Disseminated Intravascular Coagulation in Rabbits Induced by Administration of Endotoxin or Tissue Factor: Effect of Anti-Tissue Factor Antibodies and Measurement of Plasma Extrinsic Pathway Inhibitor Activity

By Thomas A. Warr, L. Vijaya Mohan Rao, and Samuel I. Rapaport

Rabbits were given polyclonal anti-tissue factor (TF) immunoglobulin G (IgG) before an injection of endotoxin to test the hypothesis that TF triggers disseminated intravascular coagulation (DIC) after endotoxin. The rabbits had been prepared with cortisone to develop DIC after one injection of endotoxin. Anti-TF IgG substantially reduced the falls in fibrinogen, factors V and VIII, and platelets noted in control rabbits given preimmune IgG before endotoxin. At autopsy 24 hours later, fibrin was present in glomerular capillaries of 4 of 5 control rabbits, but in none of 11 rabbits given anti-TF IgG. DIC was also induced in a second group of rabbits by the infusion, over 4 hours, of 1 μg/kg of purified, reconstituted rabbit brain TF. This resulted in striking falls in plasma fibrinogen, factors V, and VIII that were diminished, but not prevented by prior treatment with anti-TF IgG. Circulating activated factor VII, induced by either TF infusion or endotoxin, could not be detected after DIC. Mean plasma extrinsic pathway inhibitor (EPI) activity did not fall significantly after endotoxin, and only to about 65% of the preinfusion after infusion of TF. Thus, DIC induced by both agents proceeded despite nearly normal plasma EPI levels. Because EPI neutralizes factor Vlla/TF in vitro only after a short lag period, the DIC that persisted for up to 6 hours after injection of endotoxin suggests that TF activity continued to be generated during this period on cells to which the circulating blood was exposed. All animals given endotoxin became ill with cyanosis, tachypnea, cold ears, and diarrhea, regardless of whether they had received anti-TF IgG to attenuate DIC. Infusion of TF caused some animals to die acutely with pulmonary arterial thromboses, but surviving animals did not appear ill. The findings support the hypothesis that exposure of blood to TF triggers DIC after endotoxin, but is not important for the pathogenesis of endotoxin-induced shock.

TWO CELL TYPES normally in contact with circulating blood, monocytes1 and vascular endothelial cells,2 have been shown to express tissue factor (TF) activity after incubation with gram-negative endotoxin in vitro. Moreover, peritoneal macrophages3 and peripheral blood mononuclear cells4 from rabbits given endotoxin, and also mononuclear cells from the blood of patients with meningococcemia,5 have been shown to possess TF activity. Thus, considerable indirect evidence suggests that exposure of blood to TF triggers disseminated intravascular coagulation (DIC) in gram-negative endotoxia.

However, the literature also contains reports that could implicate reactions independent of TF in the initiation of endotoxin-induced DIC. Endotoxin has been shown to activate factor XII,6 and factor XII levels have been reported to fall both in rabbits given endotoxin7,8 and in humans with sepsis.9 Fibrinogen levels were found to fall after endotoxin in hereditary factor VII-deficient dogs.10 Thrombocytopenia was reported to protect rabbits against the generalized Shwartzman reaction after endotoxin.11 Finally, a continuing DIC has been documented12 in a patient with gram-negative infection, despite an elevated plasma level of extrinsic pathway inhibitor (EPI), a key inhibitor of the TF pathway of coagulation13 (also known as lipoprotein-associated coagulation inhibitor [LACI]).14 These observations highlight the need for direct evidence that exposure to TF is the major mechanism triggering intravascular coagulation after endotoxin. Therefore, we have performed experiments in which rabbits were infused with neutralizing antibodies to TF before attempting to induce DIC with an injection of endotoxin or, as a control, with an infusion of exogenous TF. Measurements of hemostatic factors were used to evaluate the extent of DIC. Plasma EPI activity was also measured. In addition, in the animals given endotoxin, histologic sections of the kidney were examined for evidence of deposition of fibrin in the glomerular capillary bed (generalized Shwartzman reaction). The data presented here establish that anti-TF antibodies inhibit endotoxin-induced DIC in rabbits. The data also provide clear evidence that adequate levels of plasma EPI fail to suppress the continuation of TF-induced DIC.

Materials and Methods

Rabbit TF

Rabbit brain TF apoprotein was purified to homogeneity as described elsewhere.15 A molar ratio of protein to phospholipid of 1:100,000 was used in the presence of octylglucoside to incorporate the apoprotein into mixed phospholipid vesicles containing phosphatidylserine and phosphatidylycholine in a ratio of 4:6. Aliquots stored at -80°C were thawed immediately before use. Phospholipid vesicles not containing TF were also prepared for use as a control.

