Stasis Thrombi Induced by Bacterial Endotoxin

By Duncan P. Thomas, M.D., Ph.D., and Stanford Wessler, M.D.

Previous studies in this laboratory have indicated that a combination of systemic hypercoagulability and retarded blood flow is capable of producing a red thrombus in the veins of animals.1 The hypothesis has been proposed that activation of the procoagulants involved in the formation of intrinsic thromboplastin produces massive thrombosis in large vessels in areas of markedly reduced blood flow.2 The purpose of this report is to provide evidence that single infusions of bacterial endotoxin are capable of inducing stasis thrombi in rabbits. Furthermore, data will be presented suggesting that this thrombogenic effect of endotoxin may be mediated in part through its action on a blood coagulation factor, plasma thromboplastin antecedent (Factor XI).

Methods

Glassware and needles were siliconized according to technics previously described.3

BLOOD SAMPLES

*Intact* plasma and serum refer to blood samples collected and subsequently handled so as to be exposed solely to silicone-coated surfaces. Plasma was prepared by the addition of 1 volume of 3.8% sodium citrate to 9 volumes of blood. Platelet-poor plasma was obtained by centrifuging the blood at 2500 × g at 4°C for 20 minutes, and the supernatant plasma separated (less than 20,000 platelets per mm3). Platelet-rich plasma was obtained by centrifuging at 160 × g for five minutes, and the supernatant plasma separated (approximately 1,000,000 platelets per mm3). Blood collected for serum was placed in silicone-treated glassware for 2 hours at room temperature, then stored for 18 hours at 4°C prior to centrifugation.3

THROMBOSIS

Thrombus formation was measured in white New Zealand male rabbits by a standard technic.4 Infusates diluted with isotonic saline to a constant volume were injected during a 15-second interval into a marginal ear vein. Within 15 seconds after completion of the infusion, previously freed jugular vein segments, one on each side of the neck, were isolated with silk ligatures. Only one infusate was given to each animal. Ten and 15 minutes after vein isolation, the segments were opened and examined for the presence of thrombi. Thrombus formation was measured in hens by a similar technic, except that injections were made into a brachial vein, and thrombosis was observed in the contralateral isolated brachial veins. Thrombi were scored on a scale of one to four with a score of one representing a few small strands of fibrin; a score of two, several small thrombi; a score of three, two or more large thrombi; and a score of four representing a single large thrombus, forming an entire cast of the vessel lumen.4

ENDOTOXIN

Two different, partially purified endotoxins, suspended in isotonic saline, were employed. Endotoxin derived from *Escherichia coli* 0111:B4 was generously supplied to us by Dr. Herbert A. Ravin,3 and an endotoxin prepared from *Salmonella newington* was kindly provided by Dr. P. W. Robbins.6

IN VITRO CLOTTING TESTS

Modified Lee-White clotting times7 were performed at 37°C in new, uncoated, and in new, silicone-coated, 13 × 100 mm glass tubes each containing 2 ml of whole blood. A two-tube technic was used, and the end point taken when the blood failed to flow on complete inversion of the second tube. The first tube was examined at one minute intervals and the second tube at 30 second intervals after the blood in the first tube had clotted. Clotting times were recorded to the nearest-minute.

* The word thrombus is used to denote an in vivo clot. "Thrombus — a fibrinous clot which forms in a blood vessel." Oxford Universal Dictionary, 1955.

† Purification technics are described in references 5 and 6.

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STASIS THROMBI AND BACTERIAL ENDOTOXIN

est half minute. In human subjects whole blood was obtained from a forearm vein; in rabbits, whole blood was collected from the inferior vena cava. No more than two venipunctures were performed on any one rabbit. The postinfusion sample was always obtained within 30 seconds following an infusion, from a site distal to that of the initial venipuncture.

Recalification times were determined in siliconed glass tubes measuring 10 x 75 mm. The incubation mixture for recalification times (0.2 ml of platelet-poor or platelet-rich intact plasma, 0.2 ml of dilution fluid II,* and 0.1 ml of isotonic saline with or without 10 μg E. coli endotoxin) was heated to 37°C for six minutes. 0.2 ml of 0.03 molar calcium chloride was then added and the time measured to the formation of fibrin threads.

