IMMUNOHISTOCHEMICAL DEMONSTRATION OF THE RETICULOENDOTHELIAL CLEARANCE OF CIRCULATING FIBRIN AGGREGATES*

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When thrombin is administered intravenously to rabbits for several hours at a rate just insufficient to produce intravascular thrombosis, there is nevertheless conversion of much of the animal's circulating fibrinogen to fibrin (1). In most instances, no detrimental effect is observed, and no extensive fibrin deposition is found (2). This absence of pathologic alterations in the face of extensive intravascular fibrin formation has generally been ascribed to the efficient lysis of fibrin by plasmin and other blood proteolytic enzymes. While local activation of plasminogen may be responsible in the rabbit for eliminating fibrin already deposited, recent evidence (2) has indicated that the bulk of fibrin aggregates formed in the circulating blood are rapidly removed by the reticuloendothelial system in a manner similar to the efficient clearance of foreign particulate matter and colloidal materials. It was shown that blockade of the reticuloendothelial system in rabbits receiving intravenous thrombin infusions regularly resulted in massive fibrin deposition in the terminal vascular bed. The major lesion, bilateral renal cortical necrosis, was indistinguishable from that of the generalized Shwartzman reaction which has classically been elicited by bacterial endotoxins.

In the present report immunohistochemical studies have provided direct evidence for the phagocytic clearance of fibrin by demonstrating the accumulation of this material selectively within the reticuloendothelial cells of the liver and spleen after the administration of thrombin or endotoxin. These observations further substantiate the hypothesis (2) that the generalized Shwartzman reaction in the rabbit results from the failure of the reticuloendothelial system to adequately clear the circulation of fibrin aggregates formed during systemic blood coagulation initiated by endotoxin.

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Materials and Methods

Animals.—Albino hybrid rabbits of either sex weighing 1.2 to 1.6 kilos were used.

Endotoxin.—Lyophilized lipopolysaccharide of *Escherichia coli* 0111:B4 (Difco Laboratories, Inc., Detroit) was dissolved in sterile physiologic saline solution, and 2.0 ml volumes were used for intravenous injections.

Thrombin.—Topical thrombin (Parke, Davis & Company, Detroit) was obtained as a sterile commercial preparation of bovine origin containing 5000 N.I.H. units per vial. The lyophilized material was freshly reconstituted and diluted with sterile physiologic saline solution to a final concentration of 3 units per ml just prior to use. The dilute thrombin solutions were slowly infused into the marginal ear veins of rabbits using sterile parenteral administration sets. The rate of flow did not exceed 1 ml per minute.

Heparin.—Liquaemin sodium (Organon, Inc., West Orange, New Jersey) was an aqueous solution of heparin sodium containing 10 mg per ml.

Demonstration of Heparin-Precipitable Fibrinogen (HPF).—Heparin-precipitable fibrinogen was described by Thomas, Smith, and von Korff (3) as an altered form of fibrinogen present in the blood of endotoxin-treated rabbits which formed a flocculent or stringy precipitate in the cold on the addition of heparin. HPF was not demonstrated in the circulation earlier than 1 hour after an intravenous injection of endotoxin and usually reached maximal quantity 2 to 4 hours later. Recent evidence (2, 4) has indicated that HPF probably represents the product of a transitory interaction between thrombin and fibrinogen in the circulating blood. In the present study the demonstration of HPF in endotoxin-treated animals was used as an indication of intravascular coagulation. 4 ml of blood was obtained by cardiac puncture in a syringe containing heparin in a concentration of 1.0 mg/ml of blood. The plasma was separated by centrifugation, placed in an ice bath and observed for the presence of HPF 2 hours later. The amount of precipitate was estimated visually.

