The Role of Granulocytes in the Activation of Intravascular Coagulation and the Precipitation of Soluble Fibrin by Endotoxin

By Gert Müller-Berghaus and Thomas Eckhardt

This study examines the role of neutrophils (PMN) in the pathogenesis of endotoxin-induced microclot formation. It is intended to clarify whether granulocytes are involved in endotoxin-induced activation of intravascular coagulation (generation of soluble fibrin) and/or in endotoxin-induced precipitation of soluble fibrin. Precipitation of soluble fibrin was achieved by injection of endotoxin into ancrod-infused rabbits with circulating soluble fibrin (first model). Activation of intravascular coagulation was elicited by two intravenous injections of endotoxin into rabbits (second model). Seventy-two and ninety-six hours after injection of nitrogen mustard, leukopenic rabbits had PMN counts between 0 and 50 cells per μl. Neutropenia did not prevent the occurrence of glomerular microclots after infusion of ancrod and injection of endotoxin (first model). Neutropenia influenced neither the decrease in mean fibrinogen concentrations nor the drop in mean platelet counts after ancrod and endotoxin administration. In contrast to the first model, neutropenia prevented the occurrence of glomerular microclots and of circulating soluble fibrin after two injections of endotoxin (second model). It did not, however, protect rabbits from the decrease in mean platelet counts after endotoxin administration. These data indicate that granulocytes are involved in endotoxin-induced activation of intravascular coagulation and the production of soluble fibrin but are not essential to endotoxin-induced precipitation of soluble fibrin.

THE ENDOTOXIN-INDUCED generalized Shwartzman reaction (GSR) is a multifactoral entity characterized by the development of glomerular capillary microclots. It is now established that the reaction is mediated by a process of intravascular coagulation, since treatment with heparin, dicoumarol, or streptokinase protects animals against the development of the GSR25 and since ancrod-induced hypofibrinogenemia prevents the reaction.6 The endotoxin-induced microclots present characteristics like fibrin,7,8 and coagulation factors as well as platelets are consumed in the course of the reaction.9,10 The generation of glomerular microclots is a two-phase event. First, the coagulation system has to be activated to form soluble fibrin monomer complexes (soluble fibrin), and, secondly, these complexes have to be precipitated by a process independent of the coagulation mechanism.11,12

Treatment of animals with nitrogen mustard in a dose sufficient to produce severe neutropenia prevents the development of glomerular microclots.13 Two theories are presented to explain the role of granulocytes in microclot forma-
tion. First, granulocytes are involved in triggering intravascular coagulation by releasing procoagulant thromboplastin-like material after endotoxin injection. Secondly, Thomas et al. proposed a hypothesis that endotoxin causes a release of highly charged polymers which may precipitate an endotoxin-mediated altered form of fibrinogen which has been referred to as cryoprophibrin, now termed as soluble fibrin monomer complex or soluble fibrin. The occurrence of soluble fibrin monomer complexes after endotoxin injection into rabbits has been demonstrated by several investigators. Under in vitro conditions, soluble fibrin can indeed be precipitated by the highly charged macromolecules of granulocyte origin.

The present study was designed to investigate whether granulocytes are involved in endotoxin-induced activation of intravascular coagulation (generation of soluble fibrin) and/or in endotoxin-induced precipitation of soluble fibrin.

**MATERIALS AND METHODS**

**Animals**

Male and nonpregnant female rabbits of mixed New Zealand breeds, weighing between 1.8 and 2.5 kg, were used in this study. The animals were purchased from a single local breeder and fed rabbit pellet food and water ad libitum. Four days before the provocative injection of endotoxin or the infusion of ancrod (this is the day on which nitrogen mustard was injected, see Fig. 1) a polyethylene tube was inserted into the right jugular vein for blood sampling. One hour before inducing intravascular coagulation, a second catheter was put into the marginal vein of the left ear for injecting or infusing agents. This procedure assured infusions and blood sampling over a longer period of time without anesthesia. All the rabbits compared with each other were treated in the same manner with regard to operation, timing, and blood sampling. Furthermore, all the rabbits received the same total volume of fluid by substituting isotonic saline for the injections or infusions of experimental materials.

**Production of Glomerular Microclots by Ancrod Infusion and Endotoxin Injection**

Ancrod, the purified coagulant enzyme from Agkistrodon rhodostoma venom, Arvin (Twyford Laboratories Ltd, London), was diluted in isotonic saline so that 1.5 U of ancrod per kg of
body weight were contained in 4.8 ml solution. This amount of ancrod was infused over a period of 30 min by means of an infusion pump. Five minutes after the start the ancrod infusion, 150 \( \mu \text{g} \) of *Salmonella enteritidis* endotoxin (lipopolysaccharide B from Difco Laboratories, Detroit, Mich.) were injected intravenously (groups A and B).

