multipurpose guinea pigs were by the intraperitoneal route in doses of 20 U/100 g animal weight. Experiments performed on CVF-treated guinea pigs were always done 20 h after injection, and C3-9 levels were determined on all animals before use. With this dose of CVF, titers of the C3-9 complex were less than 1% of normal.

*Preparation of complement reagents and methods for titrations.* Preparation of veronal-buffered saline (VBS), dextrose veronal-buffered saline (DVBS) containing gelatin, Ca<sup>++</sup> and Mg<sup>++</sup>, VBS with added 0.01 M ethylenediamine-tetraacetic acid (EDTA buffer), sources of antibody, prepa-

<sup>1</sup>Abbreviations used in this paper: C4D, C4 deficient; CVF, cobra venom factor.

ration of optimally sensitized sheep erythrocytes (EA) and of complement cell intermediates, and the method of C3-9 complex titrations have all been described previously (17, 18). Pooled guinea pig serum, obtained from Suburban Serum Laboratory, Silver Spring, Md. was employed as the source of C4 given to the homozygous C4D animals, and also as the serum complement source for the preparation of cellular intermediates.

C4 titrations were performed utilizing C4D serum as a reagent containing all of the components of complement in excess except C4. A 0.25 ml aliquot of the sample diluted in DVBS was added to 0.25 ml EA ( $1.5 \times 10^8$  cells/ml) in DVBS. To this mixture was added 0.25 ml C4D serum diluted 1:50 in DVBS, and after incubation for 2 h at 37°C, the reaction was stopped by the addition of 2.5 ml ice-cold EDTA buffer to each tube. The degree of lysis was recorded spectrophotometrically at 412 nm, and the C4 titer expressed as that dilution of serum which would yield one effective C4 site/cell, as calculated from the Poisson distribution and the one hit theory of complement action. A detailed rationale for this technique of C4 titration will be presented elsewhere,<sup>2</sup> but results obtained by this method are in good agreement with those derived by previously reported procedures.

*White blood cell and platelet determinations.* White blood cell (WBC) and platelet counts were made on 1 ml EDTA anticoagulated retro-orbital blood samples using an electronic particle counter (Coulter Electronics, Inc., Hialeah, Fla.) and standard techniques (19, 20). 100 cell differential leukocyte counts were done on air-dried, Wright's stained smears.

*Clotting time.* Cardiac punctures were performed with a 19 gauge small vein infusion set (Abbott Laboratories, North Chicago, Ill.). To avoid contamination with tissue fluids, blood for clotting studies was used only when the needle was placed directly into the cardiac chambers without manipulation and blood flow was immediate, rapid, and uninterrupted. 1 ml of blood was withdrawn with a 6 ml disposable polypropylene syringe and discarded. A fresh polypropylene syringe was then attached to the infusion set, and an additional 4 ml was withdrawn. 1-ml portions were placed into each of two 13 × 100-ml glass tubes and each of two 13 × 100-mm siliconized glass tubes at 25°C. A stop watch was started when blood was placed in the first tube. The first glass tube was gently tilted 45° at 1-min intervals until it could be inverted 180° without blood flowing. The tube was then gently tapped to find if the clot was solid, and formation of a solid clot was taken as the end point. The same procedure was repeated sequentially with tubes number 2, 3, and 4. The clotting times of the second and fourth tubes were recorded as the glass and silicon times respectively.

## RESULTS

*Effect of endotoxin on C4 and C3-9 levels in normal and C4D guinea pigs.* Blood samples of 1.0 ml for C4 and C3-9 were obtained from the retro-orbital sinus of 10 normal and 10 C4D guinea pigs, just before (T<sub>0</sub>) and at 15 min after the intravenous injection of *E. coli* endotoxin. Groups of 10 normal and 10 C4D guinea pigs were bled at T<sub>0</sub> and 15 min to obtain control values. All blood samples were allowed to clot at room tempera-

<sup>2</sup>Frank, M. M., and T. Gaither. Manuscript in preparation.

ture for 60 min, and were then centrifuged at 3,000 rpm for 5 min. The supernates were decanted and titrated for C4 and C3-9. The complement titers at 15 min were expressed as a percent of the  $T_0$  complement levels for each individual animal, and the mean of each experimental group determined.

Complement levels of samples obtained 15 min after the injection of endotoxin into normal guinea pigs revealed a mean decrease of  $12\% \pm 2$  SE in C4 and  $29\% \pm 4$  in C3-9 (Table I). In the C4D guinea pigs a  $16\% \pm 3$  decrease in the C3-9 complex was observed. Consumption of C3-9 in C4D animals with complement activation limited to the alternate pathway was significantly less  $P < 0.02$ ) than that observed in the normal animals with both a functional alternate and classical complement pathway. No significant decrease in C4 or C3-9 was noted in the nonendotoxin-treated control groups.

