

PREVENTION OF THE LOCALIZED AND GENERALIZED
SHWARTZMAN REACTIONS BY AN
ANTICOMPLEMENTARY AGENT,
COBRA VENOM FACTOR*

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The Shwartzman phenomenon may be defined as the pathologic manifestation of disseminated intravascular coagulation with widespread hemorrhagic necrotic lesions and bilateral renal cortical necrosis induced by the administration of two spaced injections of endotoxic lipopolysaccharide (LPS).¹ Results of previous investigations indicate that the platelet-LPS interaction with subsequent platelet adherence and release of its contents is primarily responsible for the pathologic alterations of the coagulation system (1-6). Prior treatment of rabbits which renders them thrombocytopenic or leukopenic can regularly protect these animals from the manifestation of Shwartzman lesions (7, 8). Stetson found that there was a temporal correlation between thrombocytopenia and the deposition of platelet aggregates in the lung, liver, and spleen after the administration of LPS (1). Furthermore, McKay and Shapiro observed that LPS-induced thrombocytopenia corresponded well with the appearance of a hypercoagulable state (2). Recently, Mustard et al. demonstrated the protection of rabbits from Shwartzman lesions by treatment with acetylsalicylic acid, sulfinpyrazone, or phenyl butazone which are agents that interfere with the platelet release reaction (9-11).

Although the mechanism of platelet-LPS interaction remains to be elucidated, Siqueira and Nelson suggested that the complement system is important in this interaction (12). Review of recent reports indicates that plasma factors are required for the platelet-LPS interaction and that these factors are inhibited

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¹ *Abbreviations used in this paper:* CVF, cobra venom anticomplementary factor; EACA, epsilon-aminocaproic acid; LPS, lipopolysaccharide.

by known anticomplementary agents (13). However, direct proof of the complement system's participation in the platelet-LPS interaction is lacking. The use of anticomplementary agents in vivo prevents Shwartzman lesions (14). However, it has been difficult to interpret these results because anticomplementary agents used also cause a concomitant leukopenia and thrombocytopenia, and it is known that the cellular elements are essential for the reaction (12).

A cobra venom anticomplementary factor (CVF) isolated from venom of *Naja naja* or *Naja haje* appears to be an ideal anticomplementary agent to be used for studying the role of the complement system in the Shwartzman phenomenon because the CVF administered causes leukocytosis rather than leukopenia (15). Furthermore, the duration of hypocomplementemia induced by CVF is also longer than that induced by other known anticomplementary agents. Results of studies using CVF as the anticomplementary agent, presented in this report, indicate that the complement system plays an essential role in the pathogenesis of the Shwartzman phenomenon.

Materials and Methods

Animals.—Male and female albino rabbits, weighing between 1.3 to 1.6 kg were used. These animals had free access to water and Purina rabbit pellets throughout the experiments.

LPS.—Endotoxic lipopolysaccharide was prepared from *Escherichia coli* according to the method of Boivin et al. (16). This preparation of LPS was found to have an LD₅₀ of 500 µg when tested against 20 g Swiss Webster white mice. 1.4 µg of this LPS preparation induced a 50% inhibition on the total hemolytic activity of 1 ml of 1:5 dilution of normal rabbit serum after an incubation of 60 min at 37°C.

Induction of Localized and Generalized Shwartzman Reaction.—For localized Shwartzman reaction, the preparatory dose was 100 µg of LPS given intradermally in the right upper quadrant of the abdomen followed by the provoking intravenous dose of 270 µg of LPS 24 hr later. For generalized Shwartzman reaction two intravenous doses of 270 µg of LPS were given 24 hr apart. To study both the generalized and localized Shwartzman reaction in the same rabbit, both the intradermal and intravenous dose of LPS were given simultaneously followed by the provocative dose in 24 hr. With such a scheme, we were able to induce regularly both localized and generalized Shwartzman lesions in more than 80% of the rabbits.

