A Study of the Large Heparin Requirements in the Generalized Shwartzman Reaction

By Victor Gurewich, Boguslaw Lipinski, and Robert Wetmore

The heparin requirements necessary to inhibit intravascular fibrin deposition and soluble fibrin monomer (FM) formation in the generalized Shwartzman reaction (GSR) were evaluated. Fibrin deposition was measured by a quantitative technique utilizing \(^{125}\text{I}\)-labeled rabbit fibrinogen. FM was measured semiquantitatively by gel exclusion chromatography and by the serial dilution protamine sulfate (SDPS) test. There was a fourfold increase in heparin requirement 5 min after compared with 5 min before the second dose of endotoxin. This increase in heparin requirement was not related to thrombin elaboration, since FM was not found until more than 1 hr after the second dose of endotoxin. Neither was there any evidence of diminished sensitivity to the anticoagulant effect of heparin. The heparin requirements in the GSR rabbits were found to be in excess of those needed to neutralize a defibrinating dose of thrombin. It was concluded that a potent, heparin-resistant clotting activity developed within 5 min of the second endotoxin injection. The mechanism by which the activity caused the gradual elaboration of a thrombin-like enzyme is difficult to explain on the basis of traditional coagulation reactions. The apparent role of white cells is discussed.

The mechanism by which intravascular fibrin deposition occurs in the generalized Shwartzman reaction (GSR) remains poorly understood. Thrombin elaboration is considered to be a cardinal feature of the reaction, as it is in other experimental and clinical forms of disseminated intravascular coagulation. The evidence in support of intravascular thrombin generation includes the observations that both warfarin\(^1\) and heparin\(^2\) are capable of inhibiting the GSR. However, unusually large concentrations of heparin appear to be required,\(^2\) as compared to the concentrations necessary to prevent thrombin generation in other models, such as venous stasis thrombi.\(^3\)

In the present study, previously described techniques were used for the quantitative determination of fibrin deposition in organs\(^4\) and the semiquantitative measurement of soluble fibrin monomer (FM) concentrations in blood. These measures were used to evaluate the heparin dosage required for the inhibition of intravascular thrombin elaboration and fibrin deposition in the GSR. The heparin requirements were compared with those needed to neutralize a defibrinating infusion of thrombin.

MATERIALS AND METHODS

Labeled Rabbit Fibrinogen

Rabbit fibrinogen (Fraction I, Miles Laboratories, Inc., Kankakee, Ill.) was purified as previously described.\(^4\) A 1% w/v solution (0.85% saline) was labeled with \(^{125}\text{I}\) according to the method of McFarlane.\(^5\) The specific radioactivity of the final material was 5–10 μCi/mg.
Radioisotope Counting

A well scintillation counter connected to a scaler (Spectrometer Model 530, Baird Atomic) was used. The radioactivity in the following organs was determined: heart, kidney (cortex), liver, lungs, and spleen. After removal from the animal, the organs were washed thoroughly in tap water, blotted dry, and weighed. One-gram portions were counted, and the results obtained in cpm/g for each organ were expressed as a multiple of the heart count (cpm/g heart = 1 unit) as previously described. The units of radioactivity were multiplied by the total weight of each of the organs and divided by the weight (kg) of the rabbit to obtain the units per kilogram for each organ. The sum of these organ counts was expressed as the "total organ radioactivity" for each rabbit.

Clotting Time

A modified Lee and White whole blood clotting time was performed in two 13 X 100-mm glass tubes. The normal glass clotting time was 10 ± 2 (2 SD) min. Fibrinogen was determined by the thrombin clottable protein method of Swaim and Feders.6

Soluble Fibrin Monomer (FM) Complexes

Chromatography. A siliconized glass column (15 X 900 mm) was packed with Sepharose-4B soaked in phosphate-citrate buffer, pH 7.4, containing 0.05% NaN3 and 10–3 M EACA as described by Carvalho et al.7 Two-milliliter samples of platelet-poor plasma prepared from blood collected into 4.8% citrate (9:1) were applied to the column and eluted with the same phosphate citrate buffer at a rate of 8 ml/hr. The volume of each fraction collected was 3.5 ml. The concentration of fibrinogen reactive material in each sample was determined by the staphylococcal clumping titer (SCT) method8 using a commercial preparation of staphylococci (Behring Diagnostics, Somerville, N.J.) sensitive to 0.5 μg/ml of fibrinogen. The results were plotted as a function of the SCT against the corresponding effluent volume.