**Production of Glomerular Microclots by Two Doses of Endotoxin**

The rabbits received two intravenous injections of 150 \( \mu \text{g} \) of *Salmonella enteritidis* endotoxin 24 hr apart (groups C and D).

**Induction of Neutropenia**

Ten milligrams of nitrogen mustard (Mustargen from Merck, Sharp & Dohme, West Point, Pa.) were prepared by adding 10 ml of pyrogen-free, sterile isotonic saline immediately before use. Ninety-six hours before the infusion of ancrod (groups A and B) or the second injection of endotoxin (groups C and D), a dose of 1.75 mg of nitrogen mustard per kg of body weight was injected intravenously for induction of granulocytopenia (see Fig. 1).

**Pathologic Studies**

At the end of infusions, the surviving animals were sacrificed by an overdose of sodium pentobarbital, and necropsies were performed immediately. Organs were fixed in neutral 5% formalin. Sections were stained with hematoxylin and eosin, and fibrin was identified by the dimethylaminobenzaldehyde (DMAB)-nitrite reaction for the histochemical demonstration of tryptophane according to the method of Adams.21

**Hematologic Studies**

**Blood samples.** Blood (5 ml) was serially drawn from the jugular vein catheter into siliconized centrifuge tubes using a mixture of sodium citrate (3.8%) and the proteinase inhibitor, aprotinin (1000 U/ml), as anticoagulant and antifibrinolytic agent (1 part citrate-aprotinin:9 parts blood). Plasma was prepared by centrifugation of the blood for 20 min at 1600 \( \times \) g at room temperature. Blood samples for platelet and leukocyte counts and hematocrit values were collected in capillary tubes from the anticoagulated blood.

**Platelets.** Platelets were counted directly by phase-contrast microscopy using procaine hydrochloride (3.5 g/100 ml).22

**Leukocytes.** Total leukocyte counts were performed directly by conventional method. Differential counts were obtained on 100 leukocytes in blood films stained with May-Grunwald-Giemsa stain.

**Hematocrit.** Microhematocrits were determined using standard heparinized tubes (Clay Adams, New York, N.Y.).

**Fibrinogen.** Plasma fibrinogen levels were determined by a slight modification of the method of Jacobson.23 With this method, plasma fibrinogen is clotted by thrombin in the presence of disodium ethylenediamine tetracetate (EDTA), the clot dissolved in urea, and the optical density of the fibrin solution determined at 282 nm.

**Detection of circulating soluble fibrin.** The presence of soluble fibrin in plasma samples was controlled by the ethanol gelation test as described by Godal and Abildgaard.24

**Correction factors.** Blood cells, hematocrits, and fibrinogen were always corrected for dilution of plasma by the anticoagulant mixture. The counted or measured values for blood cells and hematocrits were multiplied by the correction factor \( C_{\text{a}} = 1.11 \). Measured fibrinogen levels were multiplied by the factor

\[
C_{\text{f}} = \frac{PH_{\text{f}}}{PH_{\text{a}}} - 10
\]

where \( PH_{\text{f}} \) is the measured but not corrected plasma hematocrit value of an individual sample. The formula takes into account the different dilutions of plasma by the anticoagulant depending on the relation of different individual plasma hematocrits to a constant volume of anticoagulant.

**Experimental Protocols**

Rabbits were divided into four groups as follows.

Group A (10 rabbits) consisted of animals which received ancrod (1.5 U/kg) and endotoxin
(150 μg) intravenously. Ninety-six hours before starting ancrod infusion, nitrogen mustard (1.75 mg/kg) was injected. Blood was drawn before nitrogen mustard injection, before starting ancrod infusion, and 1, 2, 4, and 6 hr after the start of ancrod infusion (see Fig. 1A).

Group B (13 rabbits) consisted of animals which served as controls for the animals in group A and which received ancrod and endotoxin but were not injected with nitrogen mustard. Blood sampling was done as in group A.

Group C (10 rabbits) consisted of animals which received two intravenous injections of endotoxin (150 μg). The first dose of endotoxin was administered 72 hr after the injection of nitrogen mustard (1.75 mg/kg), and the second dose was given 24 hr later. Blood was drawn immediately before nitrogen mustard application, before the first and the second doses of endotoxin, and 1, 2, 4, and 6 hr after the second dose of endotoxin (see Fig. 1B).