**Effect of endotoxin on platelets and WBC in normal and C4D guinea pigs.** Base-line ( $T_0$ ) WBC and platelet counts were determined on retro-orbital blood samples from eight normal and eight C4D guinea pigs. Each animal was then injected i.v. with *E. Coli* endotoxin, and retro-orbital blood samples for platelet and WBC counts taken at intervals of 15 min, 2 h, and in some cases either 4 or 24 h after injection of the endotoxin. Differential WBC counts were performed at the 15 min interval on two animals from each group. A control group of eight normal and eight C4D guinea pigs were given 0.1 ml/100 g body weight sterile saline instead of endotoxin, and bled at similar time intervals for platelet and WBC counts. The change in platelet and WBC counts was expressed as a percent of the initial ( $T_0$ ) count for each animal, and a mean calculated for each experimental group. No differences in the initial platelet and WBC counts were noted between the normal and C4D animals; mean platelet counts were  $744,000 \pm SD 103,000$  and  $760,000 \pm SD 201,000$  respectively, mean WBC counts were  $9,000 \pm SD 2870$  and  $9,300 \pm SD 1650$ .

TABLE I  
Consumption of C4 and C3-9 after the Injection of Endotoxin\*

Animals (no.)	C4 Consumption†	C3-9 Consumption‡
	%	%
Normal (10)	$12 \pm 2$	$29 \pm 4$
Normal control§ (10)	$0 \pm 2$	$0 \pm 0.7$
C4D (10)	—	$16 \pm 3$
C4D control§ (10)	—	$0 \pm 0.6$

\* Blood for complement studies was taken just before and 15 min after the injection of endotoxin.

† Expressed as mean consumption  $\pm$  SEM.

§ Animals bled at zero time and at 15 min without receiving endotoxin.

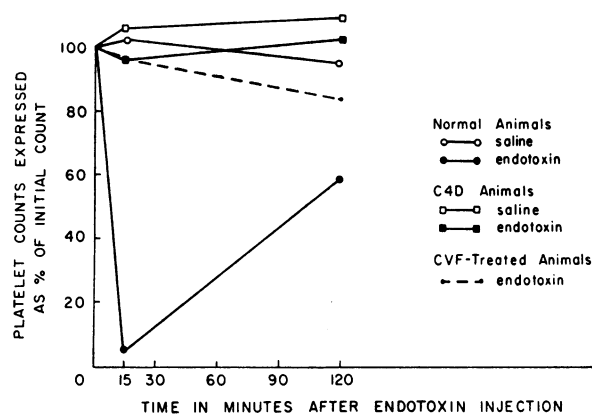


FIGURE 1 Effect of endotoxin on platelets in normal, C4D, and CVF-treated guinea pigs. The initial platelet values for each group were as follows: normal animals— $744,000 \pm SD 103,000$ , C4D animals  $760,000 \pm SD 201,000$ , CVF-treated normal— $760,000 \pm SD 74,000$ . Shown in the figure are geometric means obtained. At each point standard errors are not shown to increase clarity. The values at the 15 min point and the number of animals studied are listed below: endotoxin-treated normal animals  $5 \pm 1\%$  (8), endotoxin-treated C4D animals  $96 \pm 14\%$  (8), saline-treated normals  $103 \pm 8\%$  (8), saline-treated C4D animals  $106 \pm 11.6\%$  (8), CVF-treated normals given endotoxin  $95 \pm 5\%$  (5).

The injection of endotoxin into normal guinea pigs resulted in a decrease in platelets to  $5 \pm 1\%$  SE at 15 min with a partial return ( $59.7 \pm 14\%$ ) to normal levels by 2 h (Fig. 1). In those animals which survived the injection of endotoxin (5/8), platelet counts returned to normal by 24 h. When the same dose of endotoxin was injected into C4D guinea pigs there was no decrease in platelet counts.

The development of leukopenia after the injection of endotoxin was observed in both animal groups (Fig. 2). Differential WBC counts at the 15 min interval revealed neutropenia in both the normal and C4D animals.

**Effect of endotoxin on platelets and WBC in CVF-treated guinea pigs.** Five NIH multipurpose guinea pigs were given CVF as described above. 20 h after the injection of CVF, base-line platelet and WBC counts were performed. Base-line platelet counts were not different (mean count  $760,000 \pm SD 74,000$ ) from the normal and C4D animals, but a mild leukocytosis was observed (mean WBC count  $10,800 \pm SD 2000$ ). The animals were then given endotoxin and platelet, and WBC counts determined at 15 min, 2, and 4 h.