Polyclonal Goat Anti-Rabbit TF IgG

Purified rabbit brain TF apoprotein16 was used to raise an anti-rabbit TF antiserum in a goat. The IgG fraction (anti-TF IgG) was purified by precipitation at a 45% ammonium sulfate saturation, followed by DEAE-Affi-Gel Blue chromatography (Bio-Rad, Richmond, CA) and dialysis overnight against 10 mmol/L Hepes, 0.15 mol/L NaCl, pH 7.5. Western blots of crude Triton (Sigma, St.

From the Departments of Medicine and Pathology, University of California, San Diego.

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Address reprint requests to Samuel I. Rapaport, MD, UCSD Medical Center, 225 Dickinson St, (H-81F), San Diego, CA 92103.

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Louis, MO) extracts of rabbit brain, lung, and heart with the immunoglobulin G (IgG) preparation yielded a single band with an apparent molecular weight (mol wt) of about 45,000 daltons, which corresponds to the mol wt of purified rabbit TF apoprotein.16 IgG preparations from two different bleeds (anti-TF IgG-I and anti-TF IgG-II) were used for these experiments. With both preparations, incubation of anti-TF IgG with TF for 10 to 15 minutes substantially enhanced the ability of the antibodies to neutralize TF activity (Table 1). In two preliminary experiments in which either 5.1 mg/kg or 7.7 mg/kg of anti-TF IgG was injected into a rabbit, plasma obtained 8 hours later contained anti-TF activity (measured after incubation with TF as described in the footnote to Table 1) equivalent to 85% of the anti-TF activity of the IgG when diluted 1:40 in plasma in vitro. IgG was also prepared from preimmune goat serum to serve as an IgG control. The IgG preparations were filtered through 0.2 μm hydrophilic nylon membranes (Lida Manufacturing, Susanville, IL) just before administration to remove contaminating materials.

Endotoxin was a lyophilized powder of lipopolysaccharides from Escherichia coli serotype 0111:B4 (Sigma, St Louis, MO), dissolved in isotonic saline and stored at −20°C until use.

Animal Protocols

Female New Zealand rabbits, 1.5 to 2.5 kg, were used in protocols approved by the Animal Subjects Committee of the University of California, San Diego. The treatment groups and number of animals in each providing data for analysis are summarized in Table 2. Data obtained within a treatment group with the two anti-TF IgG preparations have been combined because of their similar in vitro activity (Table 1) and in vivo effects.

Endotoxin-Induced DIC

Rabbits were given 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.5,11 Fifty minutes after the fourth dose, 10 mg/kg of endotoxin or saline was given intravenously (IV) over 1 to 2 minutes. Serial 2-mL blood samples were obtained over the next 24 hours by inserting a 23-gauge needle into the marginal ear vein (dilated with xylene). Nine parts of blood were allowed to drip directly into marked plastic tubes containing 1 part of a balanced citrate anticoagulant.16 Platelet-poor plasma, obtained by centrifugation for 10 minutes at 15,000 × g, was stored in small aliquots in capped propylene Eppendorf micro test tubes at −20°C until used.16 After 24 hours, the animals were killed by an injection of Terminol-III (Anpro Pharmaceutical, Arcadia, CA) and subjected to autopsy. Sections of kidney and liver were fixed in formalin and stained with hematoxylin and eosin and with phosphotungstic acid hematoxylin (PTAH).

Table 1. The Ability of Two Anti-TF IgG Preparations to Neutralize TF Activity In Vitro

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Incubation Time (min)</th>
<th>Clotting Time (s)</th>
<th>Residual TF Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-TF IgG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG:1</td>
<td>0</td>
<td>26</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>IgG:2</td>
<td>0</td>
<td>27</td>
<td>76</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>48</td>
<td>12</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Preimmune IgG</td>
<td>13</td>
<td>24</td>
<td>100</td>
</tr>
<tr>
<td>Buffer</td>
<td>13</td>
<td>24</td>
<td>100</td>
</tr>
</tbody>
</table>

*Immediate antibody neutralizing ability was measured by allowing 0.1 mL of rabbit plasma and 0.05 mL of 50 ng/mL TF to stand for 3 minutes at 37°C in a plastic tube followed by the addition, in rapid succession, of 0.05 mL of 500 μg/mL anti-TF IgG and 0.1 mL of 25 mmol/L CaCl2. Neutralization after incubation of antibodies with TF was measured by incubating 0.05 mL of 50 ng/mL TF for 10 minutes at 37°C in a plastic tube with 0.05 mL of 500 μg/mL anti-TF IgG or (as controls) 500 μg/mL preimmune IgG or buffer. Then, 0.1 mL of rabbit plasma was added and, 3 minutes later, 0.1 mL of 25 mmol/L CaCl2. Percent residual TF activity was calculated from a reference curve prepared by plotting clotting times after recalcification against known concentrations of TF added to rabbit plasma.

