Inhibition of the Generalized Shwartzman Reaction by Hypofibrinogenemia

By William R. Bell, Robert E. Miller, and Jack Levin

The effect of hypofibrinogenemia on production of the generalized Shwartzman reaction (GSR) by the administration of Thorotrast (3 ml/kg) and Escherichia coli endotoxin (50 µg) to rabbits was determined. Severe hypofibrinogenemia (fibrinogen less than 15 mg/100 ml) was produced by Arvin, a proteolytic coagulant from the venom of the Malayan pit viper, Agkistrodon rhodostoma. Development of the GSR was inhibited significantly only when hypofibrinogenemia was produced 30 hr before the preparatory injection of Thorotrast and maintained for the duration of the experiment. Severe hypofibrinogenemia of 2-20-hr duration before administration of endotoxin did not block the production of the GSR. Elevated levels of fibrinogen degradation products were demonstrated in the serum of all animals that received Arvin. The concentration of fibrinogen degradation products decreased markedly prior to death only in those animals that developed the GSR. The GSR was not observed in rabbits that received only Thorotrast and Arvin, although these animals had the highest levels of fibrinogen degradation products. The data suggest an essential role for fibrinogen or fibrinogen degradation products and endotoxin in the production of the GSR.

The Generalized Shwartzman Reaction (GSR) has been produced classically in rabbits by two appropriately spaced intravenous injections of endotoxin. More recently, Thorotrast has been substituted for the first injection of endotoxin. Previous studies have demonstrated that production of the GSR can be blocked by granulocytopenia, thrombocytopenia, or anticoagulation. It is generally accepted that the GSR is the result of intravascular coagulation with deposition of fibrin in renal glomerular capillaries.

To test further the postulate that the final common pathway in the production of the GSR is intravascular coagulation, experiments were carried out to determine whether the GSR could be blocked by severe hypofibrinogenemia. Arvin, a proteolytic, coagulant enzyme obtained from the crude venom of the Malayan pit viper, Agkistrodon rhodostoma, was used to produce hypofibrinogenemia.
fibrinogenemia. Arvin produces hypofibrinogenemia by the progressive conversion of circulating fibrinogen to an abnormal fibrin monomer that is removed primarily by the reticuloendothelial system (RES). Our studies indicated that the administration of Arvin to rabbits resulted in severe hypofibrinogenemia. However, it was necessary to induce and sustain hypofibrinogenemia for an interval of 30 hr before the initial injection of Thorotrast in order to reduce the incidence of the generalized Shwartzman reaction.

MATERIALS AND METHODS

Animals

New Zealand white rabbits (1.84–2.75 kg) from a single local rabbitry were employed for all studies. The rabbits were housed in air-conditioned quarters and fedRalston Purina Rabbit Chow and water ad lib.

Production of the Generalized Shwartzman Reaction

Preparatory Injection: Thorotrast, 24%–26% stabilized, colloidal suspension of thorium dioxide (Lot No. 14430, Fellows Testagar Div., Fellows Mfg., Detroit, Mich.) was administered intravenously (3 ml/kg).

Provocative Injection: E. coli lipopolysaccharide (W, 026:B6, Westphal method, Lot No. 519567, Difco, Detroit, Mich.) was prepared freshly for each experiment in sterile, pyrogen-free 0.85% sodium chloride and pyrogen-free glassware. Endotoxin (50 μg in 5 ml of 0.85% sodium chloride) was administered intravenously during a 15-min interval.

In all experimental models, E. coli endotoxin was administered 18 hr following the injection of Thorotrast. In previous experiments in our laboratory, this sequence has been shown to produce the GSR in 66% of rabbits.13,14

Pathologic Studies

All animals were examined at the time of death. Rabbits that survived 24–30 hr after the injection of endotoxin were sacrificed by the intravenous injection of lethal doses of sodium pentobarbital and were immediately autopsied. Portions of kidney and adrenal were fixed in 10% formalin, and paraffin sections were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and phosphotungstic acid-hematoxylin (PTAH) by the usual methods.15 Histologic sections of kidney were evaluated for the presence of thrombi in glomerular capillaries and for cortical hemorrhage and necrosis. Thrombi that had a dark blue, fibrillar appearance with PTAH were considered to contain fibrin or related fibrinogen degradation products. PTAH-positive material in the glomerular capillaries and renal cortical necrosis and hemorrhage were required for the pathologic definition of the GSR.

Only rabbits that survived 12 hr or more following injection of the endotoxin were included in the tabulated analysis, in order to exclude animals that died before adequate time had elapsed for the maximum development of the GSR. However, inclusion of animals that survived more than 4 hr would not have altered the results.