As can be seen in Fig. 1 treatment of normal guinea pigs with CVF prevented the profound fall in platelets seen in normal animals after the injection of endotoxin. The leukopenia (Fig. 2) was, however, unmodified.

**Effect of endotoxin and platelets in C4D guinea pigs with reconstituted C4 levels.** Three C4D guinea pigs were injected i.v. with 4 ml of pooled normal guinea

pig serum resulting in a restoration of functional C4 activity as measured by hemolytic assay (C4 titers were in the range of 10,000-12,000). 30 min after the administration of C4, blood samples for base-line platelets were obtained. The animals were then injected with endotoxin i.v. and serial blood specimens obtained for platelet counts. Controls consisted of C4D guinea pigs with restored C4 levels given saline, and normal guinea pigs given endotoxin.

The results graphed in Fig. 3 indicate that the injection of endotoxin into C4D guinea pigs with reconstituted C4 levels did result in thrombocytopenia although the degree of fall was not as marked as in their normal counterparts.

*Effect of endotoxin on clotting time in normal and C4D guinea pigs.* Glass and silicon clotting times were performed on 15 normal and 15 C4D guinea pigs. Other groups of 12 normal and 12 C4D guinea pigs were injected i.v. with endotoxin, and the clotting times determined 15 min later. The mean clotting time for each experimental group was calculated.

No statistically significant difference ( $P > 0.20$ , Student *t* test) in the clotting time of normal and C4D guinea pigs was noted (Table II). Normal animals had a mean glass clotting time of 12.5 min  $\pm 0.5$  SE, and a silicon clotting time of 29.2 min  $\pm 1.7$ . C4D guinea pigs

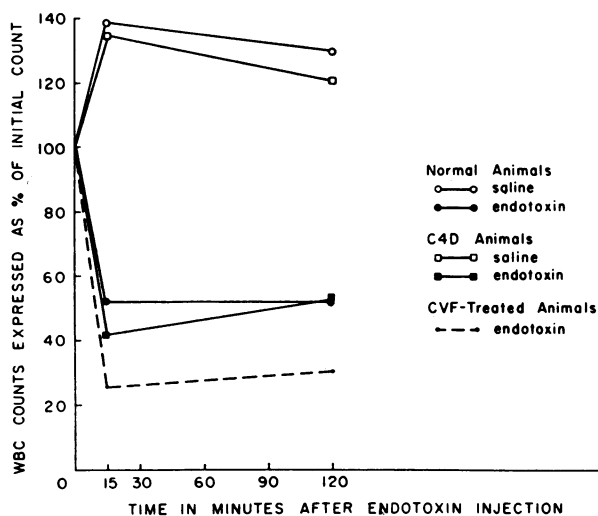


FIGURE 2 Effect of endotoxin on leukocytes in normal, C4D and CVF-treated guinea pigs. The initial WBC values for each group were as follows: normal animals—9000  $\pm$ SD 2870, C4D animals—9300  $\pm$ SD 1650, CVF-treated normal animals 10,800  $\pm$ SD 2000. Shown in the figure are geometric means obtained at each point. Standard errors are not shown to increase clarity. The values at the 15 min point and the number of animals studied are listed below: endotoxin-treated normal animals 52  $\pm$ 8% (8), endotoxin-treated C4D animals 41  $\pm$ 3% (8), saline-treated normal animals 139  $\pm$ 6% (8), saline-treated C4D animals 135  $\pm$ 30% (8) CVF-treated normal animals given endotoxin 25  $\pm$ 1% (5).

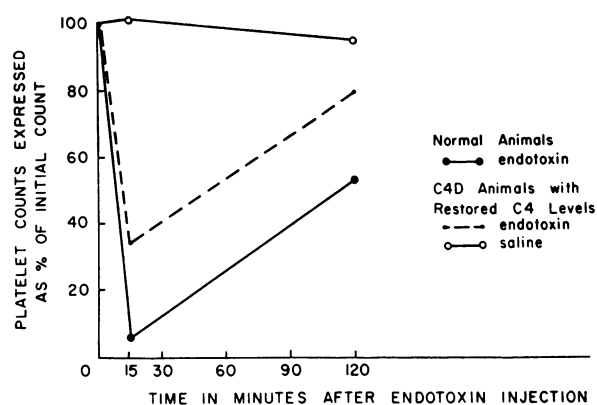


FIGURE 3 Effect of endotoxin on platelets in C4D guinea pigs with restored C4 levels. The initial platelet counts for this group of animals was 859,000  $\pm$ SD 131,000. For the restored C4D animals, the geometric mean and standard error at the 15 min point was 34  $\pm$ 6%.