Table 2. Number of Rabbits (N) in Each Treatment Group Providing Data for Analysis

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>IgG</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone/endotoxin</td>
<td>Preimmune</td>
<td>6</td>
</tr>
<tr>
<td>Anti-TF*</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td>TF infusion</td>
<td>None</td>
<td>6</td>
</tr>
<tr>
<td>Anti-TF†</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>TF infusion controls‡</td>
<td>None</td>
<td>6</td>
</tr>
</tbody>
</table>

*Anti-TF IgG-1, six animals; anti-TF IgG-2, six animals. †Anti-TF IgG-1, three animals; anti-TF IgG-2, three animals. ‡Phospholipid vesicles, three animals; saline, three animals.

TF-Induced DIC

Female New Zealand rabbits were infused through a marginal ear vein over approximately 4 hours with 100 mL of isotonic saline containing 20 to 25 ng/mL of reconstituted TF. When 0.1 mL of such a TF suspension was incubated with 0.1 mL of pooled reference rabbit plasma at 37°C in a plastic tube and recalcified with 0.1 mL of 25 mmol/L CaCl2, the mixture clotted in from 29 to 31 seconds. The total dose of TF infused was 1.0 μg/kg. Control animals were infused with either 100 mL of isotonic saline or 100 mL of isotonic saline containing the same concentration of phospholipid vesicles as received by the animals given reconstituted TF. Before, during, and after the infusion, 2-mL samples of blood were obtained from the marginal vein of the opposite ear and processed as described above.

Reagents for Clotting Assays

Rabbit brain thromboplastin was from Sigma. Automated APTT was from Organon Teknika (Durham, NC); bovine thrombin and purified human fibrinogen were from DADE (Aguada, Puerto Rico). Russell's viper venom (Wellcome, Beckenham, England), 1 part, was mixed with 9 parts of a 50% dilution of rabbit brain cephalin (Sigma) in 0.05 mol/L Tris, 0.15 mol/L NaCl, pH 7.5, containing 1 mg/mL bovine serum albumin (TBS/BSA) to make RVV/cephalin reagent. Hereditary human factor-deficient plasmas were either purchased from George King Bio-Medical (Overland Park, KA) or obtained from volunteer donors. In addition, factor X-deficient plasma and factor VII-deficient plasma for use in some experiments were prepared by passing normal human plasma over either rabbit anti-human factor X antibody or rabbit anti-human factor VII antibody immobilized on Affi-Gel 15 (BioRad). These substrate plasmas contained less than 1% residual activity. Barium-absorbed rabbit plasma for use in factor X and factor VII assays was prepared as described previously.17 Pooled citrated platelet-poor plasma18 from six rabbits was used as a reference standard and arbitrarily assigned a value of 100% activity for all clotting factors.
Clotting Factor Assays

All plasma clotting factors were measured in duplicate, and the average of the two determinations was taken as the value for the specimen. Plasma samples were diluted for assay in cold TBS/BSA, and diluted specimens stood for 5 to 30 minutes on ice before assay, as determined by the number of samples being assayed. (An exception was the fibrinogen assay in which samples were diluted in Veronal buffer [Sigma] at room temperature.) Individual assays are described below.

Rabbit Plasma EPI Assay

Like a previously described assay for EPI in human plasma, the rabbit plasma EPI assay is based on the ability of a dilution of test plasma to inhibit factor VIIa/TF-catalyzed activation of \(^3\)H-factor IX, as measured by release of the trichloroacetic acid (TCA) soluble activation peptide. Details of the rabbit plasma EPI assay, which differs from the human plasma assay in the use of a critical, reduced concentration of exogenous factor X and of a preliminary incubation period before addition of the \(^3\)H-factor IX, are provided elsewhere. Mean baseline EPI activity for the 22 young, female New Zealand rabbits used in this study was 100% of the reference plasma, with a range of 76% to 138%.

