Immunodepletion of Extrinsic Pathway Inhibitor Sensitizes Rabbits to Endotoxin-Induced Intravascular Coagulation and the Generalized Shwartzman Reaction

By Per Morten Sandset, Bonnie J. Warn-Cramer, Steven L. Maki, and Samuel I. Rapaport

We have reported earlier that immunodepletion of extrinsic pathway inhibitor (EPI) sensitizes rabbits to disseminated intravascular coagulation (DIC) induced by infusing a low concentration of tissue factor (TF). We now describe the effect of immunodepletion of EPI in rabbits administered endotoxin. Cortisone-treated rabbits were administered anti-rabbit EPI immunoglobulin (IgG) or Fab fragments or were administered control nonimmune material before an injection of endotoxin. In four of seven rabbits administered anti-EPI, plasma EPI activity levels were reduced by 70% to 80% of initial levels for 6 to 8 hours. In these rabbits the endotoxin induced extensive DIC, as evidenced by substantial decreases in fibrinogen, factor V, factor VIII, and platelet concentration of tissue factor (TF). We now describe the endotoxin-induced extensive DIC, as evidenced by substantial decreases in fibrinogen, factor V, factor VIII, and platelets, and gross hemorrhagic necrosis of the kidneys due to massive deposition of fibrin in the glomerular microcirculation (the generalized Shwartzman reaction). In three rabbits administered anti-EPI, plasma EPI levels were only transiently reduced. In these rabbits and in four rabbits administered nonimmune IgG or Fab, endotoxin induced minimal to moderate intravascular clotting and deposits of fibrin were not found in the glomerular capillaries. Because it is believed that TF expressed on monocytes triggers endotoxin-induced coagulation, these data are taken as evidence that EPI functions as a natural anticoagulant that can regulate factor VIIa/TF activity expressed on cell surfaces in vivo. They support a hypothesis that EPI prevents thrombotic complications that might otherwise result from exposure of blood to cytokine-induced generation of small amounts of TF on cell surfaces in many inflammatory and infectious disease states.

MATERIALS AND METHODS

Endotoxin. Endotoxin was a lyophilized powder of lipopolysaccharides from Escherichia coli serotype O111:B4 (Difco, Detroit, MI), dissolved in sterile, isotonic saline and stored at −80°C until use.

Polyclonal goat anti-rabbit EPI IgG and Fab fragments. Two batches of polyclonal anti-rabbit EPI IgG, referred to hereafter as anti-EPI (IgG) and anti-EPI 2(IgG), were raised against purified rabbit plasma EPI and absorbed both with immobilized rabbit IgG and with the unbound material from the factor Xa column used to purify rabbit EPI.14 A control nonimmune IgG was prepared similarly from normal goat serum (Sigma, St Louis, MO) with omission of the absorptions.

Fab fragments were prepared by a modification of the method of Nisonoff et al.17 IgG was dialyzed against 0.12 mol/L acetate buffer, pH 4.0, and then digested with pepsin (Sigma), 3 mg/100 mg IgG, by incubation at 37°C for 12 to 14 hours. After the pH was adjusted to 7.4, the Fab fragments were purified by gel filtration through Ultrogel AcA 44 (LKB Produktor, Bromma, Sweden) in 0.1 mol/L phosphate buffer, 0.15 mol/L NaCl, pH 7.4 (phosphate-buffered saline [PBS]), followed by affinity chromatography through a protein G agarose column (Cibaiochem, La Jolla, CA). Fractions were analyzed using sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE) with and without β-mercaptoethanol (all reagents for SDS-PAGE were from Bio-Rad, Richmond, CA) as described by Laemmli.18 Preparations contained mainly difragments [F(ab′)2], but also small amounts of monofragments [F(ab)] and less than 10% whole IgG (data not shown). Fab fragments were prepared on two occasions from preparations of anti-EPI (IgG). They are referred to hereafter as anti-EPI 3(Fab) and anti-EPI 4(Fab). Fab fragments were also prepared from nonimmune IgG. All purified IgG or Fab preparations were

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dialyzed against sterile 0.9% saline, filtered through a 0.2-μm hydrophilic membrane (Lida Corporation, Bensenville, IL) for sterilization, and stored in aliquots in sterile plastic tubes at −20°C.