Group D (10 rabbits) consisted of animals which served as controls for the animals of group C and which received two doses of endotoxin 24 hr apart. Ninety-six hours before the second dose of endotoxin, isotonic saline was injected instead of nitrogen mustard. Blood sampling was identical with group C.

After the end of ancrod infusion (groups A and B) or after the second dose of endotoxin (groups C and D), all the rabbits continuously received isotonic saline (6 ml/hr) with an infusion pump (model Perfusor from B. Braun, Melsungen, Germany) to prevent occlusions of catheters.

Statistical Evaluation

The results were subjected to the analysis of variance (partially hierarchical situations with three factors) and the Scheffé test using the statistical tables as described by Sachs. The levels of significance are listed under Results; a value of less than α = 0.05 was considered significant.

RESULTS

Induction of Neutropenia by Nitrogen Mustard

Total leukocyte counts and differential leukocyte counts were performed before and 72 and 96 hr after intravenous injection of nitrogen mustard or saline injections. The drop in total leukocyte counts was equally expressed 72 hr and 96 hr after nitrogen mustard application. This decrease is primarily accounted for by a drop in granulocytes (Table 1 and 2). Nitrogen mustard injection af-

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogen Mustard</th>
<th>Saline</th>
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<tbody>
<tr>
<td>Ancrod</td>
<td>Endotoxin</td>
<td>Ancrod Endotoxin</td>
</tr>
<tr>
<td>Mean relative PMN counts before ancrod infusion (% of total WBC)*</td>
<td>0.5 (0-2)</td>
<td>52.8 (45-58)</td>
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<tr>
<td>Incidence of positive ethanol gelation test† at -96 hr</td>
<td>0/10</td>
<td>0/13</td>
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<tr>
<td>Incidence of positive ethanol gelation test† at 0 hr</td>
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<td>0/13</td>
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<td>10/10</td>
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<tr>
<td>Incidence of positive ethanol gelation test† at 4 hr</td>
<td>10/10</td>
<td>13/13</td>
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<tr>
<td>Incidence of positive ethanol gelation test† at 6 hr</td>
<td>8/10</td>
<td>13/13</td>
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*Results are average of ten experiments each, with range of values given in parentheses.
†Expressed as the number of positive results over the total number of rabbits studied.
Table 2. Incidence of Renal Glomerular Microclots and Hematologic Changes After Two Injections of Endotoxin Into Nitrogen Mustard- or Saline-pretreated Rabbits

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Nitrogen Mustard</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean relative PMN counts before the second injection of endotoxin (% of total WBC)*</td>
<td>0.9 (0-2)</td>
<td>76.8 (65-87)</td>
</tr>
<tr>
<td>Incidence of positive ethanol gelation tests†</td>
<td></td>
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</tr>
<tr>
<td>at -96 hr</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>at -24 hr</td>
<td>0/10</td>
<td>0/10</td>
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<tr>
<td>at 0 hr</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>at 1 hr</td>
<td>0/10</td>
<td>3/10</td>
</tr>
<tr>
<td>at 2 hr</td>
<td>0/10</td>
<td>10/10</td>
</tr>
<tr>
<td>at 4 hr</td>
<td>0/10</td>
<td>9/10</td>
</tr>
<tr>
<td>at 6 hr</td>
<td>1/10</td>
<td>9/10</td>
</tr>
<tr>
<td>Incidence of glomerular microclots†</td>
<td>0/10</td>
<td>10/10</td>
</tr>
</tbody>
</table>

*Results are average of ten experiments each, with range of values given in parentheses.
†Expressed as the number of positive results over the total number of rabbits studied.

fected peripheral platelet counts, since 72 hr as well as 96 hr after this treatment platelet counts were slightly decreased to a mean of 70% of the initial values (Figs. 2 and 4). This decrease is significant on a level of significance of \( \alpha < 0.001 \).

Effect of Nitrogen Mustard on the Precipitation of Soluble Fibrin (Groups A and B)

Circulating soluble fibrin was produced by intravenous infusion of ancrod. As seen in Table 1, positive ethanol gelation tests were observed as soon as 1 hr after the initiation of ancrod infusion. The occurrence of circulating soluble fibrin was not influenced by nitrogen mustard treatment. Fibrinogen levels decreased pronouncedly in both groups (Fig. 2). Nitrogen mustard-pretreated rabbits, however, exhibited higher fibrinogen levels before starting ancrod infusion than normal control rabbits.

