Intravascular Clotting After Endotoxin in Rabbits With Impaired Intrinsic Clotting Produced by a Factor VIII Antibody

By S. M-C. Shen, S. I. Rapaport, and D. I. Feinstein

A rabbit model in which intrinsic clotting was selectively impaired by injection of a human factor VIII antibody was used to evaluate the mechanism of endotoxin-induced intravascular clotting in cortisone-treated rabbits. Three groups of animals were studied: a control group given factor VIII antibody followed by saline; a second control group given an inert material followed by endotoxin; and an experimental group given factor VIII antibody followed by endotoxin. The following parameters were measured: $^{125}$I-fibrinogen kinetics, fibrinogen levels, factor VIII, factor VII, factor V, WBC, platelets, and hematocrit. The kidneys were examined for deposition of fibrin. Mean values for factor VIII at the time of injection of the second test material and mean values for fibrinogen consumed in the 6 hr after the second injection were as follows: antibody-saline group, 8.5% and 11.0 mg/kg; control material-endotoxin group, 90% and 29.6 mg/kg; and antibody-endotoxin group, 7.0% and 32.7 mg/kg. Factor V, factor VII, granulocytes, and platelets fell in both groups of animals given endotoxin. One animal in each group given endotoxin developed gross renal cortical necrosis. These data establish that selective impairment of the intrinsic clotting reactions does not reduce the amount of clotting induced by a single injection of endotoxin in the cortisone-treated rabbit.

In a preceding paper, we described experiments in which the injection of a human factor VIII antibody preparation into rabbits resulted in a marked fall in the animal's plasma factor VIII activity for 12 to 24 hr. Injection of the antibody did not alter fibrinogen kinetics significantly, and adequate levels of WBC, platelets, and complement were found 6 hr after the injection. Consequently, we concluded that injection of a human factor VIII antibody could be used to produce a rabbit model suitable to test the hypothesis that selective impairment of the intrinsic clotting reactions will not reduce the amount of intravascular clotting induced by an injection of endotoxin. The results of experiments designed to test this hypothesis are described herein.

MATERIALS AND METHODS

New Zealand male rabbits, weighing 2.2 to 2.9 kg, were used. They were fed and given drinking water containing KI and NaCl as described earlier. Endotoxin was Bacto Lipopolysaccharide B Escherichia coli 0111B4 (Difco Laboratories, Detroit, Mich.). It was dissolved in a concentration of 100 µg/ml in isotonic saline just prior to injection. Animals were given 100 µg
of endotoxin per kg. Cortisone was a sterile suspension containing 25 mg of cortisone acetate per ml (Towne Paulson and Co., Monrovia, Calif.). The following materials were prepared as described earlier: factor VIII antibody fraction, control immunoglobulin fraction from normal plasma, 125I-rabbit fibrinogen, and pooled rabbit plasma reference standard. The following procedures were carried out also as described earlier: blood sampling, platelet counts, clotting factor assays, determination of radioactivity in plasma and urine, and calculation of plasma volume.

Experimental Design

Since administration of cortisone may be substituted for the first dose of endotoxin in preparing rabbits for the generalized Shwartzman reaction, we elected to evaluate the extent of clotting induced by a single injection of endotoxin in cortisone-prepared animals. Rabbits were given 25 mg of cortisone acetate intramuscularly daily for 4 days. On the second day each rabbit received 29.45 μCi of 125I-fibrinogen intravenously. The animals were divided into two control groups and an experimental group (see Table 1). Animals in each group received two injections of test materials, the first given 48 hr after the labeled fibrinogen and the second given 6 hr after injection of the first test material. Blood samples were drawn at the following times: 5 min and 30 hr after injecting 125I-fibrinogen; just before the injection of the first test material; just before the injection of the second test material, and 3, 6, 24, and 42 hr after the injection of the second test material. Animals were autopsied and the kidneys examined grossly and microscopically (Fraser-Lendrum stain) for fibrin.

RESULTS

Factor VIII Activity

The factor VIII level for each animal in each group at the time of injection of the second test material is listed in Table 2. Mean factor VIII levels for each group at this time were as follows: antibody–saline group, 8.5%; control materials–endotoxin group, 90%; and antibody–endotoxin group, 7.0%. Six hours after injection of the second test material the mean values were as follows: antibody–saline group, 10%; control materials–endotoxin group, 51%; and antibody–endotoxin group, 6%. The fall in mean factor VIII activity from 90% to 51% in the control materials–endotoxin group presumably resulted from endotoxin-induced clotting. The small difference at 6 hr between the mean of 10% for the antibody–saline group and the mean of 6% for the antibody–endotoxin group could also reflect an effect of endotoxin-induced clotting upon the small amount of factor VIII activity persisting after injection of antibody.