Anti-EPI IgG and its Fab fragments could detect the EPI present in 10 to 20 μL of normal rabbit plasma on a Western blot, ie, about 1 ng of EPI in approximately 1 mg of plasma proteins loaded onto a gel. However, under the conditions needed to demonstrate binding to plasma EPI, anti-EPI IgG was also found to bind with low affinity to several higher molecular weight materials. In contrast to EPI, these higher molecular weight materials remained in the unbound fraction when rabbit plasma was incubated with anti-EPI IgG immobilized on Sepharose beads. Anti-EPI IgG was further tested and found not to recognize microgram quantities of rabbit antithrombin III, protein C, prothrombin, or albumin on Western blots. Moreover, anti-EPI IgG had no effect on the ability of rabbit antithrombin III to neutralize factor VIIIa amidolytic activity or on the thrombin time of plasma clotted in the presence or absence of 1 U/mL of heparin.

Anti-EPI 1(IgG) was tested against a purified rabbit EPI preparation and found to neutralize more than 95% of its activity as measured in an assay for rabbit EPI based on activation peptide release from factor IXa and also in the chromogenic substrate assay described below. When the antibodies were incubated with rabbit plasma at 37°C for 45 minutes and residual plasma EPI was measured in the chromogenic substrate assay, about 50 μg of anti-EPI 1(IgG) or anti-EPI 3(Fab) was found to neutralize 70% to 80% of the EPI activity in 1 mL of rabbit plasma (1 U), whereas about 100 μg of anti-EPI 2(IgG) or anti-EPI 4(Fab) was required to neutralize 60% to 70% of the activity in 1 mL of rabbit plasma (Fig 1).

Assay of rabbit plasma EPI activity. A two-stage chromogenic substrate assay was used to measure EPI activity in multiple samples of rabbit plasma. Test samples were diluted to 1% in ice-cold buffer containing 0.05 mol/L Tris · HCl, 0.15 mol/L NaCl, pH 7.5, and bovine serum albumin (BSA; Sigma) at 1 mg/mL (TBS/BSA). In the first stage, a test sample was incubated with a limiting concentration of purified recombinant rabbit TF apoprotein, a saturating concentration of purified human factor VII, a low concentration of human factor Xa, and calcium ions. In the second stage, a high concentration of human factor Xa was added to the reaction mixture as substrate for residual factor VIIa/TF catalytic activity and the factor Xa activity generated was measured with an amidolytic substrate (Chromozym X; Boehringer Mannheim, Indianapolis, IL). Absorbance was read at 405 nm. A single rabbit plasma was calibrated against a pooled plasma from five rabbits and used as a reference plasma arbitrarily assigned a value of 100% EPI. The absorbance reading obtained with a test plasma was converted to percent EPI from standard curves obtained by plotting log absorbance against log dilution of the reference plasma (Fig 2). Details of the assay have been provided.

Measurement of fibrinogen, factor V, and factor VIII. Fibrinogen concentration was measured by the method of Clauss, which is based on determining the clotting time of plasma after the addition of a high concentration of bovine thrombin (Dade). Clotting times were converted to fibrinogen concentration from a standard curve prepared with human fibrinogen (Dade).

Factor V was determined by incubating 100 μL of a 1:100-fold dilution of the test sample with 100 μL factor V-deficient plasma (from a volunteer donor or human plasma prepared by immunoabsorption) and 100 μL of rabbit brain thromboplastin (Sigma). After 3 minutes at 37°C, clotting was triggered by the addition of 100 μL of 35 mmol/L CaCl₂ that had been warmed to 37°C.