Antiserum.—Antiserum to rabbit fibrin was prepared in the sheep by Antibodies Inc., Davis, California, according to the following procedure. Blood was withdrawn from a rabbit by cardiac puncture in 1/10 volume of M/10 sodium oxalate. The plasma was separated by centrifugation and then clotted by the addition of an equal volume of M/40 $CaCl_2$. The fibrin clot was washed twice with stirring over a period of 48 hours with isotonic saline solution maintained at pH 8 with carbonate-bicarbonate buffer. The clot was homogenized in a Waring blendor before and after the second washing. The fibrin suspension was incorporated in complete Freund's adjuvant (Difco Laboratories), and intramuscular injections of this preparation were given at multiple sites in a sheep. A total of three inoculations were made at biweekly intervals. The antiserum as received was found to contain antibodies against several serum components, as shown by double diffusion in agar. After repeated absorption with small amounts of lyophilized rabbit serum proteins, the sheep antiserum produced only a single line against rabbit plasma, and none against rabbit serum.

Conjugation of Antiserum.—A globulin fraction of sheep antiserum was prepared by precipitation with 4/10 saturated ammonium sulfate. After standing overnight in the cold the precipitate was collected by centrifugation, washed twice with 4/10 saturated ammonium sulfate, and redissolved in a small volume of distilled water. After dialysis against physiologic saline to remove the ammonium sulfate, the globulin fraction was conjugated with a commercial preparation of fluorescein isothiocyanate on celite (California Corp. for Biochemical Research, Los Angeles) using a modification of the procedure described by Rinderknecht (5). To the globulin solution in a small Erlenmeyer flask fluorescein isothiocyanate, in the amount of 22 mg per gm of protein, was slowly added with swirling to wet the material. The reaction mixture was allowed to stand at room temperature for 5 minutes with continuous stirring. The insoluble material was removed by centrifugation in the cold, and the con-

jugated antiserum was passed through a Dowex 2-X8 column to remove the unreacted fluorescein (6). 1 gm of Dowex 2-X8 (J. T. Baker Chemical Co., Phillipsburg, New Jersey) was used per ml of conjugated antiserum. Non-specific staining was next removed by three absorptions with mouse and guinea pig tissue homogenates and a final absorption with acetone-dried human liver powder.

Preparation of Tissue Sections.—Each rabbit received an intravenous dose of heparin just prior to sacrifice in an attempt to minimize postmortem clotting. Blocks of tissue from the liver, spleen, lung, and kidney were removed under pentobarbital anesthesia immediately before or after death and frozen in liquid nitrogen. The specimens were sectioned at 2 microns in an International Harris cryostat, air-dried, fixed in acetone for 10 minutes, and hydrated in phosphate buffered saline at pH 7.0. Sections were covered with conjugated anti-rabbit fibrin for 30 minutes in a moist chamber at room temperature, followed by three washings in buffered saline of 10 minutes each. The specificity of the immunological staining was shown by the virtually complete prevention of staining by the prior application to the section of unconjugated anti-rabbit fibrin serum for 45 minutes. The staining was not prevented by the prior application of a sheep antiserum containing antibodies against rabbit gamma globulin and several other serum proteins. Furthermore, absorption of the conjugated serum with a preparation of rabbit fibrin markedly reduced staining.

Optical System.—A Zeiss standard microscope was used with an Osram HBO 200 watt mercury lamp and a darkfield immersion condenser.

RESULTS

Normal Rabbits.—In order to interpret the findings in the experimental animals it was necessary to investigate the distribution of material staining with conjugated antifibrin antibody in normal rabbits, especially in the liver and spleen, which contain the bulk of the reticuloendothelial cells. Six normal rabbits were studied. In all tissues small clumps and strands of brightly staining material were seen in the lumina of some of the blood vessels. Within some splenic sinuses and in an occasional hepatic sinusoid similar material reacting with labeled antibody was found. Some alveolar capillaries in the lung and many intertubular capillaries in the kidney contained strongly fluorescent material. The material seen in these locations undoubtedly represented fibrin formed in the postmortem state. In addition, a thin layer of faintly fluorescent material was often observed lining the sinuses of the liver and spleen and outlining the glomerular loops of the kidney. Stained material was not observed within cells in any of the tissue sections examined.