CVF.—Lyophilized cobra venom (*Naja haje*) was purchased from Ross Allen Reptile Institute, Inc., Silver Springs, Fla. The anticomplementary CVF was isolated from the cobra venom by diethylaminoethyl (DEAE)-cellulose column chromatography as described by Ballow and Cochrane (17). No attempt was made to further purify the CVF by Sephadex or sodium carboxymethylcellulose (CM)-cellulose chromatography. The anticomplementary activity of the CVF was assayed according to the method of Cochrane et al. We modified their method by using 5×10^7 sensitized sheep cells, adjusting the final volume to 7.5 ml and measuring the amount of hemolysis at 412 nm (15). Our CVF preparation was found to have 80 units of anticomplementary activity/ml when one unit of anticomplementary activity is defined as the reciprocal of the dilution of the CVF preparation in 0.1 ml volumes which reduces the hemolytic activities of 0.4 ml of a 1:20 dilution of normal human serum by 50%, after an incubation period of 20 min at 37°C.

Experimental Design.—Rabbits were divided randomly into experimental and control groups. A total of 30 rabbits were studied in five separate experiments; assays on blood samples were carried out for 10 animals in each group. The experimental group received three

intravenous injections of CVF (total dose of 320 anticomplementary units/kg of rabbit) at -24, -20, and -16 hr; the control group received saline (sodium chloride injection, USP, Cutter Laboratories, Berkeley, Calif.) of identical volumes and at similar time intervals.

At zero time each rabbit was injected with 100 μ g of LPS intradermally and 270 μ g of LPS intravenously; this was followed by a provocative dose of 270 μ g of LPS intravenously 24 hr later. Blood samples from each animal were collected from the ear artery immediately before the first dose of CVF or saline; immediately before the preparatory dose of LPS; and at 28, 48, 72, and 96 hr after the initial LPS injections. On each of these blood samples the following assays were performed: white cell count, platelet count, fibrinogen and fibrin degradation products, fibrinogen level, and hemolytic complement activity.

Cell Counts.—White blood cell counts were performed manually by standard techniques. Platelet counts were performed with the use of 1% ammonium oxalate solution as the diluting fluid and a phase-contrast microscope as was described by Brecher et al. (18).

Fibrinogen and Fibrin Degradation Products.—The staphylococcal clumping test of Hawiger et al. for the determination of fibrinogen and fibrin degradation products was performed with modifications (19). 0.96 ml of blood was collected into a test tube containing 0.04 ml of epsilon-aminocaproic acid (EACA) and thrombin (EACA 5 mg and thrombin 2 units/0.04 ml). EACA was obtained from Lederle Laboratories, Pearl River, N. Y.; thrombin from The Upjohn Company, Kalamazoo, Mich. After collection, the blood sample was incubated at 37°C for 1 hr and the serum separated by centrifugation and stored at -20°C until use. The clumping positive *Staphylococcus aureus* (strain Newman D₂C) was kindly provided by Dr. J. Hawiger (Vanderbilt University School of Medicine, Nashville, Tenn.). To assay the fibrinogen and fibrin degradation products titer, the staphylococcal clumping test was carried out with the use of 0.025 ml microdiluters and microtiter plates (Cooke Engineering Co., Alexandria, Va). Lyophilized preparations of the bacteria were prepared as described (19) and suspended at a concentration of 2.5 mg/ml in 0.05 M tris (hydroxymethyl) aminomethane (Tris) buffer containing 0.01% bovine serum albumin at pH 7.4. With the use of microdiluters, serum samples were serially diluted in 0.025 ml volumes of the same buffer. An equal volume of the bacteria suspension was then added. The microtiter plate was gently agitated for 2 min and its contents allowed to settle at room temperature for 30 min before the clumping titer was scored.

Fibrinogen Determination.—A modified method of Ratnoff and Menzie for fibrinogen determination was used (20). Briefly, 0.95 ml of blood was collected into a test tube containing 0.05 ml of 0.1 M EDTA (ethylenediaminetetraacetate sodium), pH 7.4. 50 μ l of the fresh plasma were transferred to another test tube containing 2 ml of normal saline at pH 7.4, 100 μ l of thrombin (1000 units/10 ml, Upjohn), and approximately 0.5 volume of glass beads (Super-brite glass beads, 200 μ in diameter, Minnesota Mining and Manufacturing Co., St. Paul, Minn.). The tube contents were mixed and allowed to sit at room temperature for 20 min. A Vortex-Genie mixer (Scientific Industries Inc., Springfield, Mass.) was used to ensure thorough mixing so that all clottable proteins were trapped onto glass beads. The soluble proteins in the supernatant were removed by three successive washings with normal saline. After the addition of 1 ml of 10% sodium hydroxide solution, the tube was boiled in a water bath for 10 min and allowed to cool. The following reagents were added in sequence with mixing: 7 ml of distilled water, 3 ml of 20% sodium carbonate solution, and 1 ml of phenol reagent (Harleco, Philadelphia, Pa.). After a 10 min room temperature incubation, a spectrophotometric determination of the tube content at 650 nm was performed with a Coleman Junior Spectrophotometer. A standard curve was obtained by the determination of standard tyrosine solution in 0.1 N hydrochloric acid. It is possible to match the optical density values of fibrinogen determination with that of the standard curve so that corresponding amounts of tyrosine may be obtained. The amount of fibrinogen was calculated by multiplying the amount of corresponding tyrosine by a conversion factor of 11.7.