Measurement of Fibrinogen

The methods of Claus and Exner et al\(^{10,21}\) were used in which fibrinogen concentration is determined from the clotting time of plasma after addition of a high concentration of bovine thrombin. Clotting times were determined in a coagulizer (Electra 800, Medical Laboratory Automation, New York, NY), and clotting times were converted to fibrinogen concentration from a standard curve prepared with purified human fibrinogen.

Factor V

Fifty microliters of factor V-deficient plasma was incubated with 50 \(\mu\)L of rabbit brain thromboplastin for 3 minutes at 37°C. Then 25 \(\mu\)L of a 1:100 to 1:250 dilution of the test sample was added, followed by 50 \(\mu\)L of 35 mmol/L CaCl\(_2\) warmed to 37°C.

Factor VIII

Fifty microliters of human factor VIII-deficient plasma, 50 \(\mu\)L of APTT reagent, and 50 \(\mu\)L of a 1:50 to 1:100 dilution of test sample were incubated for 5 minutes at 37°C, followed by the addition of 50 \(\mu\)L of 25 mmol/L CaCl\(_2\) warmed to 37°C.

Factor X

Fifty microliters of a mixture of equal parts of factor X-deficient human plasma and a 50% dilution in TBS/BSA of barium-absorbed rabbit plasma were incubated with 50 \(\mu\)L of an RVV/cephalin reagent for 3 minutes at 37°C. Then, 25 \(\mu\)L of a 1:50 to 1:200 dilution of test sample was added, followed by 50 \(\mu\)L of 35 mmol/L CaCl\(_2\) warmed to 37°C.

Factor VII

Factor VII was measured by two methods: a clotting assay (factor VIIc) and a coupled amidolytic assay (factor VIIam). In the clotting assay, 50 \(\mu\)L of an equal part mixture of factor VII-deficient plasma and barium-absorbed rabbit plasma were incubated with 50 \(\mu\)L of rabbit brain thromboplastin for 3 minutes at 37°C. Twenty-five microliters of a 1:30 to 1:50 dilution of test sample was then added, followed by 50 \(\mu\)L of 35 mmol/L CaCl\(_2\) warmed to 37°C. The coupled amidolytic assay was performed as described by Seligsohn et al\(^{12}\) except that 50 \(\mu\)L of 0.1 \(\mu\)g/mL purified, reconstituted rabbit TF was used in place of crude human brain TF.

Accuracy of Clotting Assays Using TF When Test Samples Contained Anti-TF IgG

Anti-TF IgG was added directly to rabbit plasma in concentrations equivalent to those calculated to be present in the plasma of animals administered anti-TF IgG. The plasma was then assayed for factors V, VIIc, and VIIam; ie, for those factors in which TF is a reagent in the assay system. Test results did not differ from those obtained when buffer instead of anti-TF IgG was added to the plasma. The high concentration of TF used in these assays systems apparently eliminated potential error due to neutralization of TF during assays by the anti-TF IgG in the diluted plasma samples. Because the assay for rabbit plasma EPI requires the use of a low concentration of TF, which would be neutralized by anti-TF IgG in a test sample, EPI levels were measured only in rabbits not given anti-TF antibodies.

Hematologic Tests

Packed red cell volume (PCV) was determined by centrifugation of blood in heparinized microcapillary tubes. White blood cells (WBCs) and platelets were counted in a hemocytometer.

Statistical Methods

Data at a single time point of greatest DIC-induced change in hemostatic factors were compared for different treatment groups. When data from two groups were compared, a two-tailed Student's \(t\) test was used. When data from three groups were compared, a single factor analysis of variance was followed by a Newman Keuls test.

Endotoxin Experiments

Effects of preparation with cortisone. The mean weight of the 14 rabbits given four daily 25-mg injections of cortisol acetate increased by 8% due to fluid retention. Mean hematocrit fell from 0.40 to 0.33. Mean plasma fibrinogen concentration and plasma EPI activity fell by approximately 15%, and before infusion of endotoxin were: fibrinogen, 200 mg/dL; EPI activity, 80% of the reference standard.

Appearance of animals. All animals given endotoxin became ill with no difference in appearance between rabbits given preimmune IgG and rabbits given anti-TF IgG. Within 1 hour the rabbits become tachypneic and cyanotic. Their ears were cold, and blood was difficult to obtain. Most rabbits had profuse diarrhea. One of 7 rabbits given preimmune IgG and 1 of 13 rabbits given anti-TF IgG died approximately 2 hours after the injection of endotoxin.