Results

NATURALLY-OCcurring HYPERCOAGULABILITY IN RABBITS

We have previously observed in 100 normal rabbits that the control glass clotting time is 12 minutes, with a range of 9 to 14.5 minutes, whereas the control silicone clotting time is 37.5 minutes with a range of 22 to 52 minutes. Subsequent to these determinations, 10 rabbits with diarrhea and hyperemic intestines, presumably due to an enteric infection in the rabbit colony, were found to have markedly shortened clotting times, with a loss of the normal glass-silicone differential. In these animals the control clotting times in glass and silicone tubes were identical, with a mean of 8.0 minutes in glass and silicone (range 5 to 13 minutes). These rabbits also showed an abnormal response to the injection of intact serum. Infusion of such sera, at a dose of 1.32 ml/kg, into three of these rabbits with shortened clotting times produced thrombi after 10 minutes of stasis. In contrast, injections of samples of the same intact serum into six normal rabbits failed to produce stasis thrombi, an observation previously reported from this laboratory. These data were interpreted to mean that the rabbits with diarrhea and shortened clotting times were in a hypercoagulable state. This hypercoagulability was demonstrated by the fact that thrombosis resulted from the infusion of intact serum, which is nonthrombogenic in normal rabbits.

ENDOTOXIN-INDUCED HYPERCOAGULABILITY IN RABBITS

To determine if shortened clotting times could be produced in normal rabbits by infusions of bacterial endotoxin, E. coli endotoxin was infused into seven such animals with control silicone clotting times over 30 minutes. Blood withdrawn 30 seconds after completion of the infusion of endotoxin had a considerably shortened clotting time. The mean control silicone clotting time in these animals was reduced from 35.5 to 13 minutes, with a range of 8 to 16 minutes (table 1). It was concluded that the intravenous infusion of endotoxin was capable of rapidly reducing the silicone clotting time of rabbit blood to the range normally found in glass tubes. It has been shown previously that such reductions do not occur with the infusion of isotonic saline or 7% human albumin.

To determine whether the endotoxin-treated rabbits were in a hypercoagulable state, E. coli endotoxin was injected into 19 rabbits in doses of 0.25 and 0.75 mg/kg (table 2). After 10 minutes of stasis 12 rabbits showed no thrombi, while 7 showed partial thrombi (1 to 2+). In contrast, after 15 minutes of stasis, 5 of the 19 animals had no thrombi, 6 had partial thrombi (1 to 3+), while in 8 rabbits, the thrombi formed complete casts of

TABLE 1

Effect on Rabbit Silicone Clotting Times of Intravenous Injection of 0.75 mg/kg of E. Coli Endotoxin

<table>
<thead>
<tr>
<th>Control silicone clotting time (minutes)</th>
<th>Postinfusion silicone clotting time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32.0</td>
<td>12.0</td>
</tr>
<tr>
<td>32.5</td>
<td>8.0</td>
</tr>
<tr>
<td>39.0</td>
<td>12.0</td>
</tr>
<tr>
<td>40.0</td>
<td>16.0</td>
</tr>
<tr>
<td>41.0</td>
<td>15.0</td>
</tr>
<tr>
<td>32.0</td>
<td>13.0</td>
</tr>
<tr>
<td>39.0</td>
<td>12.0</td>
</tr>
<tr>
<td>35.0</td>
<td>12.0</td>
</tr>
</tbody>
</table>

* Dilution fluid II contains 200 ml of veronal buffer, 200 ml of 25.66 mm sodium citrate dihydrate solution, and 600 ml 0.85% sodium chloride solution.

Circulation Research, Volume XIV, June 1964
TABLE 2
Effect of Varying Dose of E. Coli Endotoxin and Length of Stasis on Production of Stasis Thrombi in Nineteen Rabbits

<table>
<thead>
<tr>
<th>Dose of endotoxin (mg/kg)</th>
<th>Stasis thrombi* (10 minutes)</th>
<th>Stasis thrombi* (15 minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.50</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0.75</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0.75</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>1.00</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>2.00</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

* Scale of thrombosis = 0 to 4.

Effect of Varying Dose of Endotoxin and Stasis Duration on Production of Stasis Thrombi

It is apparent that under the conditions of the experiment the duration of stasis was more important than the dose of endotoxin in determining the extent of the thrombotic response. However, considerable rabbit to rabbit variation occurred with the same dose of endotoxin and the same duration of stasis. When the control silicone clotting times, instead of the dose of endotoxin, were compared with the degree of thrombosis, a positive correlation was noted (table 3). In those 10 rabbits with control silicone clotting times greater than 33 minutes, no animal demonstrated any thrombosis after 10 minutes of stasis. On the other hand, in 11 rabbits with clotting times below 34 minutes, only 3 rabbits did not have some thrombosis after 10 minutes of stasis.