Hemolytic Complement Determination.—The preparation of buffers and sheep erythrocytes sensitized with rabbit hemolysin have been described previously (21). Rabbit serums were serially diluted in 0.5 ml volumes of glucose-gelatin-barbital-buffered saline containing calcium and magnesium at pH 7.4. After the addition of 0.5 ml of sensitized sheep erythrocytes at 1×10^8 ml⁻¹, the mixtures were incubated at 37°C for 60 min. The amount of hemolysis was determined at 412 nm and the CH₅₀ value calculated.

RESULTS

Pathogenic Examination.—Both the experimental and control groups of animals were examined for kidney, skin, liver, and lung lesions 96 hr after

TABLE I
Pathologic Assessment of CVF or Saline-Treated Rabbits Subjected to the Induction of Shwartzman Reactions by Endotoxin Injections

Treatment	CVF				Saline			
	Kidney	Localized skin	Liver	Lung	Kidney	Localized skin	Liver	Lung
Hemorrhagic necrosis*	1/15	1/15	0/15	6/15	12/15	12/15	9/15	13/15
Severity of pathology	+‡	10 mm§	0‡	+‡	3+	17 mm	1+	2+

* Expressed as the No. of positive responses over the No. of rabbits studied.

‡ Severity of pathology expressed on a scale of 0 to 4+; where

0 = no gross lesion

+ = fine petechial hemorrhage

2+ = prominent but discrete "flea-bite" hemorrhage

3+ = coalescent hemorrhagic areas

4+ = massive hemorrhagic lesion.

§ Average diameter of the hemorrhagic necrotic area.

initial LPS injection when they were sacrificed or at an earlier time if they died spontaneously. Observations in the gross were checked by histologic examination for fibrin deposition. The characteristic blue color on phosphotungstic acid hematoxylin staining was considered diagnostic of fibrin deposition. Results of these experiments are presented in Table I. It is apparent that animals treated with CVF are protected from both the localized and generalized Shwartzman reactions. Of the CVF-treated group only one of 15 rabbits developed the localized and generalized Shwartzman reactions and they were mild. However, fine petechial hemorrhages of lungs were noted in approximately 40% of the CVF-treated animals. The control group responded to the LPS challenge more frequently and also more vigorously. Histologic examinations of the skin biopsies taken 20 hr after the preparatory intradermal dose of LPS revealed

identical characteristics for both groups of animals. However, the control animals were susceptible to the challenging dose of LPS and manifested hemorrhagic necrosis at the prepared skin site. By contrast, the CVF-treated animals were resistant to these pathologic changes.

White Blood Cell Count.—In view of the protective role of leukopenia against the development of localized and generalized Shwartzman reaction, it is important to determine the white cell count after the administration of CVF. Furthermore, the changes in the circulating white blood cells during the course

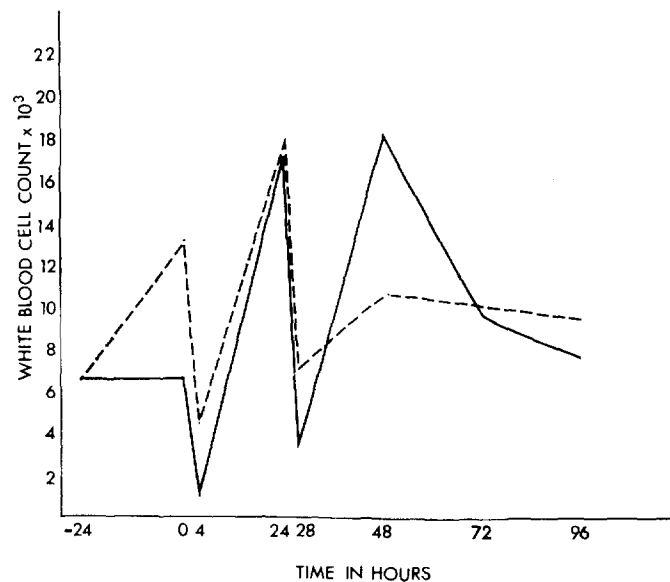


FIG. 1. White blood cell counts of rabbits pretreated with CVF (---) or saline (—) and followed by endotoxin injections for inductions of localized and generalized Shwartzman reactions. Pretreatment injections were given at -24, -20, and -16 hr; LPS injections given at 0 and 24 hr.