had a glass clotting time of 12.60 min  $\pm 0.5$  and a silicon clotting time of 32.0  $\pm 1.8$ . When endotoxin was injected into the normal guinea pigs a significantly accelerated mean silicon clotting time (19.2 min  $\pm 1.5$ ) was noted at 15 min ( $P < 0.001$  when compared with normal animals given saline rather than endotoxin). The clotting time of C4D guinea pigs was not accelerated by the injection of endotoxin and did not statistically ( $P > 0.20$ ) differ from the clotting time of C4D guinea pigs not given endotoxin.

*Effect of endotoxin on clotting time in CVF-treated normal animals.* To determine if exhaustion of the components of the alternate pathway and C3-9 had an effect on the normal clotting time, clotting times were performed on eight CVF-treated normal guinea pigs. Six additional CVF-treated normal animals were injected with endotoxin and their clotting times determined 15 min later. There was no statistical difference ( $P > 0.20$ ) in the mean glass and silicon clotting times of CVF-treated animals when compared with the normal group (Table II). The administration of CVF, however, prevented the acceleration in clotting time noted in the normal animals after endotoxin administration.

*Effect of endotoxin on the clotting time in C4D guinea pigs with reconstituted C4 levels.* 4 ml of pooled normal guinea pig serum (C4 titer 80,000) was injected i.v. into each of 12 C4D guinea pigs. Clotting times were determined on six animals 15 min after the administration of normal guinea pig serum. The second group of six animals was given endotoxin 15 min after receiving normal serum, and clotting times performed 15 min later. Reconstitution of C4 levels in C4D guinea pigs resulted in a significant ( $P < 0.001$  when compared with C4D guinea pig controls) acceleration of

TABLE II  
Clotting Time in Normal C4D, and CVF-Treated Animals: Alteration with Endotoxin

Animals	Clotting time (min) before endotoxin		Clotting time 15 min after injection of endotoxin	
	Glass	Silicon	Glass	Silicon
Normal	12.53±0.46* (15)	29.2±1.71 (15)	11.18±0.46 (12)	19.25±1.49 (12)
C4D	12.60±0.49 (15)	30.2±1.81 (15)	13.0±0.52 (12)	33.91±2.25 (12)
CVF-treated normal	13.37±0.56 (8)	27.5±1.83 (8)	11.66±0.21 (6)	32.0±3.34 (6)
C4D with restored‡ C4	12.16±0.54 (6)	34.1±2.21 (6)	10.83±0.49 (6)	22.66±0.71 (6)

The number of animals are indicated in parentheses.

\* Expressed as the mean±SE; C4D, animals deficient in C4; CVF, cobra venom factor.

‡ See text.

clotting when the animals were given endotoxin (Table II). A mean silicon clotting time of 34.1 min ±2.2 was obtained in the reconstituted animals not given endotoxin, while the reconstituted animals given endotoxin had a mean silicon clotting time of 22.7 min ±0.7.

#### DISCUSSION

In this study the in vivo effects of bacterial endotoxins on the development of thrombocytopenia, leukopenia, and the hypercoagulable state have been examined. The role of the two major known complement pathways in production of these effects has been explored. These studies were made possible by the availability of guinea pigs with a genetically controlled inability to synthesize C4, one of the early components in the classical complement pathway (12). Incubation of the sera of these animals with endotoxin leads to consumption of the late components of complement (8). Since the C4D animals have a complete block in function of the classical complement pathway, these studies (8) provided the first unequivocal evidence of the function of an alternate complement pathway, bypassing the early components. Some of the other biologic reactions induced by endotoxin are normal in these C4D animals. For example, incubation of their serum with endotoxin leads to the generation of normal amounts of chemotactic factors, and the kinetics of generation of chemotactic factors are normal.<sup>8</sup> Serum of these animals can opsonize gram-negative bacteria for phagocytosis, and the serum is able to mediate the bactericidal reaction, although kinetic studies show the reaction to be abnormally slow (21). These and other studies have suggested that endotoxin is a potent activator of the al-

ternate complement pathway and produces its biological effects by this means.