Factor VIII was determined by incubating 100 μL of a 1:2.5- to 1:50-fold dilution of the test sample with 100 μL factor VIII-deficient plasma (from a volunteer donor) and 100 μL APTT reagent (Organon Teknika, Durham, NC) for 6 minutes at 37°C. Clotting was then triggered by the addition of 100 μL of 25 mmol/L CaCl₂ that had been warmed to 37°C. In both the factor V and factor VIII assays samples were diluted in ice-cold TBS/BSA and...
clotting times were determined using a semiautomatic coagulometer (Lancer; Sherwood Medical Industries, St Louis, MO).

Hematologic tests. Hematocrit was determined by centrifugation of blood in heparinized microcapillary tubes. Platelets and white blood cells (WBC) were counted in a Coulter Model ST counter (Coulter, Hialeah, FL).

Animal protocols. Female New Zealand rabbits, weighing 1.4 to 2.4 kg, were used in a protocol approved by the Animal Subjects Committee of the University of California, San Diego. The rabbits were administered daily intramuscular injections of 25 mg cortisone acetate (Merck, Sharp and Dohme, West Point, PA) to prepare them for development of DIC after a single injection of endotoxin.\textsuperscript{14,15} Fifty minutes after the fourth dose, 10 mg/kg of control or immune IgG or Fab in a 3 to 5 mL volume was injected into a marginal ear vein. Ten minutes after the injection of IgG or Fab fragments, 100 \mu g/kg of the endotoxin solution was administered intravenously over approximately 1 minute.

Seven rabbits received immune IgG or Fab and four rabbits received control IgG or Fab (Table 1). The first two rabbits administered Fab (an experimental animal administered immune Fab [rabbit no. 7, Table 1] and a control animal administered nonimmune Fab [rabbit no. 11, Table 1] received only a single injection. It was noted that the decrease in plasma EPI in the experimental animal was not sustained. It was then found, by autoradiography of plasma samples from these animals using \textsuperscript{125}I-labeled donkey anti-IgG (Sigma) for detection of injected Fab, that the Fab fragments had disappeared rapidly from the circulation (data not shown). Hence, in the next two rabbits administered anti-EPI Fab (rabbits no. 3 and 4 of Table 1) a second dose of 10 mg/kg was administered 2 hours after the injection of endotoxin to assure the continuing presence of anti-EPI in the circulation.

Serial 2 mL blood samples, obtained by inserting a 23-gauge needle into the marginal ear vein, were collected before the injection of IgG or Fab fragments, and 1, 2, 4, 6, 8, and 24 hours after the injection of the endotoxin. Nine parts of blood were allowed to drip into marked Eppendorf tubes containing one part of a balanced citrate anticoagulant (sodium citrate, 0.06 mol/L, and citric acid, 0.04 mol/L). Platelet-poor plasma was prepared by centrifugation at 10,000g for 10 minutes at 4°C and stored in aliquots at −80°C.

After 24 hours, the animals were killed by an injection of Terminol-III (Anpro Pharmaceutical, Arcadia, CA) and subjected to autopsy. Tissue sections of the kidneys were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin and with phosphotungstic acid hematoxylin (PTAH).

Data processing. All values reported for each group of rabbits are means ± SEM. When values for clotting factors and EPI activity for each animal were converted to percent of initial values (measured in the blood sample collected before infusion of IgG or Fab fragments), the samples were corrected for the decrease in hematocrit and for the dilution of the blood samples through the addition of citrate anticoagulant as described earlier.\textsuperscript{16}

**RESULTS**

*Effects of preparation with cortisone.* Treatment of the 11 rabbits with four daily intramuscular injections of 25 mg cortisone acetate caused mean weight to increase by 3% and mean hematocrit to decrease from 0.40 to 0.35. Mean plasma clotting factor levels decreased from pretreatment mean levels by 24% for EPI, by 12% for factor VIII, and by 3% for fibrinogen. The mean factor V level increased by 9%.