of lesion development were studied. The mean values of 10 blood samples from each of the experimental and control groups are presented in Fig. 1. As reported earlier, the administration of CVF induced a leukocytosis response so that circulating white blood cell count was approximately doubled within 24 hr. After the injection of LPS, both experimental and control animals responded similarly within the first 28 hr. However, the leukopenia was less for the CVF-treated group than that for the control. By the 48th hr the control group reached a peak level of leukocytosis while the CVF-treated animals responded only slightly. Such a leukocytosis response by control animals probably represents an acute phase or response to inflammatory reaction directed against the

disseminated intravascular coagulation. Protection conferred by CVF from Shwartzman reaction is certainly not due to a leukopenia.

Platelet Count.—The platelet count is a valuable assay for the assessment of Shwartzman reaction since changes in circulating platelet levels correspond to presumed pathogenetic events in Shwartzman reaction. Furthermore, platelet-LPS interaction is believed to be primarily responsible for the initiation of Shwartzman lesions (1-6). The mean values of 10 platelet counts are plotted

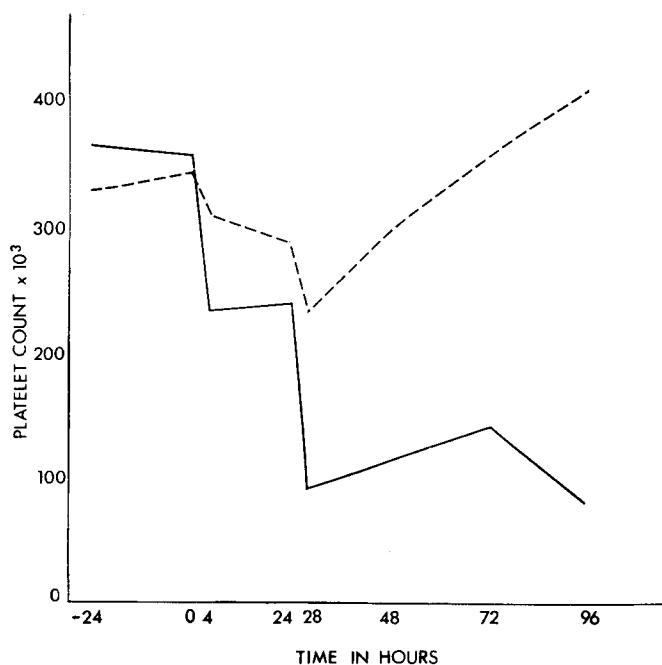


FIG. 2. Platelet counts of rabbits pretreated with CVF (---) or saline (—) and followed by endotoxin injections for inductions of localized and generalized Shwartzman reactions. Pretreatment injections were given at -24, -20, and -16 hr; LPS injections given at 0 and 24 hr.

against time in Fig. 2. No significant changes occurred in circulating platelet levels after CVF administration. When LPS was given intravenously, unprotected animals showed precipitous drops in their circulating platelet levels and little evidence of recovery from the thrombocytopenic state even as late as the 96th hr after the first LPS injection. By contrast to controls, CVF-treated animals showed only a mild decrease in circulating platelet levels after LPS administration, and they were able to respond rapidly with thrombocytosis.