Fibrinogen, factor V, and factor VIII levels. Animals administered preimmune IgG and then endotoxin had clear-cut evidence of DIC, as manifested by a substantial fall in the levels of fibrinogen, factor V, and factor VIII. As judged by fibrinogen levels, clotting appeared to begin within 1 hour after the injection of endotoxin and continued for a total duration of at least 6 hours. At that time, the mean plasma fibrinogen level was about 50% of the value before endotoxin (Fig 1A). Factors V and VIII also fell during this period to a level of about 30% of the value before endotoxin (Fig 1 B and C).

DIC was attenuated in animals administered anti-TF IgG and then endotoxin. Mean fibrinogen levels fell only minimally (Fig 1A) to 93% of the value before injection. Mean
levels of factor V and of factor VIII fell to between 60% and 70% of the value before injection. Mean values for fibrinogen, factor V, and factor VIII at 6 hours after the injection of endotoxin were significantly higher in the animals administered anti-TF IgG than in the animals given preimmune IgG ($P < .005$).

Factor VII and factor X levels. Mean plasma factor VII concentration, as measured in the coupled amidolytic assay (Fig 2A), remained above 90% of the initial value during the 6-hour period after endotoxin injection both in the animals administered preimmune IgG and in the animals given anti-TF IgG. A later fall in mean factor VII concentration in the animals administered anti-TF IgG was observed (Fig 2A) and may reflect the more severe liver damage noted in these animals at autopsy (see below). Factor VII activity was also measured in a clotting assay and yielded no evidence for circulating activated factor VII; ie, VIIc/VIIam ratios were 1 or less (Table 3). Mean factor X levels (Fig 2B) fell to about 65% to 75% of pretreatment values in both the animals administered preimmune IgG and animals given anti-TF IgG.

EPI levels. Mean plasma EPI activity in the six rabbits receiving preimmune IgG and endotoxin fluctuated between 80% and 110% of the pretreatment level (Fig 3A) over the first 6 hours; ie, while the fibrinogen level was falling. Mean EPI activity 6 hours after endotoxin was 90% of the preinjection mean; individual values at 6 hours were 66% to 127% of preinjection values.

Hematologic values. WBC counts fell within 1 hour after endotoxin to about 500 per $\mu$L both in rabbits administered anti-TF IgG and in rabbits given preimmune IgG (Fig 4A). The WBC count remained markedly depressed for 6 hours and then rose to reach higher than normal levels within 24 hours. Platelet counts fell only modestly within the first hour, continued to decline for 8 to 12 hours, and remained depressed at 24 hours (Fig 4B). In contrast to the WBC count, the fall in platelet count was less pronounced in the
Table 3. Ratio of Factor VII Activity as Measured in the Clotting Assay (VIIc) to Factor VII Activity as Measured in the Coupled Amidolytic Assay (VIIam) During DIC Induced in the Different Treatment Groups

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>IgG</th>
<th>Experimental Time (h)</th>
<th>Mean VIIc/ Mean VIIam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone/endotoxin</td>
<td>Preimmune</td>
<td>1</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Anti-TF*</td>
<td>1</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
<td>1.0</td>
</tr>
<tr>
<td>TF infusion</td>
<td>None</td>
<td>1</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>0.88</td>
</tr>
<tr>
<td></td>
<td>Anti-TF†</td>
<td>1</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>1.3</td>
</tr>
<tr>
<td>TF in vitro‡</td>
<td>25 ng/mL</td>
<td>—</td>
<td>25.0-3.0</td>
</tr>
<tr>
<td></td>
<td>1 μg/mL</td>
<td>—</td>
<td>16.0</td>
</tr>
</tbody>
</table>

*Data from six animals.
†Data from three animals.
‡Rabbit plasma containing an equal volume of rabbit TF in the concentrations listed was recalcified and assayed for factor VII 1 hour after clotting.

animals administered anti-TF IgG. The mean platelet count at 8 hours after endotoxin was significantly higher for animals administered anti-TF IgG than for animals given preimmune IgG (P < .025).

The mean hematocrit before endotoxin was 0.33, which reflected expansion of plasma volume due to cortisone. Mean hematocrit values fell further after endotoxin and multiple blood sampling, and 24 hours after endotoxin they were: preimmune IgG animals, 0.27; anti-TF IgG animals, 0.24.

Pathologic findings. One rabbit receiving preimmune IgG and one rabbit receiving anti-TF IgG group died within 2 hours after the injection of endotoxin. Each animal had marked pulmonary edema and thrombi in pulmonary vessels. The remaining animals were killed 24 hours after the injection of endotoxin and, except for one animal in each group, were subjected to autopsy examination.