Rabbits Infused with Thrombin.—Nine rabbits received intravenous infusions of 150 to 400 units of thrombin over a period of 1½ to 2½ hours without apparent deleterious effects. Blocks of tissue were removed immediately following termination of the infusion. In all animals many Kupffer cells (Fig. 1) and splenic macrophages (Fig. 2) contained brightly stained material within their cytoplasm. The stained material was present in either homogeneous or granular form. In the lung an occasional phagocytic cell contained material which reacted with conjugated antibody. Greater amounts of intracellular material were found in rabbits receiving 400 units than in those given 150

units of thrombin. In the hepatic and splenic sinuses clumps and strands of fluorescent material were present, in larger quantities than were seen in normal animals. The findings in the kidneys did not differ from those seen in normal rabbits except for one animal in which moderate amounts of brightly stained material were present within some glomerular capillaries. As in normal animals, strands of fluorescent material were observed in the lumen of an occasional blood vessel. In contrast to normal rabbits, the thin layer of faintly stained material lining the hepatic and splenic sinuses was seen less frequently in animals injected with thrombin. A possible explanation for the latter observation is that sinus walls are normally coated by a thin layer of plasma fibrinogen which disappears when circulating fibrinogen is substantially depleted by thrombin administration.

Rabbits Injected with Endotoxin.—There is ample evidence indicating that bacterial endotoxins activate the blood coagulation mechanism *in vivo* (2, 7–10). It was previously postulated that the absence of appreciable fibrin deposition after a single injection of endotoxin was, as in the case of thrombin infusions, the result of efficient removal of circulating fibrin aggregates by the reticuloendothelial system (2). Accordingly, tissues from endotoxin-treated rabbits were examined for the presence within reticuloendothelial cells of material staining with conjugated antifibrin antibody. Since the occurrence of heparin-precipitable fibrinogen (HPF) in the circulation following endotoxin injection is believed to be indicative of intravascular coagulation (2–4), an attempt was made to correlate its presence in these animals with the finding of specifically stained material in the phagocytic cells.

Six rabbits received single intravenous injections of 0.4 mg of *E. coli* endotoxin. Tissues were removed from two rabbits 1 hour later and from four rabbits at 3 to 5 hours after injection of endotoxin. Just prior to sacrifice, 4 ml of heparinized blood was obtained from each animal to determine the presence of HPF.

In one of the rabbits examined 1 hour after endotoxin administration, only a few Kupffer cells and splenic macrophages showed bright cytoplasmic staining. In this rabbit HPF was present in small quantity in the circulation. In the other rabbit studied at 1 hour, no intracellular staining was observed, and in this animal HPF was not demonstrated in the blood drawn. In the four rabbits studied at 3 to 5 hours after injection of endotoxin, brightly staining material was found within the cytoplasm of a moderate number of Kupffer cells (Fig. 3) and splenic macrophages (Fig. 4). Compared to normal tissues, increased staining was observed within the sinuses of the spleen and sinusoids of the liver, in the animals treated with endotoxin. However, the amount of material reacting with conjugated antiserum within cells or sinuses was somewhat less than in the rabbits given 400 units of thrombin. A thin layer of fluorescent staining was seen lining the hepatic and splenic sinuses, but this material appeared fainter than in normal rabbits.

Rabbits Treated with Heparin Prior to the Administration of Thrombin or Endotoxin.—Two rabbits were given an intravenous infusion of 300 units of thrombin over a 2 hour period. Heparin (40 mg/kilo of body weight) was administered intravenously just before the start of the thrombin infusion and repeated 1 hour later. Tissues were removed immediately after termination of the infusion. In addition, two rabbits received single intravenous injections of 0.4 mg of endotoxin. Heparin was injected intravenously just prior to endotoxin administration and repeated 1½ hours later. 3 hours after injection of endotoxin, heparinized blood was obtained to demonstrate HPF, and tissues were removed for staining with conjugated antiserum.

In tissues from both groups of animals the distribution of fluorescent staining was the same as that found in normal rabbits. HPF was not present in the blood samples.