Fibrinogen Levels

Mean plasma fibrinogen levels fell during the 6 hr after injection of the second test material in the control materials–endotoxin group and in the antibody–endotoxin group, whereas mean fibrinogen levels rose during the same
Table 2. Factor VIII Level and Fibrinogen Consumption of Individual Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Animal No</th>
<th>Factor VIII Level (%)</th>
<th>Fibrinogen Consumption (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antibody-</td>
<td>1</td>
<td>6.5</td>
<td>8.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>6.5</td>
<td>7.1</td>
</tr>
<tr>
<td>Saline</td>
<td>3</td>
<td>10.0</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11.0</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.5</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>9.0</td>
<td>12.1</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>8.5 ± 0.8</td>
<td>11.0 ± 1.4</td>
</tr>
<tr>
<td>± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>90</td>
<td>21.6</td>
</tr>
<tr>
<td>Material-</td>
<td>2</td>
<td>88</td>
<td>32.4</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>3</td>
<td>96</td>
<td>45.6§</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>88</td>
<td>22.5</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>115</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>64</td>
<td>27.7</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>90 ± 6.7</td>
<td>29.6 ± 3.6</td>
</tr>
<tr>
<td>± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Antibody-</td>
<td>1</td>
<td>10.5</td>
<td>25.6</td>
</tr>
<tr>
<td>Endotoxin</td>
<td>2</td>
<td>8.5</td>
<td>37.4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.0</td>
<td>12.3</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.5</td>
<td>23.0</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>5.5</td>
<td>73.9§</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>6.0</td>
<td>23.8</td>
</tr>
<tr>
<td>Mean</td>
<td></td>
<td>7.0 ± 0.8</td>
<td>32.7 ± 8.9</td>
</tr>
<tr>
<td>± SE</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Level at the time of injection of the second test material expressed as per cent of a reference standard made by pooling plasma from five normal rabbits.
† Mg/kg body weight of fibrinogen consumed during the 6 hr after injection of the second test material.
‡ Animals 1-3 in this group received saline as the control material; animals 4-6 received immunoglobulin as the control material.
§ Gross renal cortical necrosis.

period in the antibody-saline group (see Fig. 1). However, because of the small number of animals and large values for SE, the fall in fibrinogen level was not statistically significant for either group receiving endotoxin (p > 0.10).

**Fibrinogen Radioactivity**

Plasma fibrinogen radioactivity, plasma nonclottable protein radioactivity, total body pool of $^{125}$I not bound to protein, and urinary excretion of radioactivity were determined for each animal in each group. Mean values are plotted in Figs. 2-4. As expected, injection of saline into animals previously treated with factor VIII antibody did not affect the disappearance of plasma $^{125}$I-fibrinogen (Fig. 2). In contrast, the injection of endotoxin into animals previously injected with either a control material or factor VIII antibody (Figs. 3 and 4) resulted in an excess disappearance of plasma $^{125}$I-fibrinogen over the 6-hr period after injection of the endotoxin. Plasma nonclottable protein radioactivity was low, below 1.5% of the injected dose, at the time of injection of the second test material and did not increase after the injection of endotoxin.
Fig. 1. The effect of injection of test materials upon mean fibrinogen levels. In this and subsequent figures the first arrow indicates the time of injection of the first test material: either factor VIII antibody or control material. The second arrow indicates the time of injection of the second test material: either endotoxin or normal saline. In all figures, values represent means for six animals in each group. Brackets delineate SE.