Gross evidence of organ damage was confined to the liver, which contained areas of focal hemorrhagic necrosis. These were noted in 1 of 5 animals administered preimmune IgG and 7 of 11 animals given anti-TF IgG. In two of the latter, areas of hemorrhagic necrosis occupied 70% to 80% of the liver surface. On microscopic examination, hemorrhage was present in an acinar pattern, suggestive of occlusion of presinusoidal vessels. However, PTAH stains failed to show deposits of fibrin in terminal branches of the portal vein or hepatic artery. There was no evidence of pre-existing infectious or inflammatory disease in the areas of hemorrhagic necrosis. In addition to the areas of hemorrhagic necrosis seen in some animals, prominent centrilobular hydropic degeneration of hepatocytes was present in all animals.

No animal had gross evidence of renal cortical necrosis. Histologic examinations of the kidneys were performed without knowledge of an animal’s treatment group. Four of five rabbits autopsied in the preimmune group had prominent deposits of fibrin within glomerular capillaries. These four animals also had histologic evidence of acute tubular necrosis. None of the 11 animals autopsied in the anti-TF IgG group had fibrin demonstrable by PTAH stain within glomerular capillaries. One animal had evidence of acute tubular necrosis.

Infusion of Tissue Factor

Appearance of animals. Six of 12 animals not administered anti-TF IgG and 3 of 9 animals given anti-TF IgG died during the infusion of TF; at autopsy, thrombi were found in the pulmonary arteries. Surviving animals did not appear ill.

Fibrinogen, factor V, and factor VIII levels. Mean fibrinogen levels fell during the 4 hours of infusion of TF: to 12% of the initial value in the animals not administered anti-TF IgG and to 44% of the initial value in the animals given anti-TF IgG (Fig 5A). This difference was significant (P < .025). However, the mean fibrinogen level of 44% at 4
Fig 4. WBC counts (A) and platelet counts (B) after endotoxin in animals administered either preimmune IgG or anti-TF IgG. Values shown are mean counts (± SEM) at each time point. Symbols are: (●), six rabbits administered preimmune IgG before endotoxin; (○), 12 rabbits administered anti-TF IgG before endotoxin.

Hours in animals administered anti-TF IgG also differed significantly from the mean fibrinogen level of 101% at 4 hours in the control animals infused with either phospholipid vesicles or saline (P < .005).

By the end of the infusion of TF, the mean factor V level had decreased to 12% of the initial value in animals not administered anti-TF IgG and to 42% in the animals given anti-TF IgG (Fig 5B). This difference was significant (P < .025). The mean factor VIII level had decreased to 18% in the animals not administered anti-TF IgG and to 35% in the animals given anti-TF IgG (Fig 5C). This difference was not significant (P > .05). In the animals administered anti-TF IgG, the mean factor V and VIII levels after the TF infusion were significantly lower than the mean factor V (80%) and mean factor VIII (90%) levels in the control animals infused with either phospholipid vesicles or saline (P < .005).

Factor VII and factor X levels. Factor VII levels, measured in both the coupled amidolytic and clotting assay, changed minimally, if at all, in the six animals infused with TF and not administered anti-TF IgG. For example, mean values at the end of the 4-hour infusion were: factor VIIa, 100%; factor VIIc, 88% of the initial value. Mean factor VIIc level in the animals receiving anti-TF IgG was 96% at the end of the 4-hour infusion. Factor VIIa levels were also measured in 3 of the 6 rabbits administered anti-TF IgG and were consistently lower than the initial value; eg, the mean
level at the end of the infusion was 70%. The reason is unknown but not a result of interference by anti-TF IgG with the assay (see Materials and Methods).

Mean factor X levels fell progressively for both groups of animals infused with TF: for the animals not administered anti-TF IgG to 77% and for the animals given anti-TF IgG to 71%. The mean value of control animals infused with phospholipid vesicles or saline also fell to 88% of the initial value. These mean values are not significantly different (P > .05).

**EPI levels.** Mean plasma EPI activity fell modestly during infusion of TF in the six rabbits not given anti-TF IgG (Fig 3B). The range of values in the sample drawn at the end of the TF infusion varied from 42% to 90% of the pretreatment value. Mean plasma EPI activity also appeared to fall slightly in three control animals infused with phospholipid vesicles not containing TF (Fig 3B). The mean value of 65% after infusion of TF did not differ statistically from the mean value of 84% after infusion of phospholipid vesicles not containing TF, possibly because of the small number of animals in each group. In three rabbits infused with saline (data not shown in Fig 3B for clarity), serial mean values of EPI levels did not differ significantly from the preinfusion mean.