When endotoxin is injected into C4D guinea pigs, no precipitous fall in platelet count is observed, although normal animals sustain a 95% decrease in platelet levels. Platelet levels are not lowered even though the alternate complement pathway is activated in C4D animals as evidenced by the fall in the serum level of C3-9. If the level of circulating C4 is restored in C4D animals, the expected decrease in platelet count is observed. These results demonstrate that the thrombocytopenia observed in guinea pigs on injection of endotoxin is indeed complement related and, moreover, despite the fact that endotoxin is a potent activator of the alternate pathway, that the classical pathway is requisite for the production of thrombocytopenia. In these studies, there was a clear dissociation between the effects of endotoxin on platelets in C4D animals and the effect of endotoxin on circulating granulocytes. Leukopenia was seen in all animal groups and did not appear to be complement related.

When endotoxin is administered to CVF-treated normal guinea pigs, a similar failure to develop thrombocytopenia, but not leukopenia, is observed. CVF has been shown to deplete guinea pigs of the late components of complement but does not affect levels of the early components C1, C4, and C2 (22). The data suggest that thrombocytopenia is a consequence of activation of the late complement components, but that activation of these components must proceed through activation of the classical pathway. Activation of the alternate pathway does not produce this effect.

The second part of this study examined the role of complement in the production of the hypercoagulable state. To do this it was necessary to establish normal

<sup>8</sup> Clark, R. A., M. M. Frank, H. S. Shin, and H. R. Kimball. Manuscript in preparation.

values for glass and silicon-surface clotting times for C4D and CVF-treated normal animals, and to relate these to values obtained in normal guinea pigs of the same strain. These observations are of additional importance because of the recent observations which implicate complement activation in the normal clotting mechanism (23, 24).

C4D guinea pigs had clotting times equal to normals of the same strain, both in glass and silicon-coated glass tubes. Thus, C4 did not play a role in normal clotting. CVF-treated animals also had normal clotting times. Thus, no contribution of the late components of complement was demonstrated. When normal animals were given endotoxin, there was a marked shortening of the clotting time. However, when endotoxin was administered to C4D animals, there was no acceleration of clotting; endotoxin did not lead to the generation of the hypercoagulable state. Thus, as in the production of thrombocytopenia, the sequential activation of C1, C4, and C2 was requisite for the acceleration of clotting. It seems likely that these two effects occur together and the shortened clotting time may be secondary to platelet damage. These findings contrast with those of Zimmerman, Arroyave, and Müller-Eberhard who showed a defect in clotting in rabbits genetically deficient in C6 (23). Moreover, activation of the alternate pathway was felt to be required for the shortening of the clotting time observed on the addition of complement-activating factors (24). Although the reasons for these differences are unknown, it must be emphasized that species differences may be important, and that the data presented here were obtained in an *in vivo* model while those reported by Zimmerman, Arroyave, and Müller-Eberhard were obtained *in vitro*.

A number of possible explanations for the findings reported here have been considered. It is possible that the alternate complement pathway cannot mediate the immune adherence reaction, which has been thought to contribute to platelet sequestration (5, 6). This possibility has been directly tested using C4D or normal guinea pig serum, endotoxin, and C4D or normal guinea pig platelets as the target cell. In these studies immune adherence reactivity was generated by both pathways (25).

A second possible explanation for these findings is provided by our observation that the alternate pathway is not directly activated by antibodies directed at antigens localized on intact mammalian membranes and thus does not damage the membrane of antibody-sensitized cells (26, 27). A portion of injected endotoxin is known to attach to formed elements in the blood. In the rabbit, evidence has been presented that circulating platelets become rapidly coated with injected endotoxin (28). Perhaps this portion of the injected endotoxin is re-

sponsible for the coagulopathy. The endotoxin which is not cell bound may activate the alternate pathway but does not produce platelet damage. Although C4D and normal guinea pigs utilized in this study did not have antibody detectable by the bentonite flocculation test, they undoubtedly had low levels of "natural" antibodies to endotoxins (29). Presumably, in normal animals, these antibodies, interacting with membrane-bound endotoxin, activated the classical complement sequence and produced thrombocytopenia, either through an immune adherence mechanism (5, 6) or by direct damage to the platelet membrane. Because anti-membrane antibodies did not activate the alternate complement pathway, C3 and the late components of complement were not fixed to the endotoxin-coated membrane in the C4D animals and platelets were neither sequestered nor destroyed.

One may attempt to relate these findings to those obtained in human diseases, however, it should be stressed that there are marked differences in the biological reactivity of various species to LPS. In clinical gram-negative bacteremia, a state considered by some to be analogous to endotoxemia in animals, hypercoagulability, and mild thrombocytopenia are not infrequently seen (30). These latter findings may be related to activation of complement, and on the basis of the data presented here the distinct possibility exists that only one of the two complement pathways is involved in the production of these phenomena. If such is the case, selective manipulation of the complement system in endotoxemia may be of some clinical importance.

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