Fibrinogen and Fibrin Degradation Products.—The detection of fibrinogen and fibrin degradation products in circulation reflects the occurrence of fibrino-

lysis. Results of these studies using the staphylococcal clumping test are presented as mean values of 10 determinations and expressed as \log_2 of reciprocal dilutions in Fig. 3. No significant amounts of fibrinogen and fibrin degradation products were detected in CVF-treated animals throughout the experiment. However, in the control animals, fibrinogen and fibrin degradation product titers became markedly elevated by the 24th hr and continued to rise to the peak level by the 48th hr. Over the last 2 days of the experiment, a gradual decline of this titer was observed. Thus in the phase of disseminated intravascular coagulation, control rabbits were able to respond with fibrinolysis

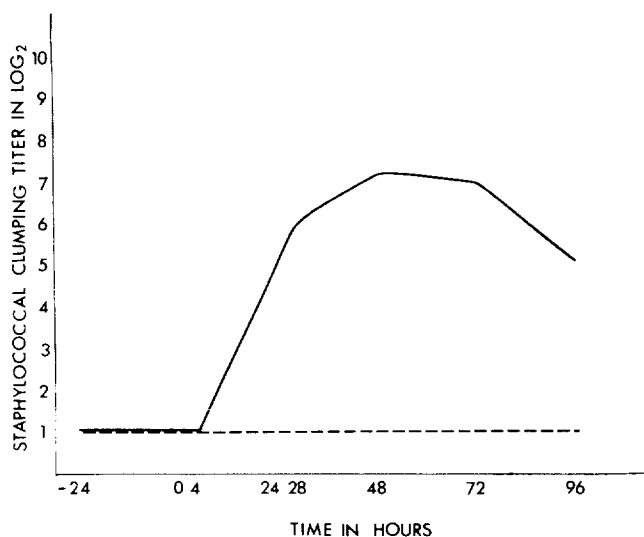


FIG. 3. Fibrinogen and fibrin degradation titers of rabbits pretreated with CVF (----) or saline (—) and followed by endotoxin injections for inductions of localized and generalized Shwartzman reactions. Pretreatment injections were given at -24, -20, and -16 hr; LPS injections given at 0 and 24 hr.

but this response was insufficient to prevent tissue damage as a consequence of the intravascular coagulation.

Fibrinogen.—Fibrinogen plays an important role as the final common pathway for Shwartzman reaction or any disseminated intravascular coagulopathy in that fibrin deposition is responsible for tissue damage whereas fibrinogen deprivation or fibrin removal provides protection. The mean fibrinogen values of 10 samples are plotted against time in Fig. 4. After the administration of CVF an increase of fibrinogen level occurred. The preparatory dose of LPS induced a similar decrease of circulating fibrinogen levels in both groups of animals. However, control animals responded with hyperfibrinogenemia within 24 hr while CVF-treated animals had only a slight increase of fibrinogen level.

In control animals the provocative dose of LPS produced a precipitous drop of fibrinogen level which was again followed by rapid development of hyperfibrinogenemia. By contrast, CVF-treated animals showed only a small drop of fibrinogen level after the second dose of LPS, and no reactive hyperfibrinogenemia was observed.

Hemolytic Complement Assay.—The mean CH_{50} values of 10 samples of each group of animals were plotted against time in Fig. 5. Injections of 320 CVF

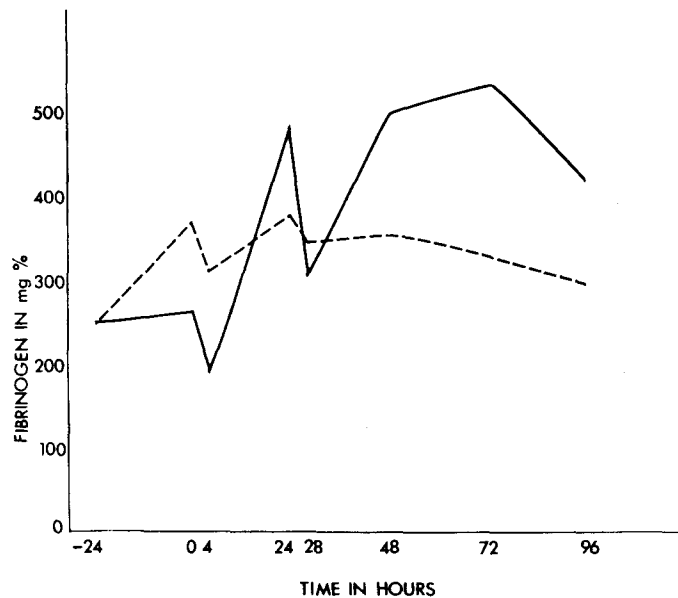


FIG. 4. Fibrinogen levels of rabbits pretreated with CVF (---) or saline (—) and followed by endotoxin injections for inductions of localized and generalized Shwartzman reactions. Pretreatment injections were given at -24, -20, and -16 hr; LPS injections given at 0 and 24 hr.

anticomplementary units/kg of rabbit given in three divided doses by four hourly intervals failed to produce signs of hemoglobinuria or hemoglobinemia. 24 hr after the initial dose of CVF, the total hemolytic complement activity was regularly less than 10% of pretreatment values. The preparatory dose of LPS induced a mild increase of hemolytic complement activity in both experimental and control animals. After the provocative dose of LPS, a rapid decrease of hemolytic complement activity in the control group occurred which contrasted with an increase of hemolytic complement activity in the CVF-treated group. Little variation in the CH_{50} values in both groups of animals occurred during the last 3 days of the experiment.