**Hematologic values.** Mean WBC counts and hematocrit values did not change significantly during the infusion of TF or control materials. Mean platelet counts decreased during the 4-hour infusion with TF, to about one third of the initial value in animals not administered anti-TF IgG and to about two thirds of the initial value in animals given anti-TF IgG (Fig 6). The fall in platelet count in these two groups of animals differed significantly (P < .025). The fall in platelet count during TF infusion in animals administered anti-TF IgG differed significantly (P < .01) from the mean platelet count of controls, which did not decrease during the infusion.

**DISCUSSION**

The data presented provide direct in vivo evidence that endotoxin-induced intravascular coagulation stems primarily from exposure of the circulating blood to TF. In rabbits prepared with cortisone and administered preimmune goat IgG, a single IV injection of endotoxin resulted in the consumption, over the ensuing 6 hours, of at least 50% of the circulating fibrinogen, a decrease in plasma factor V and VIII levels and platelet counts to about one third of initial values, and deposition of fibrin within the glomerular capillary bed (generalized Shwartzman reaction). In rabbits prepared with cortisone and administered goat anti-rabbit TF IgG, a single IV injection of endotoxin resulted in only a minimal decrease in fibrinogen level, a significantly reduced decrease in factors V and VIII levels and platelet count, and no recognizable deposition of fibrin within the glomerular capillary bed.

Although the anti-TF IgG attenuated endotoxin-induced coagulation, it did not abolish the coagulation. One of the 13 rabbits administered anti-TF IgG died 2 hours after endotoxin and thrombi were found in pulmonary vessels. Mean factor V and VIII levels decreased to some extent (Fig 1, B and C), which is strong presumptive evidence that some thrombin was generated in all animals. Moreover, one should not discount the minimal decrease in mean fibrinogen levels in the animals administered anti-TF IgG (Fig 1A), since increased fibrinogen production after endotoxin has been shown to prevent plasma fibrinogen levels from falling, despite modestly increased fibrinogen consumption after endotoxin.23 The limited coagulation in the rabbits administered anti-TF IgG could reflect the inability of the anti-TF IgG to neutralize TF activity immediately or totally (Table 1). This explanation receives support from the experiments in which the anti-TF IgG reduced but could not prevent DIC in rabbits infused with exogenous TF (Fig 5). Nevertheless, the possibility cannot be excluded that initiating events other than exposure of blood to TF also contributed to the limited activation of coagulation after endotoxin observed in the animals administered anti-TF IgG.

Considerable evidence13,15 supports the hypothesis that monocytes activated by endotoxin provide the TF that functions as the trigger for endotoxin-induced intravascular coagulation. Although many investigators have confirmed the original observation1 that cultured human umbilical vein endothelial cells develop TF activity after incubation with endotoxin, Wilcox et al24 could not demonstrate synthesis of TF messenger RNA in endothelial cells in vivo in rats injected with endotoxin. It remains to be determined whether endotoxin, directly or indirectly, can induce the expression of TF activity on endothelial cells in vivo in the rabbit.

The experiments with infused TF provide perspective on the concentration of TF to which circulating blood was exposed in the rabbits administered endotoxin. If the orientation of TF apoprotein molecules in phospholipid vesicles is random,25 then the concentration of functional infused TF
was one half of the apparent concentration of the material in the infusion bag, or 275 to 300 pmol/L (about 12.5 ng/mL). The decrease in plasma fibrinogen level of animals infused with exogenous TF exceeded substantially the fall in plasma fibrinogen level of the animals injected with endotoxin (compare Figs 1A and 5A). In contrast, in a preliminary experiment in which two rabbits were infused with TF at a functional concentration of 3.1 ng/mL, plasma fibrinogen concentration decreased only minimally (data not shown). Consequently, we assumed that the circulating blood of rabbits administered endotoxin was exposed to the functional equivalent of a concentration of TF substantially lower than 275 to 300 pmol/L, but higher than 75 pmol/L.