Production of Hypofibrinogenemia

Arvin, prepared as previously described16 from a single batch of crude venom, was used to produce and maintain hypofibrinogenemia. One unit of Arvin (containing 2 μg of protein) is that amount of material necessary to clot a standard solution of fibrinogen in the same time as 1 NIH unit of thrombin. Arvin was administered intravenously (in 5% dextrose in water) using a constant infusion pump (Model 600-95, Harvard Apparatus, Dover, Mass.), according to the experimental protocols indicated below.

The initial dose of Arvin was 0.8–1 U/kg, administered slowly during a 30-min period. Hypofibrinogenemia was maintained by the repeated injection of the same dose every
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12 hr for the duration of the experiment. Arvin was given continuously via infusion pump over 18 hr (total dose 2–6 U/kg) following the injection of endotoxin in six animals in model 4 because of a tendency for fibrinogen levels to increase following injection of endotoxin.

Evaluation of Blood Coagulation

Blood Samples: Blood (2 ml) was obtained serially by venipuncture from the marginal ear vein and was collected in siliconized glassware (G.E. Silicone S.C.-87 Dri-Film, General Electric Co., Waterford, N.J.), using 3.8% sodium citrate (1 part citrate: 9 parts whole blood) as anticoagulant. Plasma was prepared by centrifugation of whole blood for 15 min at 1100 g at room temperature. Blood samples for platelet counts and hematocrit values were collected in capillary tubes (Drummond Capillary Tubes, Drummond Scientific, Broomall, Pa.) from the same venipuncture. Tubes used for platelet counts were treated with EDTA and heparin; tubes for microhematocrit values were double oxalated.

Platelets: Blood platelets were counted by the method of Bull et al.17 by use of a Coulter electronic counter.

Fibrinogen: Fibrinogen concentration (thrombin-clottable protein) in plasma was measured by a modification of the thrombin time, as described by Clauss.18 Low concentrations were also measured periodically by the method of Ratnoff and Menzie19 to confirm the validity of the first technique.14

Prothrombin Time and Partial Thromboplastin Time: These were measured using standard techniques and reagents. Thromboplastin for prothrombin determination was prepared from acetone-dried rabbit brain. Thrombofax reagent (Ortho Diagnostics, Raritan, N.J.) was used to determine the partial thromboplastin time.

Fibrinogen Degradation Products (FDP): Plasma was clotted at 4°C for 4 hr with an equal volume of a solution containing 1.1 M epsilon aminocaproic acid, 0.025 M calcium chloride, and 5 NIH units of thrombin. The resultant serum was tested for FDP. Plasma and serum not tested immediately were frozen at −20°C. Fibrinogen degradation products were measured by the tanned red cell hemagglutination inhibition immunooassay of Merskey et al.,20 as modified for rabbit FDP in our laboratory. Results are expressed as the highest dilution of serum that inhibited agglutination of fibrinogen-coated red cells by antirabbit fibrinogen serum. Normal levels in our laboratory are equal to or less than 1:8.

Preparation of Guinea Pig Antirabbit Fibrinogen Serum: Guinea pigs received injections of rabbit fibrinogen (Fraction I, 60% clottable protein, Pentex, Kankakee, Ill.) or rabbit fibrin, obtained by clotting rabbit fibrinogen with thrombin. With an equal volume of Freund's adjuvant, 0.01 mg of fibrinogen or fibrin was emulsified and injected initially into each footpad and then subcutaneously at weekly intervals. Two weeks after the fourth weekly subcutaneous injection of antigen, the animals were bled, and serum was prepared from blood that had been allowed to clot for 2 hr at 37°C. The antisera was adsorbed three times with aged, rabbit serum at room temperature, during a 36-hr period. This antisera, when reacted with rabbit fibrinogen, gave a single band on immunodiffusion. The antirabbit fibrinogen serum was titered by agglutination of rabbit red cells that had been coated with rabbit fibrinogen. A 1:2000 dilution of antirabbit fibrinogen serum was capable of agglutinating these red cells.

Experimental Protocols

Model 1 (Thorotrast 18 hr→ Endotoxin): Endotoxin (50μg) was administered 18 hr after the preparatory injection of Thorotrast (3 ml/kg).

Model 2 (Thorotrast 16 hr→ Arvin 2 hr→ Endotoxin): Arvin was administered initially 16 hr after the preparatory injection of Thorotrast (and 2 hr before the injection of endotoxin).

Model 3 (Arvin 2 hr→ Thorotrast 18 hr→ Endotoxin): Arvin was administered initially 2 hr before the preparatory injection of Thorotrast.