Serial dilution protamine sulfate (SDPS) test. A slight modification of the method previously published was used.9 Protamine sulfate, 1% (Eli Lilly Co., Indianapolis, Ind.) was mixed with 0.05 M Tris-buffered saline (pH 6.5) in the following dilutions: 1:10, 1:20, 1:40, 1:80. Two-tenths milliliter of each of the dilutions were placed into 12 X 75-mm glass test tubes. Three drops (Pasteur pipette) of aprotinin (Trasylol, FBA Pharmaceuticals, Inc., New York, N.Y.) were added to 1 ml platelet-poor plasma, and 0.2-mI volumes were added to each of the test tubes containing the protamine sulfate dilutions. The tubes were mixed gently and allowed to stand at room temperature for 30 min. Only the presence of fibrin strands (fs) or a gel (g) in any of the tubes was considered a positive reaction. A granular precipitate was considered negative, being due to precipitated fibrinogen. The dilution of the last tube showing a positive reaction was recorded.

Animal Models

All experiments were performed on New Zealand white rabbits weighing 2-2.5 kg. Blood was collected at hourly intervals from a polyethylene catheter (PE 90) in the carotid artery. Each experiment was terminated at 4 hr at which time the animal was killed and the organs removed.

In five control rabbits, 125I-labeled fibrinogen was given alone and the organ radioactivity determined 4 hr later.

In six rabbits, endotoxin (25 μg/kg) (lipopolysaccharide B, Escherichia coli 0 III:B4, Difco Laboratories, Mich.) was given through a marginal ear vein. Twenty-four hours later, the animal was anesthetized with Nembutal, given 125I-fibrinogen (1 mg) intravenously, and 6 min later, the second challenging dose of endotoxin (75 μg/kg) was given. In these rabbits, 5 ml saline (0.85%) was given intravenously 5 min after the second dose of endotoxin. Heparin sodium (10,000 USP units/ml; Upjohn Co.) diluted in 0.85% saline was given either 5 min before or 5 min after the second endotoxin injection. Heparin (300-1500 U/kg) was administered by a single injection through a marginal ear vein in a constant volume (5 ml). There were three rabbits in each of the heparin groups.

Clotting time. Heparin (400 U/kg) was given to three rabbits not given endotoxin, and clotting times were determined at hourly intervals and compared to those found in three heparinized groups of endotoxin treated animals.
**Heparin sensitivity.** Activated partial thromboplastin times (PTT) were performed on rabbit plasma according to the directions accompanying Platelin plus Activator (Warner Lambert Co., Morris Plain, N.J.). Prior to performing the PTT, 0.5 ml of plasma was mixed with either 0.1 ml saline (0.85%) or 0.1 ml heparin (1 U/ml), giving a final heparin concentration of 0.170 U/ml. Blood samples were obtained from three rabbits before the second dose of endotoxin, and at 15, 60, 120, and 180 min after the second dose. Heparin and nonheparinized PTTs were performed and the results expressed as a percentage of the saline PTT for each sample.

In three rabbits given the first dose of endotoxin, thrombin (100 NIH/U/kg/hr) was infused for 4 hr instead of the second dose of endotoxin. Three other rabbits were similarly treated but also given a loading dose of heparin (100 U/kg) just before the thrombin infusion, followed by a continuous intravenous infusion of heparin (100 U/kg). All infusions were given with an infusion pump (Harvard Apparatus Co., Dover, Mass.) in a constant volume of 4 ml/hr. In three rabbits given two doses of endotoxin, a heparin dosage schedule identical to the thrombin-infused rabbits was given.

**RESULTS**

In the control rabbits given $^{125}$I-fibrinogen alone, the total organ radioactivity (mean and range) was 75 (69–82) units. The SDPS test remained negative in these rabbits until the fourth hour, at which time a positive reaction (1:20 fs–1:40 fs) was found in four of five rabbits. This change is commonly found and believed to be related to the effect of prolonged anesthesia.

The total organ radioactivity (mean and range) in the endotoxin rabbits given saline was 410 (285–580) units. Changes in the SDPS test and plasma chromatogram in these animals were invariably delayed for about 1 hr. Thereafter, the baseline SDPS test (negative—1:20 fs) became strongly positive, reaching a peak of 1:80 fs–1:80 g in each animal (Fig. 1). The plasma chromatograms before 1 hr showed a single fibrinogen peak with little or no SCT positive material prior to effluent volumes 60–70. Thereafter, a major shift to effluent volumes 40–60 occurred. The amount of fibrinogen reactive material in the earlier effluent volumes corresponded to the SDPS titer (Fig. 2). A 40%–50% fall in plasma fibrinogen was found at the end of 4 hr in this group.

In rabbits given heparin 5 min before the second dose of endotoxin, there was a progressive reduction in total organ radioactivity reaching a significant ($p < 0.05$) level at a heparin concentration of 200 U/kg. The reduction in organ radioactivity was accompanied by a lower peak SDPS titer and correspondingly less fibrinogen reactive material in the early effluent volumes on the chromatograms. At a heparin concentration of 400 U/kg, the total organ radioactivity was not significantly different than in the control rabbits given $^{125}$I-fibrinogen.