The effect of endotoxin in this experimental model is reflected in platelet as well as leukocyte counts (Fig. 2). In the control rabbits, the mean leukocyte counts decreased from 6576 cells to 912 cells per \( \mu l \) at 2 hr after endotoxin injection, whereas, in nitrogen mustard-pretreated animals, the leukocyte number did not drop significantly because of a lack of granulocytes. Nitrogen mustard injection did not influence the decrease in platelet counts after ancrod and endotoxin administration, as no significant difference in the shape of the curves between nitrogen mustard-pretreated and control rabbits could be computed. Nevertheless, the mean platelet numbers before ancrod infusion were lower in the neutropenic rabbits than in the controls, most likely because nitrogen mustard impaired platelet production in the bone marrow to some degree.

In this model, precipitation of soluble fibrin was produced by intravenous infusion of ancrod and injection of endotoxin. Nitrogen mustard-induced neutro-
penia did not protect the animals against the precipitation of soluble fibrin in the glomerular capillaries. Nine out of ten rabbits pretreated with nitrogen mustard revealed fibrin-rich microclots after ancrod and endotoxin administration, whereas, in the control group, eight out of 13 rabbits exhibited micro clot formation (Table 1). The mean relative polymorphonuclear leukocyte count of the nitrogen mustard-treated animals amounted to 0.5%, in comparison to 52.8% in the control group. The microclots in this experimental model demonstrated the same histologic and histochemical characteristics as the microclots observed in the classic generalized Shwartzman reaction induced by two doses of endotoxin.

Effect of Nitrogen Mustard on the Activation of Intravascular Coagulation by Endotoxin (Groups C and D)

Since nitrogen mustard-induced neutropenia did not prevent micro clot formation after ancrod and endotoxin administration, the effectiveness of nitrogen mustard in protecting rabbits against the activation of intravascular coagulation after two doses of endotoxin had to be evaluated. The decrease in plasma fibrinogen concentrations as well as the occurrence of positive ethanol gelation tests, indicative of the presence of circulating soluble fibrin, corresponded to the drop in leukocyte counts after endotoxin injection into untreated rabbits. If, however, animals had been pretreated with nitrogen mustard, endotoxin injec-
Second injection of endotoxin

First injection of endotoxin

Fig. 3. Changes in mean fibrinogen levels after two intravenous injections of endotoxin into nitrogen mustard- or saline-pretreated rabbits. Before the first injection of endotoxin, the mean fibrinogen concentration of the nitrogen mustard-treated rabbits was significantly higher than the mean value of the control animals (α < 0.001). Each curve represents ten animal experiments.

tion caused neither a decrease in fibrinogen levels nor positive ethanol gelation tests (Fig. 3 and Table 2). The occurrence of positive ethanol gelation tests was independent of the changes in platelet counts, as platelet numbers decreased in nitrogen mustard-pretreated rabbits as well as in the controls (Fig. 4). None of ten animals pretreated with nitrogen mustard and injected with two doses of endotoxin exhibited microclots, whereas all ten untreated control animals revealed renal glomerular microclots.

DISCUSSION

The data reported herein confirm earlier observations by Thomas and Good,13 Horn and Collins,27 Lerner et al.,28 and Forman et al.29 that granulocytes are essential to the production of glomerular microclot formation. If rabbits are injected with nitrogen mustard 96 hr before administration of a challenging dose of endotoxin, the resulting neutropenia apparently protects against the occurrence of microclots and the development of the generalized Shwartzman reaction. It remains speculative, however, at which precise site of action granulocytes are essential in the pathogenesis of endotoxin-induced microclot formation.30,31 As Thomas et al.16 observed that nitrogen mustard did not protect against the development of the generalized Shwartzman reaction induced by synthetic acidic polymers, these authors suggested that leukocytes may provide substances with biologic functions of acidic polymers. Thomas et al.16 as well as Horn and Spicer25 and Horn30 postulated that these acidic polymers, possibly acid mucopolysaccharides or basic proteins, might be released from the leukocytes after endotoxin injection and might precipitate soluble fibrin. According to these studies, two theories may be presented on the role of
granulocytes in the production of endotoxin-induced glomerular microclot formation: (1) Granulocytes are essential to the activation of intravascular coagulation with formation of circulating soluble fibrin and/or (2) granulocytes are necessary for the precipitation of soluble fibrin. In the present investigation, both theories were examined in a comparative study.