*Clinical effects of endotoxin.* None of the rabbits became tachypneic or cyanotic after the injection of endotoxin. However, all rabbits developed signs of endotoxemia as manifested by diarrhea and cold ears, and it became difficult to obtain blood samples from the marginal ear vein.

*EPI activity levels.* In our earlier study,\textsuperscript{16} the injection of anti-EPI equivalent to what is referred to here as anti-EPI 1 (IgG) was found to depress the EPI activity of plasma from blood drawn 10 minutes after the injection by more than 80% of the initial value. In the present experiments, plasma EPI activity was reduced 1 hour after endotoxin in

<table>
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<th>Rabbit Group/No.</th>
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<th>EPI Activity (%)</th>
<th>Fibrinogen (mg/dL)</th>
<th>Glomerular Fibrin*</th>
<th>Gross Hemorrhagic Cortical Necrosis*</th>
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*Glomerular fibrin deposition and gross hemorrhagic renal cortical necrosis graded from 0 (no fibrin deposition/no cortical renal necrosis) to 4+ (massive fibrin deposition/severe cortical renal necrosis).
†Polyclonal IgG against rabbit EPI, anti-EPI batches 1 and 2. The rabbits were administered a single injection of 10 mg/kg intravenously 10 minutes before the injection of endotoxin.
‡Polyclonal Fab fragments against rabbit EPI, anti-EPI batches 3 and 4. Two of the rabbits (nos. 3 and 4) were administered a repeated dose of 10 mg/kg 2 hours after the injection of endotoxin.
§Nonimmune, control IgG.
| Nonimmune, control Fab fragments.
all rabbits injected before the endotoxin with 10 mg/kg of either anti-EPI IgG or anti-EPI Fab. Individual values are listed in Table 1, in which the rabbits have been divided into two experimental groups and the control group. Group I consists of two rabbits administered a single injection of anti-EPI 1(IgG) and two rabbits administered two doses of anti-EPI 4(Fab). In these rabbits, plasma EPI activity remained low over the 8 hours after endotoxin that it was measured. Mean plasma EPI activity was 36% of the reference standard 1 hour after the injection of endotoxin, and 27% 6 hours after the endotoxin (Fig 3). Group II consists of two rabbits administered a single injection of anti-EPI 2(IgG) and one rabbit administered a single dose of anti-EPI 3(Fab). In these rabbits the decrease in EPI activity was less pronounced to a mean of 44% 1 hour after endotoxin, and plasma EPI increased thereafter to return to near normal within 6 to 8 hours after the endotoxin. In group III, the control group consisting of three rabbits administered a single injection of nonimmune IgG and one rabbit administered a single injection of nonimmune Fab, mean plasma EPI activity did not decrease significantly.

Fibrinogen, factor V, and factor VIII levels. Mean levels of these clotting factors decreased after endotoxin in all three groups of animals but to different extents (Figs 4 and 5). Consumption of fibrinogen, factor V, and factor VIII was substantially greater in group I, ie, in the rabbits in which anti-EPI had induced a pronounced, prolonged decrease in plasma EPI activity. Fibrinogen concentration decreased from a mean of 224 mg/dL before treatment to a mean of 57 mg/dL at 8 hours after endotoxin (Fig 4), ie, to 31% of the mean value before endotoxin. Individual values for these rabbits are listed in Table 1. Factor V (Fig 5A) decreased from a mean of 131% to a mean of 15% of the reference standard 8 hours after endotoxin, and factor VIII (Fig 5B) decreased from a mean of 88% to a mean of 24% of the reference standard. Expressed as percent of the mean before endotoxin, the values 8 hours after endotoxin were, for factor V, 12%, and for factor VIII, 30%.