DISCUSSION

As a defense mechanism for clearing the blood of extraneous matter, the reticuloendothelial system has been shown to function efficiently in the phagocytic removal of a variety of macromolecular substances, ranging from carbon particles and bacteria to aggregated proteins and immune complexes (11). Recently, evidence (2) was presented which indicated that fibrin aggregates in the circulating blood, like foreign colloidal materials, were subject to rapid phagocytosis by the reticuloendothelial system. Furthermore, it appeared that in the rabbit it was this phagocytic mechanism rather than fibrinolysis which was chiefly responsible for the elimination of circulating fibrin, since blockade of the reticuloendothelial system permitted the development of massive fibrin deposition during intravascular coagulation.

In the present study direct evidence in support of this hypothesis was obtained by the demonstration of the selective uptake of fibrin by the Kupffer cells of the liver and the macrophages of the spleen and lung following administration of thrombin or endotoxin. While fibrin and fibrinogen cannot be discriminated on the basis of immunofluorescence, several observations during this study suggest that the material phagocytized was indeed fibrin and not fibrinogen altered by some reaction other than the blood coagulation process. First of all, this material was found in greatest amounts within the phagocytic cells of rabbits receiving infusions of thrombin in which circulating fibrinogen was obviously converted to fibrin. Specific staining material was also seen within reticuloendothelial cells following endotoxin injection. Although endotoxin produces *in vivo* a wide spectrum of effects, there is good evidence that the blood coagulation mechanism is activated (2, 7–10). Furthermore, the appearance of this material within macrophages after an injection of endotoxin was correlated with the presence of HPF in the blood which is probably an early step in the conversion of fibrinogen to fibrin (2–4). Finally, animals pretreated with heparin in doses rendering the blood incoagulable did not

exhibit intracellular accumulation of this material following administration of thrombin or endotoxin. On the basis of the present evidence, however, it cannot be determined to what extent polymerization must occur following thrombin-fibrinogen interaction before phagocytosis takes place.

The finding of fibrin within reticuloendothelial cells after an intravenous injection of endotoxin provides further clarification of the role of the blood coagulation mechanism and of the reticuloendothelial system in the pathogenesis of the generalized Shwartzman reaction. It was postulated (2) that in normal rabbits an intravenous injection of endotoxin gives rise to circulating fibrin aggregates which are efficiently removed by an active reticuloendothelial system. However, in rabbits with depressed phagocytic function fibrin aggregates persist in the circulation and are gradually deposited in the terminal vascular bed, particularly, in glomerular capillaries with development of bilateral renal cortical necrosis. The results of this study are in accord with this hypothesis.

SUMMARY

In rabbits given an intravenous infusion of thrombin or an injection of endotoxin, immunohistochemical examination of the tissues, using conjugated antiserum against rabbit fibrin, showed bright intracytoplasmic staining in many of the phagocytic cells of the liver and spleen. In normal rabbits as well as in animals injected with large doses of heparin prior to thrombin or endotoxin administration, no such intracellular staining was observed. The findings of this study substantiate the hypothesis that fibrin aggregates formed in the circulating blood during low grade intravascular coagulation are largely removed by the reticuloendothelial system.