The presence of soluble fibrin in the plasma was determined by the ethanol gelation test. The quantity of fibrin monomer generated after endotoxin injection could not be measured, since the clot that formed in this test system after the addition of ethanol contained fibrinogen as well as other proteins besides fibrin monomer. This assay, however, gives reliable qualitative results if the fibrinogen level is below 500 mg/100 ml. Nitrogen mustard-induced neutropenia protected against the activation of intravascular coagulation since neutropenic rabbits did not exhibit circulating soluble fibrin after endotoxin injection. As activation of intravascular coagulation did not take place in neutropenic rabbits, microclots did not develop in the glomerular capillaries. However, thrombocytopenia did occur in neutropenic rabbits to the same degree as in normal control rabbits. This indicates that endotoxin-induced platelet aggregation in vivo is independent of the presence of granulocytes and the activation of intravascular coagulation. Furthermore, this observation supports the view that endotoxin-induced platelet aggregation itself does not trigger intravascular coagulation.

Nitrogen mustard-induced neutropenia did not prevent the increase in fibrinogen concentration after the first injection of endotoxin, demonstrating that...
this increased fibrinogen synthesis rate after endotoxin injection\textsuperscript{35} is not mediated by products of intravascular coagulation as fibrin monomer or fibrinopeptides split off during the conversion of fibrinogen to fibrin.

To examine the second theory, soluble fibrin was induced by ancrod infusion without generating thrombin. When ancrod acts on fibrinogen, it splits off fibrinopeptide A, but not peptide B, whereas thrombin splits off peptides A and B.\textsuperscript{36,37} Ancrod differs further from thrombin in that it does not activate the enzyme that cross-links fibrin.\textsuperscript{38} If ancrod is infused intravenously, hypofibrinogenemia, with plasma fibrinogen levels of less than 50 mg/100 ml occurs within a few hours.\textsuperscript{39,40} Yet the generalized Shwartzman reaction can be prevented if animals are pretreated with ancrod 30 hr before the preparatory injection of Thorotrast to produce severe and long-standing hypofibrinogenemia.\textsuperscript{6} The intention of this investigation was to use the phase of fibrinemia which precedes hypofibrinogenemia. After ancrod infusion, fibrinogen concentration decreased, and soluble fibrin occurred equally pronouncedly in both normal and nitrogen mustard-treated rabbits, indicating that granulocytes are not involved in the in vivo action of ancrod. However, nitrogen mustard-pretreated animals exhibited significantly higher fibrinogen levels before ancrod infusion, an observation already described by Shen et al.\textsuperscript{41} This increase in fibrinogen concentration may be caused by an acceleration of the fibrinogen synthesis rate. If endotoxin was injected into rabbits with ancrod-induced fibrinemia, the animals showed microclots in the glomerular capillaries typical of the generalized Shwartzman reaction.\textsuperscript{11,12} Since the generation of microclots by a single injection of endotoxin in ancrod-infused rabbits could not be prevented by heparin treatment, precipitation of soluble fibrin was suggested. The data of this investigation demonstrate that the precipitation of soluble fibrin does not depend on the presence of granulocytes, since nitrogen mustard-induced neutropenia did not prevent the occurrence of glomerular microclot formation.

With this study, the second theory on the role of granulocytes in the development of the generalized Shwartzman reaction can be excluded. Precipitation of soluble fibrin by endotoxin may be caused by the release of basic proteins from platelets, since nitrogen mustard treatment did not decisively decrease circulating platelet counts. After endotoxin injection, the mean platelet number dropped in nitrogen mustard-pretreated rabbits as pronouncedly as it did in control rabbits receiving ancrod and endotoxin. Furthermore, the precipitation of soluble fibrin by endotoxin may be mediated by stimulation of alpha-adrenergic receptor sites. It has been shown recently that infusions of norepinephrine into ancrod-infused rabbits result in microclot formation.\textsuperscript{42} In addition, it might be conceivable that endotoxin-induced endothelial damage represents a surface for the precipitation and polymerization of soluble fibrin. Recently, it was demonstrated by Gaynor\textsuperscript{43} that nitrogen mustard-mediated neutropenia does not protect rabbits from endothelial injury due to endotoxin.

The role of granulocytes in the generation of microclots and the development of the generalized Shwartzman reaction concentrates on the release of procoagulant tissue thromboplastin-like material.\textsuperscript{14,15} Indeed, the infusion of peritoneal leukocytes derived from endotoxin-primed rabbits caused activation
of intravascular coagulation with consumption of coagulation factors. Thus, granulocytes are essential to the activation of intravascular coagulation and formation of soluble fibrin, but are not necessary for the second step in micro clot formation, the precipitation of soluble fibrin.

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REFERENCES

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