Fig. 2. Mean values for parameters of $^{125}$I-fibrinogen catabolism in the antibody-saline group. The solid line identified by the symbol $q(t)$ is the terminal portion of the mean plasma fibrinogen radioactivity curve. The line identified by the symbol $1-u(t)$ represents the mean difference between the injected dose of radioactivity (which has been called $I$) and the cumulative urinary excretion of radioactivity at time $t$. The line identified by the symbol $y(t)$ is mean radioactivity of nonclottable plasma proteins. The line identified by the symbol $z(t)$ represents mean total body $^{125}$I not bound to protein.
Excretion of radioactivity in the urine decreased during the 24 hr after injection of endotoxin, during which time the animals looked ill and their urine volumes were reduced. Reduced urinary excretion of radioactivity was associated, in turn, with a sharp increase in calculated radioactivity in the total body pool of \(^{125}\)I not bound to protein. In both groups of animals given endotoxin the sum of the mean value for urinary excretion of radioactivity and for increase in total body \(^{125}\)I not bound to protein approached the mean value for loss of plasma...
fibrinogen radioactivity. For example, mean loss of plasma fibrinogen radioactivity in the antibody-endotoxin group during the 30 hr following the injection of antibody equalled 22.6% of the injected dose, whereas the mean value for urinary excretion during this period was 12%, and the mean value for increase in total nonprotein bound-iodide was 7.3%.

The amount of fibrinogen disappearing from the plasma during the 6 hr after injection of the second test material was calculated from the following formula:

\[ F/kg = \frac{V/kg \times (c_1 + c_2)}{2} \times \frac{q_1 - q_2}{q_1} \]

where \( F/kg \) equals mg of fibrinogen per kg body weight disappearing during this 6 hr period, \( V/kg \) equals the plasma volume (ml) per kg body weight, \( c_1 \) and \( c_2 \) equal the plasma fibrinogen concentration (mg/ml) at the time of the second injection and at 6 hr after the injection, and \( q_1 \) and \( q_2 \) equal plasma fibrinogen radioactivity (per cent injected dose) at the time of the second injection and at 6 hr after the injection.

The value obtained from this calculation for each animal in each group is listed in Table 2. Mean values for each group were as follows: antibody-saline group, 11.0 ± 1.4 mg/kg (SE); control materials–endotoxin group, 29.6 ± 3.6 mg/kg; and antibody-endotoxin group, 32.7 ± 8.9 mg/kg. The difference between the mean value for the group receiving saline as the second material and the mean value for each of the groups receiving endotoxin as the second material was statistically significant (\( p < 0.05 \)), i.e., excess plasma fibrinogen beyond that expected from normal catabolism was lost in each group given endotoxin.

Factor V and Factor VII Activity

Mean values for factor V and for factor VII in each group are listed in Table 3. Note first that, as in our initial study, injection of factor VIII antibody resulted in slight falls in factor V activity. After injection of the second test material, factor V activity did not fall further in the antibody–saline group but did fall further in the antibody–endotoxin group. Factor V levels also fell after the injection of endotoxin in the control materials–endotoxin group.

Factor VII activity fell slightly in both groups of animals given factor VIII antibody, but the decrease in activity following injection of the antibody was not statistically significant for either group. After injection of the second test material the factor VII level rose in the antibody–saline group, whereas the factor VII level fell further in the antibody–endotoxin group. The factor VII level also fell in the control materials–endotoxin group after the injection of endotoxin.

The falls in factor V and VII levels after endotoxin were statistically significant for both the control materials–endotoxin group and the antibody–endotoxin group, but for the latter only when the data were first corrected for change in hematocrit and anticoagulant dilution and expressed as percent of the value at the time of injection of endotoxin (\( p < 0.02 \)).

Platelets

Mean platelet counts for the three groups of animals are plotted in Fig. 5. Despite some reduction in the count attributable to injection of factor VIII anti-
Table 3. Effect of Injection of Test Materials Upon Mean Factor V and Mean Factor VII Levels

<table>
<thead>
<tr>
<th>Factor</th>
<th>Measured Value</th>
<th>Corrected Value</th>
<th>Measured Value</th>
<th>Corrected Value</th>
<th>Measured Value</th>
<th>Corrected Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Antibody-Saline Group</td>
<td>Antibody-Endotoxin Group</td>
<td>Control Mat-Endotoxin Group</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hr After Second Material</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>6: 166 ± 16.5</td>
<td>145 ± 8.0</td>
<td>131 ± 10.5</td>
<td>0: 111 ± 8.0</td>
<td>107 ± 6.5</td>
<td>136 ± 16.5</td>
</tr>
<tr>
<td>VII</td>
<td>6: 163 ± 13.5</td>
<td>141 ± 15.0</td>
<td>147 ± 8.0</td>
<td>0: 138 ± 10.5</td>
<td>115 ± 13.5</td>
<td>155 ± 13.5</td>
</tr>
</tbody>
</table>

* Measured value is percent of standard rabbit reference plasma. Corrected value is corrected for anticoagulant dilution and hematocrit change and expressed as percent of the value at the time of injection of the second material.