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**Fig. 1.** Total organ radioactivity (mean) and maximum positive SDPS titer after second endotoxin injection in relation to heparin dose given either 5 min before or 5 min after the endotoxin. The dotted line indicates the baseline total organ radioactivity (mean) found in control rabbits not given endotoxin.
alone. At this heparin concentration, the SDPS test remained negative and only a single fibrinogen peak was found on the chromatograms (Figs. 1, 2).

In rabbits given heparin 5 min after the second dose of endotoxin, no significant reduction in organ radioactivity occurred until a heparin concentration of 400 U/kg was given. At this concentration, the SDPS peak titer was still 1:80 fs and a major shift to earlier effluent volumes was found by chromatography. A significant reduction in SDPS titer occurred only when the heparin dosage was increased to 1000 U/kg. At 1500 U/kg but not less, the SDPS test remained negative and organ radioactivity was comparable to that found in the 125I-fibrinogen control rabbits (Fig. 1).

**Clotting time.** Prolongation of the clotting time by heparin and duration of effect was proportional to the heparin dosage. At the higher concentration used, the clotting time was in excess of 4-5 hr even in the 4-hr sample. The duration of anticoagulant effect was greater in the endotoxin-treated animals than in the saline controls (Fig. 3).

**Heparin sensitivity.** The percentage prolongation of the PTT by heparin (mean and range) for each sample was as follows: baseline—157% (94-220); 15’—147% (98-193); 60’—150% (100-198); 120’—246% (170-322); 180’—243%

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Fig. 2. Representative chromatograms and corresponding SDPS test results (inscribed). Baseline plasma (left) showing narrow fibrinogen peak (white) with very little fibrinogen reactive material in earlier effluent volumes (hatched). Plasma from GSR rabbit giving a positive SDPS test (1:20 fs) and showing a major shift to earlier effluent volumes on chromatogram (middle). Plasma from GSR rabbit given no heparin with a strongly positive SDPS test and showing most of the fibrinogen reactive material in earlier effluent volumes (right).

Fig. 3. Clotting times 1-4 hr after a single intravenous injection of heparin 600 (x), 400 (x), and 300 (e) U/kg. The endotoxin-treated rabbits (-----) showed a more sustained prolongation of clotting time than the saline controls (---).
Fig. 4. Total organ radioactivity (mean) and SDPS test (maximum) in rabbits given endotoxin followed in 24 hr by thrombin (100 U/kg/hr) or a second dose of endotoxin. The effect of heparin (100 U/kg/hr) on these two models is shown. Heparin was completely inhibiting only in the thrombin-infused animals.

(190–300). Therefore, after endotoxin the PTT prolongation by a standard concentration of heparin tended to increase, indicating some increase in heparin sensitivity in the blood.

In the thrombin-infused animals, the mean and range of total organ radioactivity was 475 (385–651) units, greater than in the GSR rabbits. The SDPS test was positive in all samples reaching a maximum of 1:80 fs or 1:80 g in each rabbit. A 65%–80% fall in fibrinogen occurred in these animals at the end of the infusion. In the thrombin-infused animals given heparin, there was little organ radioactivity found and the SDPS test remained negative throughout the 4 hr. By contrast, there was relatively little inhibition of either the SDPS test or organ radioactivity in the endotoxin-treated rabbits given a similar infusion of heparin (Fig. 4).

DISCUSSION

In the present study, organ radioactivity rather than renal cortical necrosis was used to identify the GSR. We have previously shown that the radioactive material in this model has the characteristics of fibrin, based on its solubility characteristics. Therefore, the method permits quantitation of the fibrin deposits in the kidney as well as in other organs. Since renal cortical necrosis probably develops as a result of intravascular thrombosis, the present technique is believed to provide a reliable measure of the pathologic process responsible for the GSR.

Intravascular coagulation leading to thrombin generation is believed to be one of the cardinal features of the GSR. Large molecular weight soluble complexes of FM are formed in the blood as a consequence of the enzymatic action of thrombin on fibrinogen. Their measurement therefore provides an indirect index of thrombin elaboration. The SDPS test provides a semiquantitative measure of FM based on the principle that nonenzymatic fibrin formation develops when these complexes are exposed to protamine sulfate. Gel-exclusion chromatography is another method for identifying FM complexes based on their molecular weight. The chromatographic method of Carvalho et al. gives a narrow fibrinogen peak which is easily separated from the larger FM complexes. It has recently been found that when the percentage area occupied by
FM on the chromatogram is plotted against the SDPS titer, a highly significant \((p < 0.001)\) correlation is obtained. It has been shown that the GSR does not develop unless FM is formed as determined by the SDPS test.\(^4\)