DISCUSSION

Antigen-antibody complexes may be used to replace LPS in the induction of pathologic responses in animal models (22). The resemblance of many of the biological activities mediated by LPS to those produced by antigen-antibody complexes led investigators to study the complement system which might act as the common pathway for amplification of biologic responses (13, 23). It has been well established that LPS reacts with serum cofactor(s) in the presence of magnesium to form a molecular complex which consumes the classical

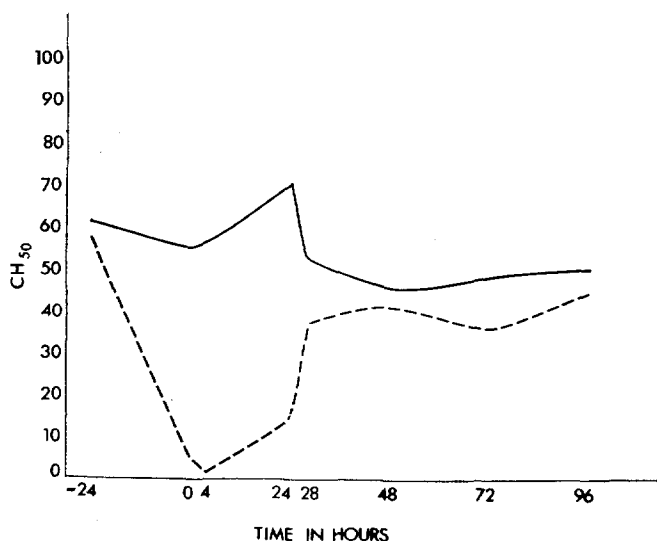


FIG. 5. Total hemolytic complement activities of rabbits pretreated with CVF (----) or saline (—) and followed by endotoxin injections for inductions of localized and generalized Shwartzman reactions. Pretreatment injections were given at -24, -20, and -16 hr; LPS injections given at 0 and 24 hr.

terminal complement components (C3-C9). During this interaction biologically active fragments are generated and released (13, 24). This report represents an attempt to modify LPS-mediated Shwartzman reactions by prior consumption of terminal complement components with a cobra venom anticomplementary factor. Our results show that CVF-treated animals are protected from the manifestations of disseminated intravascular coagulation and bilateral renal cortical necrosis. Since the CVF-induced hypocomplementemia is a relative but not an absolute deficiency, it is not surprising to find that 40% of the treated animals had petechial hemorrhages in their lungs. Untreated animals showed lesions that were qualitatively and quantitatively different from those of the CVF-treated group.

Prior studies have revealed that both platelets and white blood cells are essential to the expression of both localized and generalized Shwartzman reactions (7, 8). The use of CVF as the anticomplementary agent had the advantage of permitting study of animals responding with leukocytosis. Furthermore, neutrophils isolated from CVF-treated rabbits showed no deficit in their capacity to respond to a chemotactic stimulus (15). The depletion of terminal complement components, mainly C3, by CVF appears to influence the white blood cell responses towards LPS administration only moderately. Thus no histologic differences of skin biopsies were noted in control or CVF-treated rabbits 20 hr after the intradermal injection of LPS. This observation suggests that LPS might utilize noncomplement-dependent chemotactic mechanisms or be responsible for generating an adequate quantity of chemotactic factors (e.g. C5a or C567) using the small amount of C3 available.