† Time of injection of second test material.
body, adequate numbers of platelets were present in all animals at the time of injection of the second test material. Platelet counts fell after injection of endotoxin in both the antibody-endotoxin and control materials-endotoxin groups. Platelet counts also fell after the injection of saline in the antibody-saline group, presumably reflecting a delayed effect of factor VIII antibody upon the platelet count as observed in our earlier study.1

**Granulocytes**

Normal or increased numbers of granulocytes were present in all animals at the time of injection of the second test material (see Fig. 6). Injection of endotoxin produced a striking granulocytopenia at 3 hr in the control materials-endotoxin group. In contrast, injection of endotoxin produced only a slight granulocytopenia at 3 hr in the antibody-endotoxin group. The animals in the antibody-saline group had markedly elevated granulocyte counts 3 hr after injection of the saline, presumably due to a delayed effect of the antibody.1 The difference between the granulocyte counts at 3 hr in the control materials-endotoxin group and in the antibody-endotoxin group probably stemmed from increased release of granulocytes into the circulation due to antibody in the latter group.

**Hematocrit**

Mean hematocrit values for all three groups of animals were between 29% and 30%, just before injection of the second test material. These low values probably reflect the effects of two processes—an increase of plasma volume secondary to cortisone-induced fluid retention3 and blood loss from prior sampling. Mean hematocrit values 6 hr after injection of the second test mate-
rial were as follows: antibody saline group, 29°ⁿ; control materials endotoxin group, 26°ⁿ; and antibody endotoxin group, 29°ⁿ.

Pathologic Findings

One animal in the control materials endotoxin group and one animal in the antibody endotoxin group developed gross bilateral renal cortical necrosis (see Fig. 7) associated with microscopic evidence of deposition of fibrin throughout the glomerular capillaries. The animal in the control group had a calculated fibrinogen consumption after endotoxin of 45.6 mg/kg (mean for the group, 29.6 mg/kg); the animal in the antibody group had a calculated fibrinogen consumption after endotoxin of 73.9 mg/kg (mean for the group, 32.7 mg/kg). The factor VIII levels at the time of injection of endotoxin for the two animals developing renal cortical necrosis were as follows: for the control animal, 96°ⁿ; for the antibody-treated animal, 5.5°ⁿ.

Fig. 6. Effect of injection of test materials upon mean granulocyte counts.

Fig. 7. Gross appearance of the kidney of an animal in the antibody-endotoxin group which developed renal cortical necrosis.
None of the other animals given endotoxin had definite histologic evidence of fibrin deposition in the glomerular capillaries.

DISCUSSION

The data reported herein confirm earlier observations from this laboratory that a single injection of endotoxin in cortisone-treated rabbits produces significant excess disappearance of intravascular radioactive fibrinogen over the ensuing 6 hr. The reasons for accepting that this excess disappearance of radioactive fibrinogen stems from endotoxin-induced intravascular clotting may be summarized as follows:

1. It is associated with a decrease in platelets, factor V and factor VIII as expected with intravascular clotting.

2. It is reduced by treatment of animals with warfarin.

3. It is not associated with an increase in nonclottable protein radioactivity (see Figs. 3 and 4) as should occur with consumption due to activation of plasmin and formation of nonclottable degradation products.

4. The greatest excess consumption of intravascular fibrinogen was found in the present experiments in two rabbits with diffuse deposits of fibrin in the glomerular capillaries and gross renal cortical necrosis.

Consequently, we believe that the amount of intravascular fibrinogen disappearing in the 6 hr following an injection of endotoxin provides a valid estimate of the degree of intravascular clotting induced by endotoxin.

It is not known whether this clotting proceeds primarily by way of the intrinsic clotting reactions, primarily by way of the extrinsic clotting reactions, or equally by way of both reactions. Two kinds of data have been interpreted as evidence for intrinsic clotting: the reported prevention of the generalized Shwartzman reaction after endotoxin by thrombocytopenia and the fall in plasma factor XII activity observed after the injection of endotoxin. However, neither of these observations represent clear-cut evidence that endotoxin triggers clotting primarily by way of the intrinsic pathway. Thus, thrombocytes could be needed for the generalized Shwartzman reaction, not to generate thrombin in intrinsic clotting, but to provide cationic proteins (platelet factor 4 activity) which facilitate precipitation of fibrin monomer in the glomerular capillary bed. Treatment of rabbits with a vitamin K antagonist prevents the fall in factor XII level after administration of endotoxin. Yet vitamin K antagonists should not impede direct activation by endotoxin of factor XII and its subsequent clearance. Consequently, one has difficulty in accepting activation of factor XII in intrinsic clotting as the reason for the fall in factor XII activity after administration of endotoxin to rabbits not treated with vitamin K antagonists. Moreover, inhibition of activation of factor XII by infusion of lysozyme failed to prevent the generalized Shwartzman reaction after endotoxin in Thorotrast-treated rabbits.