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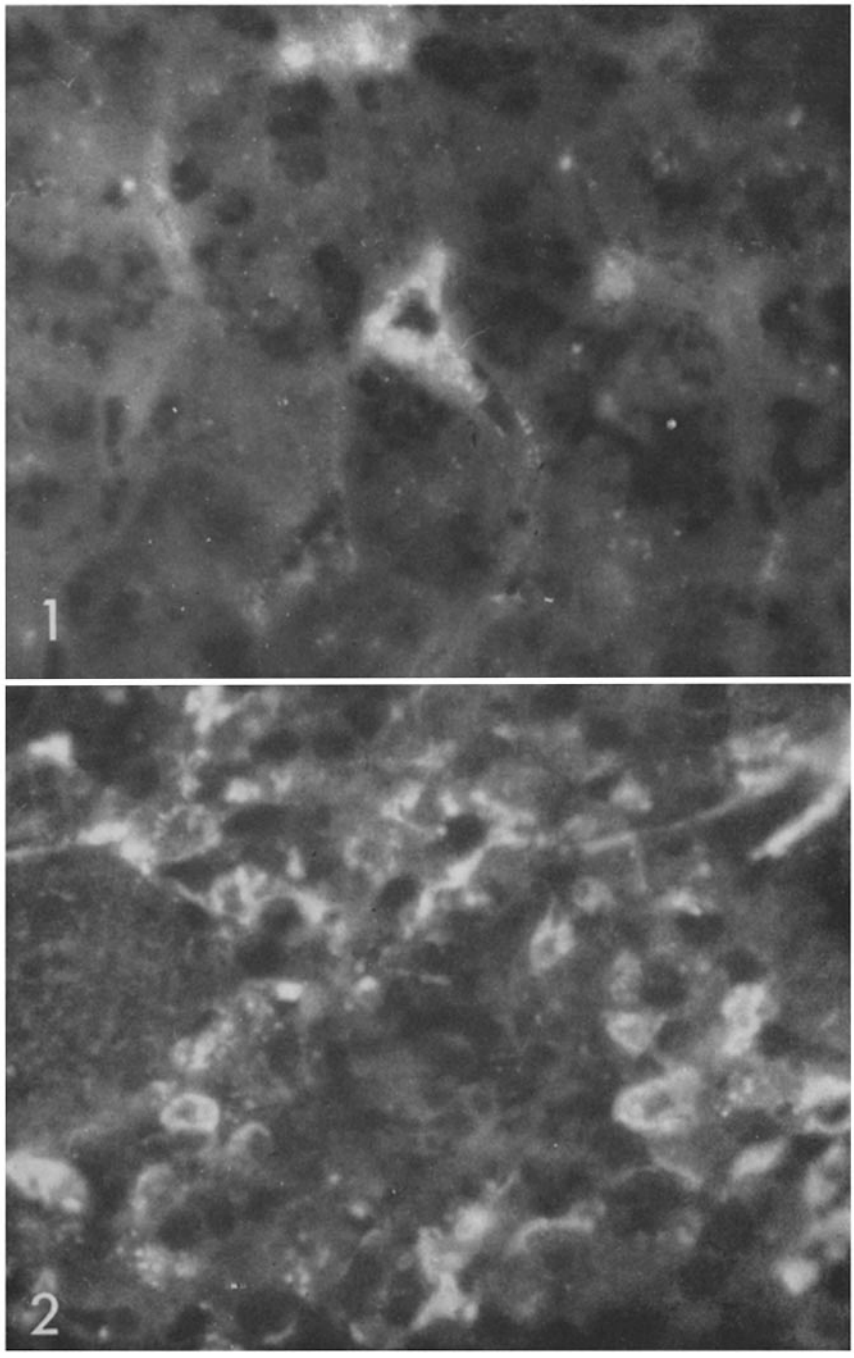
EXPLANATION OF PLATES

FIGS. 1 to 4. Photomicrographs of sections stained with fluorescein-labeled sheep anti-rabbit fibrin antiserum.

PLATE 83

FIG. 1. Section of liver from rabbit infused with 400 units of thrombin over a period of 2½ hours. Tissue was removed immediately after termination of the infusion. A Kupffer cell is shown with bright, granular, intracytoplasmic staining. × 1280.

FIG. 2. Section of spleen from rabbit infused with 400 units of thrombin. Numerous phagocytic cells show bright intracytoplasmic staining. × 1280.