In 15 additional rabbits in which Salmonella endotoxin was infused, the extent of thrombosis was again influenced more by the control silicone clotting time and the duration of stasis, than by the dose of endotoxin (table 4). It was concluded that rabbits with prolonged silicone clotting times were relatively resistant to the thrombogenic effect of endotoxin. Conversely, shortened silicone clotting times were associated with an increased...
tendency to thrombosis. Within the dose range studied, no consistent correlation could be determined between the dose of endotoxin and the extent of thrombosis.

**EXPLORATORY STUDIES OF EFFECT OF ENDOTOXIN ON HUMAN CLOTTING FACTORS**

Recalcification times with and without *E. coli* endotoxin were performed on the platelet-poor plasma of ten normal subjects, as well as on plasma of subjects with congenital deficiencies of Factor XII (Hageman), XI (PTA), and IX (PTC) (table 5a). The data indicate that 10 μg of endotoxin shortened the recalcification times of all plasma,
TABLE 6

<table>
<thead>
<tr>
<th>Control clotting time</th>
<th>Postheparin clotting time</th>
<th>Thrombosis After 15' stasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glass</td>
<td>Silicone</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>29.5</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>24</td>
<td></td>
</tr>
<tr>
<td>10.5</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>12.5</td>
<td>27.5</td>
<td></td>
</tr>
<tr>
<td>11.5</td>
<td>27</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>27</td>
<td></td>
</tr>
</tbody>
</table>

Mean: 11 (10.5 to 12.5) 26 (21 to 29.5) 14 (11 to 16.5) 43.5 (25 to 65)

with the exception of plasma obtained from patients deficient in Factor XI. The degree of shortening ranged between 10 and 21%, and varied from subject to subject, and in the same subject on different days (subjects 1 and 2, table 5a). In platelet-poor Factor XI-deficient plasma there was, in fact, a 7 to 10% lengthening of the recalcification time. When, however, in two Factor XI-deficient patients (no. 2 and 3) concurrent recalcification times were measured using platelet-rich plasma, it was found that endotoxin now produced a shortening of the recalcification time (table 5b).

EFFECT OF HEPARIN ON ENDOTOXIN-INDUCED STASIS THROMBI

Ten units of heparin * per kilogram injected intravenously into six rabbits prolonged the silicone clotting time, although the glass time was not significantly altered. Twenty μg/kg of Salmonella endotoxin were then injected into each of these rabbits and neck vein segments isolated. After 15 minutes of stasis, the vein segments were opened and examined for the presence of thrombus formation. In none of the animals did thrombosis occur (table 6), despite control silicone clotting times that were all in a range in which endotoxin produced thrombosis after 15 minutes' stasis, in the absence of heparin (table 4). It was concluded that a small dose of heparin, insufficient to prolong markedly the glass clotting time, was capable of preventing the thrombogenic effect of Salmonella endotoxin.

EFFECT OF E. COLI ENDOTOXIN IN HENS

E. coli endotoxin was given intravenously to eight hens in doses of 0.25 to 2.0 mg/kg and segments of the brachial veins isolated for 10, 15, and 20 minutes following injection. Thrombosis did not occur in any of the venous segments, and it was concluded that E. coli endotoxin was incapable of producing stasis thrombi in hens in the dose range employed.

Discussion

The observation that prompted this study was the finding of abnormally short control silicone clotting times in several rabbits with diarrhea. Correlated with this observation was the additional finding that intact serum, not thrombogenic in normal rabbits, was capable of producing stasis thrombi in such animals. We have previously suggested that an increased tendency to thrombus formation, coupled with silicone clotting times in the glass range, represents a hypercoagulable state. The finding of diarrhea and hyperemic intestines suggested the possibility that these rabbits were suffering from a naturally acquired enteritis, which was associated with abnormally shortened clotting times. Evans et al. have described a disease of young rabbits characterized by the frequent association of enteritis and polyagglutinable red cells. They did not, however, implicate endotoxin in this condition. Diarrhea in rabbits may, however, be related to the hemorrhagic enteritis syndrome in mice, which Mims has suggested may be mediated through an endotoxin.

* 'Lipo-hepin', kindly supplied by Riker Laboratories, Inc., Northridge, California.
The length of time required to form a venous thrombus in rabbits following endotoxin injection appears to be related in part to the control silicone clotting time. The shorter the clotting time, the shorter was the stasis time required to produce a thrombus. In contrast, rabbits with clotting times in excess of 33 minutes (table 3) were relatively resistant to endotoxin-induced thrombosis. In the dose range studied, the thrombogenic effect appeared to be independent of the dose of endotoxin. It is possible that in these rabbits with shortened control silicone clotting times, endotoxin had already entered their systemic circulation. Ravin et al. 5 have presented evidence for a continuous, although not necessarily constant, absorption of endotoxin from the gastrointestinal tract of the normal rabbit. The considerable variation in control silicone clotting times of apparently normal rabbits may thus be attributable to a varying degree of absorption of endotoxin into their systemic circulation, and which in turn affects their response to intravenously injected endotoxin. Specific data are required to demonstrate that endotoxins absorbed into the systemic circulation from the gut are responsible for the observed variations in the silicone clotting time. There seems little doubt, however, that endotoxin has the potential to produce such variations. The present study has demonstrated that entry of endotoxin into the systemic circulation induces a demonstrable hypercoagulable state in rabbits.