When the sequence of events involved in the pathogenesis of Shwartzman reaction in control and CVF-treated animals were considered, there could be no question that hypocomplementemia was associated with a protective role towards circulating platelets. In CVF-treated rabbits, no substantial alterations in circulating fibrinogen levels were observed, a finding that corresponded well with the minor changes in platelet levels seen in these rabbits. Furthermore, the CVF protection did not appear to act via the induction of fibrinolytic activities, because no significant amount of circulating fibrinogen and fibrin degradation products was detected. In control animals, the drop of circulating platelet counts correlated well with changes of fibrinogen levels. Furthermore, in control rabbits after the first injection of LPS, we demonstrated the appearance of fibrinogen and fibrin degradation products. The level of fibrinogen and fibrin degradation products was increased by the provocative dose of LPS and persisted in circulation for at least 3 days. Since McKay and Shapiro could not demonstrate any circulating fibrinolytic activity in rabbits manifesting Shwartzman reaction (2), these results indicated that rabbits were able to respond with local fibrinolytic activities to the disseminated intravascular coagulation by releasing fibrinogen and fibrin degradation products but without demonstrating significant amounts of circulating plasmin activity.

In normal rabbits after the preparatory dose of LPS, a mild increase of the hemolytic complement activity, probably as a consequence of an inflammatory response, occurred. Upon the injection of the provocative dose of LPS, a decrease in the hemolytic complement activity occurred which correlated with analogous changes in white blood cell, platelet, and fibrinogen levels. The hemolytic complement activity then remained at this lower level from 28th to 96th hr of the experiment. For CVF-treated rabbits, LPS appeared to stimulate resulting increased hemolytic complementary activity. At the time of the provocative LPS dose, hemolytic complement activities were about 25% of those observed before CVF treatment.

It seems likely to us that prevention of Shwartzman reaction by CVF-induced hypocomplementemia reflects an essential role of the complement system in mediating platelet-LPS interaction. Whether the mode of complement participation is like that involved for particulate antigen and antibody interactions as described by Henson and Cochrane (25) remains conjectural. The concept that LPS activates the complement system through very avid antibodies and by an extremely efficient use of early acting complement components would favor this view of complement-mediated platelet-LPS interactions (13, 26). However, prior immunization of animals to LPS protects against rather than to enhance Shwartzman lesions. That complement-independent immune platelet aggregation (27) is operative in Shwartzman reaction seems unlikely because CVF-induced hypocomplementemia is associated with such striking protection.

Dodds and Pickering demonstrated alterations of hemostasis in guinea pigs after a single intraperitoneal injection of CVF (28). Possible mechanisms entertained by these investigators were a direct effect of CVF on platelets and an indirect induction of intravascular hemolysis. However the period of alterations of hemostasis in these guinea pigs was less than 21 hr. We could detect no evidence of intravascular hemolysis in our animals after the administration of CVF. Furthermore, the provocative dose of LPS was given 40 hr after the last dose of CVF so that any occurrence of hemostatic alterations would not have persisted to that time. Thus the prevention of Shwartzman reaction by CVF almost certainly operated by the hypocomplementemia which protects circulating platelets from reacting with LPS. Recently the interaction of coagulation and immune systems has been emphasized (29, 30). Indeed, the substitution of LPS by antigen-antibody complexes in the induction of Shwartzman reactions was well established (31, 32). Understanding of immune-mediated coagulopathy may help us gain further insight in the nature of immunologic injury and may offer new approaches for immunologic manipulation.

SUMMARY

Both localized and generalized Shwartzman reactions were induced in the same rabbits by simultaneous administration of preparatory intravenous and intradermal doses of endotoxin followed in 24 hr by the provocative dose. Control rabbits with more than 80% positive responses showed corresponding changes of platelet, white blood cell, fibrinogen, and hemolytic complement levels. Circulating fibrinogen and fibrin degradation products were detected shortly after the preparatory dose and persisted for at least 3 days. Rabbits given cobra venom anticomplementary factor showed hypocomplementemia (less than 10% of normal), leukocytosis, and elevated fibrinogen levels. After the administration of endotoxin, only one of 15 CVF-treated animals developed a Shwartzman reaction and that was mild. These rabbits showed only minor changes of platelet and fibrinogen levels throughout the experiment although

their white blood cell responses were similar to those of the control group. No detectable fibrinogen and fibrin degradation products appeared in circulation, and the hemolytic complement activity increased gradually beginning with the preparatory dose of endotoxin. Thus depletion of terminal complement components (mainly C3) in rabbits is protective against the development of both localized and generalized Shwartzman reactions; its mechanism of action is probably through the sparing of platelets by inhibiting platelet-endotoxin interaction. The essential role of the complement system in Shwartzman reaction indicates that this coagulopathy probably represents a manifestation of immunologic injury.

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