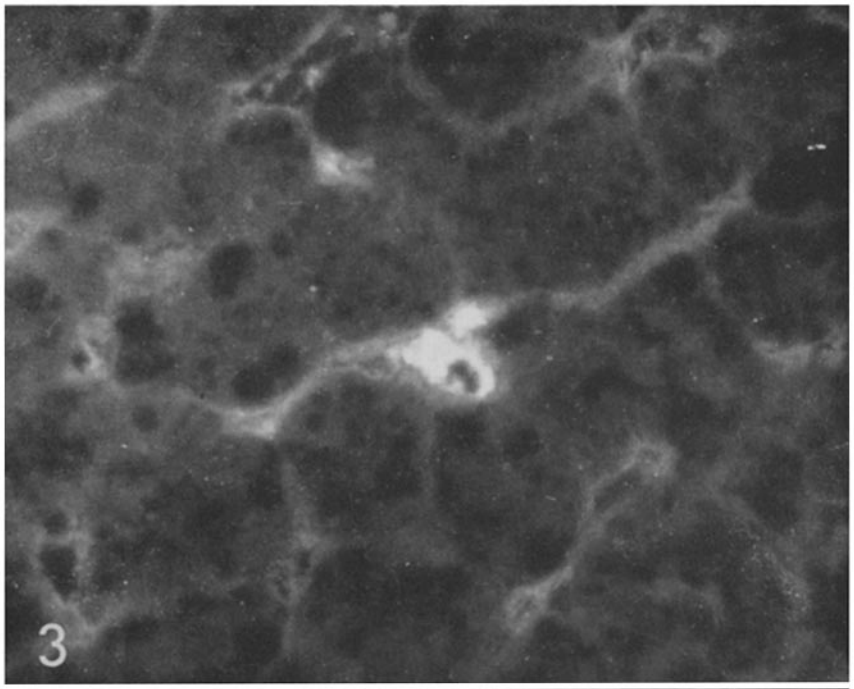


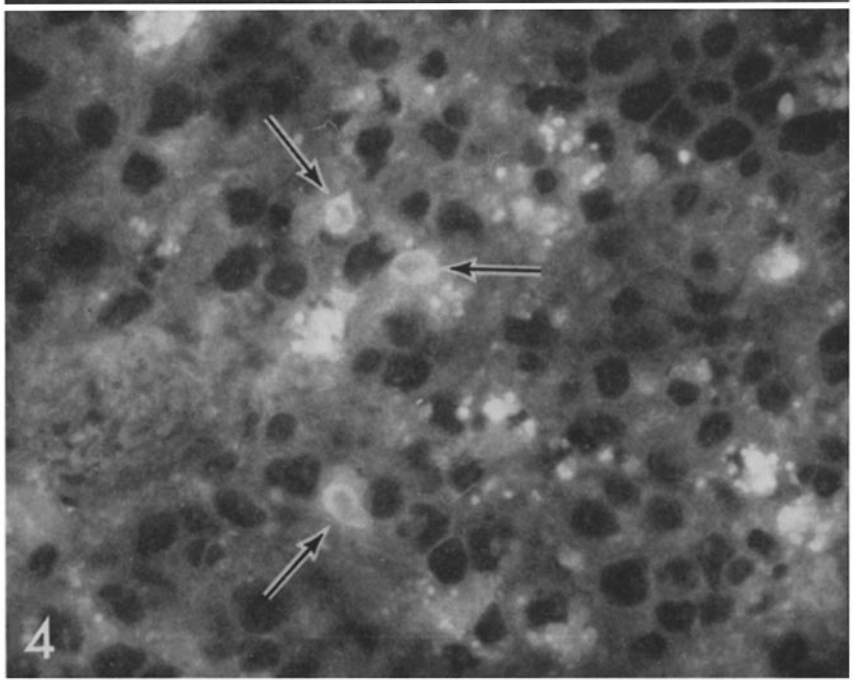
(Lee and McCluskey: Phagocytic clearance of fibrin)

PLATE 84

FIG. 3. Section of liver from rabbit killed 4 hours after intravenous injection of 0.4 mg of endotoxin. Brightly stained material is seen within the cytoplasm of a Kupffer cell. × 1280.

FIG. 4. Spleen from rabbit given 0.4 mg of endotoxin 4 hours before sacrifice. Several macrophages show specific fluorescent staining in their cytoplasm (arrows). In addition, several macrophages contain granular, fluorescent yellow pigment. × 1280.





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