The site of action of endotoxin on the coagulation mechanism has been studied by several investigators. McKay et al. 12 demonstrated that endotoxin accelerated coagulation of the blood independently of leukocytes and red blood cells. In one subject, they showed that endotoxin shortened the clotting time of native, platelet-poor plasma. Although McKay et al. did not study the immediate effect of endotoxin on the silicone clotting time of animals, they showed a shortening four hours after an injection of endotoxin, from a mean control time of 27 minutes to 19 minutes. Of particular interest was their observation that the overall incidence of microthrombi in the lungs, liver, spleen, and kidneys was doubled by a second injection of endotoxin. These findings of McKay et al. are consistent with our suggestion that rabbits with low silicone clotting times form stasis thrombi more readily when given endotoxin, because endogenous endotoxin has previously entered the animal's circulation.

Shimamoto et al. 13 and Des Prez et al. 14 have shown that endotoxin acts on platelets, with the release of serotonin. Des Prez et al. 14 found that the platelet-endotoxin interaction did not occur in the absence of a heat-labile plasma factor. Spink and Vick 15 have also demonstrated that a heat-labile serum or plasma factor is essential for endotoxin to produce peripheral vascular failure in dogs and suggested that complement may be involved. Our data on the recalcification times with and without endotoxin of normal platelet-poor plasma, and of platelet-poor plasma from patients congenitally deficient in Factors XII, XI, and IX suggest that one of the plasma moieties required for endotoxin to accelerate coagulation may be Factor XI or some related activity.

Although the absence of thrombogenicity may be related to a species difference, it is nevertheless true that the hen is congenitally lacking in Factor XI, as well as Factors XII and IX. 17 Our data suggest that endotoxin accelerates the formation of intrinsic thromboplastin by an initial action on Factor XI, or on some other moiety associated with Factor XI. The in vitro studies (table 5a) imply that the presence of Factor XII is not required.
The observed lengthening of the recalcification time of platelet-poor Factor XI-deficient plasma by endotoxin suggests that the role of Factor XI is obscured. It is well recognized that Factor XI-deficient patients do not have a total deficiency of this clotting factor. It is possible that only when there is a paucity of platelets does the level of Factor XI influence the ability of endotoxin to accelerate coagulation.

The efficacy of small doses of heparin in preventing endotoxin-induced thrombosis is in keeping with the observation of Gans and Krivit that heparin prevents endotoxin from causing death in dogs. It is unlikely that the amounts of heparin used in the present experiments could have prevented agglutination of platelets, for there is evidence to suggest that large doses of heparin are required (greater than 10 units per ml) to inhibit this reaction. However, traces of thrombin cause platelet agglutination, and it would seem more likely that the effect of the heparin is due to its potent antithrombin action, and not to any direct action on the platelets themselves. It is also possible that heparin acts by preventing endotoxin-activated Factor XII from attacking its normal substrate, Factor IX. Des Prez and Yamamoto have recently shown that the platelet-endotoxin interaction in vitro is not blocked by less than 50 units of heparin per ml of platelet-rich plasma. However, this is a dose of heparin greatly in excess of that required to block endotoxin-induced stasis thrombi in rabbits (10 units per kg). The exact site of action of heparin in preventing endotoxin-induced thrombosis is therefore still unclear.

Summary

1. In normal rabbits, single intravenous doses of E. coli and Salmonella endotoxin produce an immediate, transient, hypercoagulable state. Venous stasis occurring during such a state allows the formation of massive red thrombi at sites of obstructed blood flow. The duration of stasis required to produce thrombi following endotoxin administration depends in part on the preinfusion silicone clotting time of the rabbit.

2. E. coli endotoxin shortens the recalcification time of platelet-poor plasma obtained from normal subjects and from patients with heredofamilial coagulation defects of Factor XII (Hageman) and Factor IX (plasma thromboplastin component). In contrast, the recalcification time of platelet-poor plasma obtained from patients with Factor XI (plasma thromboplastin antecedent) deficiency is lengthened by endotoxin. It is suggested that the heat-labile plasma factor known to be required for the platelet-endotoxin interaction may be related to Factor XI.

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References


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