In group II, ie, the three rabbits administered anti-EPI who had a lesser decrease and early recovery of plasma EPI activity, mean fibrinogen levels decreased only minimally to 88% of the initial value at 4 to 6 hours (Fig 4). Eight hours after endotoxin, mean factor V activity was 43% and mean factor VIII activity was 54% of the mean value before endotoxin. In group III, ie, the control rabbits administered nonimmune IgG or Fab who had insignificant decreases in plasma EPI levels, the mean fibrinogen level decreased minimally by 4 hours to 81% of the mean initial value and then began to increase. The mean factor V level decreased to 60% and the mean factor VIII level to 63% of the mean level before endotoxin.

Hematologic values. WBC counts 1 hour after endotoxin were 0.5 to 1 x 10^3/kL in all rabbits. Counts remained markedly depressed for 6 hours but increased to reach higher than normal levels at 24 hours. Platelet levels decreased progressively after endotoxin and were still reduced after 24 hours. The decrease in the platelet count was more pronounced in the rabbits of group I (Fig 6).
Pathologic findings. All the rabbits were killed and autopsied 24 hours after the injection of endotoxin. Findings are summarized in Table 1. All of the rabbits in group I had gross evidence of hemorrhagic renal cortical necrosis (Fig 7A). The kidneys of the remaining rabbits appeared normal (Fig 7B).

Microscopic sections were reviewed without knowledge of the treatment group of the rabbits. Massive deposition of fibrin in the glomerular capillary bed was noted in the sections from the four rabbits in group I. Fibrin was not seen in glomerular capillaries in the sections from the rabbits in groups II and III.

Fig 6. Mean values for platelet counts for rabbits administered anti-EPI IgG or Fab or nonimmune IgG or Fab. Values are expressed as the mean (±SEM) platelet count. Number of animals and symbols are as described in the legend of Fig 3.

Fig 7. (A) The gross appearance of hemorrhagic necrosis of the kidney (generalized Shwartzman reaction) from a rabbit administered antirabbit EPI Fab, 10 mg/kg, before and 2 hours after the injection of endotoxin. (B) The normal gross appearance of the kidney from a rabbit injected with nonimmune IgG, 10 mg/kg, before the injection of endotoxin.
**DISCUSSION**

The data presented provide evidence that immunodepletion of EPI sensitizes rabbits to endotoxin-induced intravascular coagulation and the generalized Shwartzman reaction. In four of seven rabbits, anti-EPI induced a sustained decrease of plasma EPI levels (Fig 3). In each of these rabbits, an intravenous injection of endotoxin caused marked consumption of fibrinogen, factor V, factor VIII, and platelets and gross hemorrhagic necrosis of the kidney due to massive deposition of fibrin within glomerular capillaries.

In three rabbits, anti-EPI induced a lesser, unstable decrease in plasma EPI activity. The reason is unknown but probably reflects a failure to maintain an adequate level of plasma antibody in these animals. In these three rabbits and in four control rabbits administered nonimmune IgG or Fab, an intravenous injection of endotoxin caused only a minimal or modest decrease in fibrinogen, factor V, factor VIII, and platelet levels (Figs 4 through 6). The kidneys of these animals appeared normal on gross and microscopic examination.

It is unlikely for two reasons that reactions triggered by anti-EPI/EPI immune complexes contributed significantly to the clotting and kidney damage after endotoxin of the animals with a sustained decrease in plasma EPI activity. First, in our earlier study, fibrinogen, factor V, and factor VIII levels did not decrease when rabbits were injected with antirabbit EPI IgG and then infused with inert control materials. Second, anti-EPI/EPI immune complexes formed with Fab should not activate the complement cascade. It can be seen in Table 1 that plasma EPI was immunodepleted in two rabbits of group I with anti-EPI IgG and in the other two rabbits of group I with anti-EPI Fab. Yet, endotoxin caused a marked decrease in plasma fibrinogen level and gross hemorrhagic necrosis of the kidneys in all four rabbits.