In the GSR, FM elaboration based on these determinations occurs 1–2 hr after the second dose of endotoxin. Correspondingly, there is little fibrin deposition in organs within the first 2 hr.\(^{13}\) Both FM and fibrin deposition are inhibited by 400 U/kg of heparin if given before the second injection of endotoxin. However, if heparin is delayed until 5 min after the endotoxin, almost 4 times as much heparin is needed. This finding suggests that intravascular coagulation is triggered during this time interval, although thrombin elaboration is not evident until 1 hr later. A progressive effect related to heparin dosage has been demonstrated at both intervals, a finding characteristic of the anticoagulant action of heparin.

In the endotoxin-treated rabbits, the clotting time prolongation by heparin was greater than that previously reported.\(^{14}\) A greater and more protracted prolongation of the clotting time was found, which may be related to the effect of endotoxin treatment on the excretion or catabolism of heparin. Based on its anticoagulant effects, heparin appeared to be potentiated rather than inhibited by endotoxin. Therefore, the cause of the heparin resistance in these animals could not be attributed to an effect of endotoxin per se. Rather, it is more apt to be related to the secondary effects of endotoxin on intravascular coagulation.

Heparin requirements for the inhibition of intravascular fibrin formation are known to vary according to the site in the coagulation sequence that is activated. For example, it has been shown that a two- to threefold prolongation of the clotting time by heparin is sufficient to arrest deep vein thrombosis in man\(^{15}\) and to prevent venous stasis thrombi in rabbits infused with serum, kaolin, or endotoxin.\(^3\) By contrast, minidose heparin is effective in the prophylaxis of most postoperative deep vein thrombosis.\(^{16}\) In some patients, disseminated intravascular coagulation has been treated successfully by low-dose heparin,\(^{17}\) whereas even high doses have resulted in therapeutic failure in other patients.\(^{18}\) In vitro studies have shown that in the presence of co-factor (antithrombin III), little heparin is needed to prevent the activation of factor \(X\), whereas higher concentrations are required to neutralize thrombin.\(^{19}\) The success of minidose heparin when given prior to thrombus formation has been attributed to this observation.\(^{16}\) In the present study, heparin was given to all of the animals at least 1 hr before thrombin elaboration occurred based on measurements of FM formation and fibrin deposition.\(^{13}\) Nevertheless, exceptionally large concentrations of heparin were required to inhibit endotoxin-induced thrombin elaboration.

The heparin concentrations which were sufficient to inhibit FM formation and fibrin deposition in thrombin-infused animals failed to prevent intravascular clotting in the endotoxin rabbits. The amount of fibrin deposition and defibrination was greater in the thrombin rabbits. Therefore, excessive thrombin elaboration in the blood did not appear to be responsible for the large heparin requirements in the endotoxin animals.

The heparin-resistant thrombotic activity responsible for these observations seems to be initiated immediately after the second dose of endotoxin. It is at
this time that a fourfold increase in heparin requirements occurs. Coincident with this change, there is a sharp drop in circulating platelets and leukocytes but little evidence of activated blood clotting. Both platelets and leukocytes are known to contain antiheparin activity. However, antiheparin activity liberated during the GSR has been shown to be weak and difficult to detect and therefore unlikely to explain the observed heparin resistance.

Leukocytes appear to be essential to the development of endotoxin-induced intravascular coagulation. Rabbits made leukopenic but not thrombocytopenic by pretreatment with nitrogen mustard develop neither intravascular fibrin deposition nor detectable FM. Procoagulant activity of granulocytes sufficient to induce intravascular coagulation has been demonstrated by several investigators. By contrast, platelet procoagulant activity appears to be weak. However, it is likely that the concentration of heparin used would have neutralized any thromboplastic material released into the blood. The findings in the thrombin-infused rabbits supports this belief. Alternatively, certain protease activities in granulocytes may be capable of clotting fibrinogen. This possibility however is inconsistent with the observation that the GSR does not develop in warfarin-treated animals or with the present findings of a progressive, dose-related inhibition by heparin. These findings indicate that thrombin rather than some other protease is responsible for fibrin formation. The role of leukocytes in this process remains poorly understood but appears to be as unique as it is potent.

In conclusion, a heparin-resistant procoagulant activity is initiated in rabbits immediately after the second dose of endotoxin. After a delay of 1 or 2 hr, progressive intravascular fibrin deposition takes place. Leukocytes have been shown to be essential to this process by a mechanism that cannot readily be explained by current concepts of intravascular coagulation. Clarification of this mechanism may be useful to our understanding of intravascular coagulation in man.

REFERENCES

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