If the factor VII concentration of rabbit plasma, like human plasma, is 10 nmol/L, and if the formation of factor VII(a)/TF complexes is stoichiometric, then at most only 3% of the circulating factor VII would have formed such complexes in the animals infused with TF, and less than 3% in the animals given endotoxin. This makes understandable our inability to measure significant changes in the plasma factor VII level during TF-induced DIC in either animals given endotoxin or animals infused with TF. These results add to the evidence that the low levels of plasma factor VII found in patients with meningococcemia cannot be accounted for by DIC secondary to endotoxemia.26

Moreover, elevated VIIc/VIIam ratios, indicative of the presence of circulating activated factor VII,22 could not be demonstrated either in the rabbits administered endotoxin or in the rabbits infused with TF (Table 3). This fits with an earlier in vitro observation in which activated factor VII was not demonstrable in serum from normal human plasma clotted with a low concentration of TF.27 Although normal human blood may possibly contain traces of factor VIIa,28 it appears increasingly evident that readily measurable amounts of free factor VIIa are not generated in plasma even after extensive TF-induced DIC.

The decrease after endotoxin in mean plasma factor X levels to 65% to 75% of initial mean values (Fig 2B) is presumed to reflect activation of factor X and its subsequent clearance from plasma. If, as all of our other data indicate, factor VIIa/TF is primarily responsible for initiating endotoxin-induced DIC, then it is difficult to understand why the animals administered anti-TF IgG and the animals given preimmune IgG had essentially equivalent falls in mean plasma factor X level at 6 hours after endotoxin.

Thrombocytopenia in rabbits administered endotoxin has been thought to stem primarily from platelet damage unrelated to intravascular coagulation and thrombin generation.29,30 However, the finding of significantly higher platelet counts (Fig 4B) in animals administered anti-TF before endotoxin suggests that thrombin-induced platelet activation also contributes to thrombocytopenia after endotoxin in rabbits. The decrease in platelets counts of animals infused with TF (Fig 6) supports this conclusion.

Because EPI is thought to be the primary inhibitor of factor VIIa/TF,14 it was of particular interest to measure serial plasma EPI levels in our experiments. In agreement with our earlier observations in patients with DIC,13 plasma EPI levels were not depleted under controlled experimental conditions in rabbits with DIC induced by either the injection of endotoxin or the infusion of TF (Fig 3). EPI is thought to inhibit factor VIIa/TF complexes stoichiometrically.14,15 Hence, it is not surprising that plasma EPI activity decreased only moderately, since only pico-molar concentrations of TF induced the continuing DIC observed in our experiments.

However, one may ask: why did DIC continue despite nearly normal levels of plasma EPI activity? We had previously observed a 2.5-minute lag in EPI-induced suppression of factor VIIa/TF-catalyzed activation of factor X, when human plasma containing a 5% dilution of a crude human TF preparation was recalcified in vitro.31 Such a lag could explain why fibrinogen continued to decrease throughout the 4-hour period of TF infusion in our rabbits; ie, why a steady supply of newly formed factor VIIa/TF complexes replacing factor VIIa/TF complexes inhibited by EPI/factor Xa could result in a continuing generation of small amounts of a low concentration of factor Xa sufficient to support continuing coagulation. This would also explain why plasma EPI failed to suppress continuing coagulation for up to 6 hours after endotoxin, if one assumes that new TF continued to be generated during this period on cells to which the circulating blood was exposed.

Strong suggestive evidence that intravascular coagulation and shock are independent manifestations of endotoxemia was summarized more than 20 years ago.32 However, a recent report that activated protein C prevents both the coagulopathic and lethal effects of E coli infusion in the baboon33 has raised anew the possibility of a link between endotoxin-induced intravascular coagulation and endotoxin-induced shock. Although our observations were limited to evaluating the appearance of the rabbits, they are pertinent to this issue. All animals administered endotoxin, including those animals in which anti-TF IgG had attenuated the intravascular coagulation, appeared ill and in some degree of shock. Because intact polyclonal anti-TF antibodies were used the possibility exists that the antibodies could have triggered complement-mediated cellular damage; eg, the areas of hemorrhagic necrosis of the liver that were found primarily in the animals administered anti-TF IgG. This might have contributed to the ill appearance of the anti-TF-treated animals given endotoxin. However, it is important to emphasize that whereas DIC induced by the infusion of TF caused some of the animals to die with pulmonary arterial thrombi, animals that survived appeared normal with no evidence of shock despite extensive DIC. Therefore, we believe that our experiments with endotoxin provide not only unequivocable evidence that exposure of blood to TF activity is the major cause for intravascular coagulation after endotoxin but also suggestive evidence that exposure of blood to TF activity plays a little, if any, role in the pathogenesis of shock after endotoxin.

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Disseminated intravascular coagulation in rabbits induced by administration of endotoxin or tissue factor: effect of anti-tissue factor antibodies and measurement of plasma extrinsic pathway inhibitor activity

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