In work from this laboratory establishing that anti-TF IgG substantially reduces intravascular coagulation after endotoxin, cortisone-treated rabbits were administered the same dose of endotoxin as used in the present experiments (100 µg/kg). The six control rabbits not administered anti-TF IgG in this earlier study had significant consumption of clotting factors. Although no control animal had gross evidence of hemorrhagic necrosis of the kidneys, four of the five rabbits autopsied had microscopic evidence of fibrin deposition in glomerular capillaries. Therefore, for the present study we had planned initial experiments to select a dose of endotoxin that would cause only minimal clotting in control animals administered nonimmune IgG. However, we found that the 100 µg/kg dose of endotoxin used earlier now caused only minimal consumption of clotting factors, and this dose of endotoxin was used for the present study. The reason for this difference from previous experience is not clear. It could reflect variability in properties of different batches of endotoxin, variability in sensitivity of rabbits to endotoxin due to unknown environmental conditions, or both.

Monocytes exposed to endotoxin in vitro, mononuclear cells isolated from rabbits administered endotoxin, and mononuclear cells from the blood of patients with meningococcemia have all been found to express TF activity. Thus, considerable evidence supports the conclusion that TF expressed on monocytes triggers endotoxin-induced intravascular coagulation. Consequently, we interpret the present data to mean that normal levels of EPI activity, present in plasma and endothelial cell surface pools, can prevent a limited concentration of factor VIIa/TF complexes formed on cell surfaces after endotoxin from inducing extensive intravascular coagulation and resultant gross hemorrhagic renal cortical necrosis (Fig 7A). However, one should not yet conclude that this protective effect of EPI stems solely from inhibition of factor VIIa/TF catalytic activity because EPI also neutralizes factor Xa activity.

As already mentioned, TF-dependent coagulation may cause continuing DIC in patients with gram-negative endotoxemia despite normal or even elevated plasma levels of EPI. Moreover, in the earlier study cited above, endotoxin-induced DIC continued over 6 hours in rabbits with nearly normal plasma EPI levels, as did DIC induced by an infusion over 4 hours of purified, relipidated rabbit TF apoprotein at a concentration of 12 ng/mL and a rate of 25 mL per hour. Thus, in both patients and experimental animals it appears that normal levels of EPI can not suppress DIC induced by a continuing exposure of blood to a concentration of TF in the range of that achieved in the TF infusion experiment.

However, we have now also established that infusion of a lesser concentration of purified, relipidated rabbit TF apoprotein induces DIC in rabbits immunodepleted of EPI but not in rabbits with normal levels of EPI. These data provide convincing evidence that EPI can function in vivo to dampen coagulation induced by exposure of blood to small amounts of infused exogenous TF. Moreover, in a converse experiment, Day et al administered very high doses of a recombinant human EPI preparation to rabbits and found that this suppressed the fibrinogen consumption induced in control rabbits by the intravenous injection of a commercial rabbit brain thromboplastin.

Yet, experiments with infused exogenous TF preparations may be viewed as unphysiologic in that the TF is infused as a suspension containing large amounts of anionic, procoagulant phospholipid. The present data establish that EPI can also dampen factor VIIa/TF activity formed in vivo under more physiologic conditions, ie, with TF expressed on the outer layer of the surface membrane of a cell. In our view, these data significantly strengthen the evidence that EPI functions as a key natural anticoagulant. Whereas EPI can not prevent TF-induced DIC in patients with extensive gram-negative endotoxemia, we believe that in less serious infectious states EPI does protect against coagulation that might otherwise be triggered by a low concentration of TF expressed on cells to which the blood is exposed.

**NOTE ADDED IN PROOF**

In July 1991 The Scientific and Standardization Committee of the International Society on Thrombosis and Haemostasis recommended that the name tissue factor pathway inhibitor (TFPI) be used in the future instead of extrinsic pathway inhibitor (EPI) or lipoprotein-associated coagulation inhibitor (LACI).
REFERENCES

Immunodepletion of extrinsic pathway inhibitor sensitizes rabbits to endotoxin-induced intravascular coagulation and the generalized Shwartzman reaction

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