Two kinds of data support the hypothesis that endotoxin triggers extrinsic clotting. First, endotoxin has been shown to damage vascular endothelium, and this damage could result in the exposure of blood to tissue materials with thromboplastic activity. Second, granulocytes are required for endotoxin to initiate intravascular clotting. Granulocytes possess tissue thromboplastic
activity \(^{13-15}\) but do not possess a demonstrable activity initiating intrinsic clotting. \(^{13}\) However, an abstract has recently been published \(^{16}\) which mitigates against a primary role for the extrinsic pathway in endotoxin-induced clotting. In this abstract Garner and Evensen report that fibrinogen levels fall after administration of endotoxin to dogs with hereditary factor VII deficiency.

The data reported herein establish that marked reduction of factor VIII activity in the rabbit failed to prevent excess disappearance of intravascular fibrinogen after administration of endotoxin. Just before the injection of endotoxin, the mean factor VIII level in the control materials-endotoxin animals was 90\%, whereas the mean factor VIII level in the antibody-endotoxin animals was only 7\% of a pooled rabbit plasma reference standard. Yet, the mean values for consumption of intravascular fibrinogen in the 6 hr after injection of endotoxin were very similar for the two groups: 29.6 mg/kg for the control materials-endotoxin animals and 32.7 mg/kg for the antibody-endotoxin animals. From knowledge of levels of factor VIII activity in patients with hemophilia A and from the increased bleeding tendency we observed during blood sampling from animals given antibody, one may reasonably conclude that a decrease in factor VIII level to 7\% of normal in the rabbit significantly impedes the animal’s intrinsic clotting reactions. If endotoxin triggered clotting primarily by way of the intrinsic pathway, then a mean value for intravascular fibrinogen consumption in the antibody-endotoxin group should have been found which was, at the most, intermediate between the mean value of 11.0 mg/kg for the antibody-saline group and the mean value of 29.6 mg/kg for the control materials-endotoxin group. Interestingly, the greatest fibrinogen consumption after endotoxin in any rabbit (73.9 mg/kg) occurred in a rabbit with a factor VIII level of 5.5\%. This rabbit developed gross renal cortical necrosis (see Fig. 7).

In calculating fibrinogen consumption after injection of the second test material, we used values for plasma volume which were obtained earlier in the experiment at the time of the injection of the \(^{125}\)I-fibrinogen. Thus, the possibility arises that the values for fibrinogen consumption in both the control materials-endotoxin group and the antibody-endotoxin group contain an error due to change in plasma volume after administration of endotoxin. However, the hemodynamic effect of endotoxin should have been similar in the two groups of animals given endotoxin. Consequently, the lack of a significant difference between the calculated values for fibrinogen consumption in the control materials-endotoxin group and the antibody-endotoxin group leads us to conclude that intravascular clotting after endotoxin does not require an intact intrinsic clotting mechanism.

Since endotoxin damages platelets, granulocytes, and vascular endothelium, multiple possible mechanisms exist whereby endotoxin could trigger intravascular coagulation. Because anticoagulation with either heparin or a vitamin K antagonist prevents the generalized Shwartzman reaction after endotoxin, one assumes that simultaneous impairment of both the intrinsic and extrinsic clotting reactions prevents the intravascular coagulation. The preliminary report of Garner and Evensen \(^{16}\) suggests that the selective impairment of extrinsic clotting imposed by hereditary factor VII deficiency fails to prevent intravascular
clotting after endotoxin in the dog. The data from our experiments indicate that the selective impairment of intrinsic clotting imposed by injection of a factor VIII antibody fails to prevent intravascular clotting after endotoxin in the rabbit. Thus, one is tempted to postulate that endotoxin triggers clotting by way of both the extrinsic and intrinsic clotting reactions and that selective impairment of either pathway alone will not prevent the resultant excess consumption of plasma fibrinogen.

ACKNOWLEDGMENT

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