Model 4 (Arvin 30 hr→ Thorotrast 18 hr→ Endotoxin): Arvin was administered initially
Fig. 1. Kidney of an animal in which Shwartzman reaction was produced with Thorotrast and endotoxin. Sections were stained with hematoxylin and eosin (H & E), periodic acid-Schiff (PAS), and phosphotungstic acid-hematoxylin (PTAH) (left to right). Microthrombi are present in glomerular capillaries. Positive PTAH reaction with fibrillar appearance indicates that glomerular thrombi contain fibrin or fibrinlike material. Original magnification × 220.

30 hr before the preparatory injection of Thorotrast.

Model 5 (Thorotrast 18 hr → Arvin): Arvin was administered initially 18 hr after Thorotrast. This is the only protocol in which endotoxin was not administered. Arvin was readministered at 12-hr intervals throughout the duration of experimental models 2, 3, 4, and 5.

RESULTS

Production of the Generalized Shwartzman Reaction

The intravenous injection of endotoxin 18 hr after the administration of Thorotrast (model 1) produced the generalized Shwartzman reaction in 83% of six rabbits. This confirmed previous observations that indicated the effectiveness of this model.13-14 Renal lesions were characterized histologically by widespread glomerular capillary thrombi (Fig. 1). The material filling the glomerular capillaries had a hyaline, eosinophilic appearance with H&E and was PAS positive with a similar amorphous character. Positive PTAH staining and a fibrillar appearance of much of the thrombotic material suggested fibrin or fibrinogen-related breakdown products. These lesions were not present in normal, untreated rabbits.

Effect of Hypofibrinogenemia on Development of Generalized Shwartzman Reaction

Model 2 (Thorotrast 16 hr → Arvin 8 hr → Endotoxin): Arvin was administered initially 16 hr after the injection of Thorotrast and 2 hr before the infusion of endotoxin. Three of five animals (60%) developed the characteristic lesions of the GSR, despite the presence of severe hypofibrinogenemia at the time of administration of endotoxin. The incidence of the GSR was only slightly less than the group of control animals (model 1) that received only Thorotrast and endotoxin. There was no significant difference
Fig. 2. Effect of administration of Arvin 16 hr after Thorotrast and 2 hr before endotoxin (model 2). Arvin was readministered every 12 hr for duration of experiment. Each point represents the mean of five animals. Time in hours after administration of Thorotrast is shown. Sixty per cent of animals developed GSR, despite production of hypofibrinogenemia. Platelet counts and fibrinogen levels of animals that developed GSR did not differ from those of animals that were negative, and results were pooled.

in either the platelet counts or fibrinogen levels of positive and negative animals (Fig. 2).

Model 3 (Arvin \(2\) hr \(\rightarrow\) Thorotrast \(18\) hr \(\rightarrow\) Endotoxin): To determine whether hypofibrinogenemia preceding the administration of Thorotrast would inhibit the development of the GSR, Arvin was administered initially 2 hr before Thorotrast. The level of fibrinogen promptly fell to zero, and the mean fibrinogen levels remained below 2 mg/100 ml until after the administration of endotoxin (the maximum individual fibrinogen level during this period was 5 mg/100 ml) (Fig. 3). Nevertheless, three of seven animals (42%) developed the GSR. Although this incidence was less than when Arvin had been administered initially only 2 hr before endotoxin, it was not significantly lower than the control animals (model 1). The platelet counts and fibrinogen levels were similar in positive and negative animals. However, prior to sacrifice the concentrations of fibrinogen degradation products in serum were higher.
in rabbits that did not develop the GSR (mean = 1:4256) than in GSR-positive rabbits (mean = 1:200).

Histologic sections of the kidneys of a representative animal that developed the GSR are shown in Fig. 4. The material filling many capillary loops was hyaline and eosinophilic with H&E and was PAS positive with similar amorphous character. The PTAH stain also was positive, and much of the thrombotic material showed a fibrillar appearance suggesting the typical staining reaction of fibrin. These findings were compatible with deposition of fibrinogen and FDP in the kidney following the administration of endotoxin, despite the continuously low levels of circulating fibrinogen.

Model 4 (Arvin $^{30}$ hr → Thorotrast $^{18}$ hr → Endotoxin): The moderate decrease in the incidence of the GSR when the duration of hypofibrinogenemia
Fig. 4. Sections of kidney from a positive experimental animal (model 3) showing extensive fibrin thrombi in glomerular capillaries (H&E, PAS, and PTAH, left to right). Staining of thrombotic material is identical to that observed in Shwartzman reaction (Fig. 1). Original magnification × 220.

Fig. 5. Effect of administration of Arvin 30 hr before Thorotrast and 48 hr before endotoxin (model 4). Arvin was readministered every 12 hr throughout experiment. Each solid symbol represents mean value of three animals that developed GSR. Open symbols represent mean value for 15 animals that were negative for GSR. Animals that developed GSR did not have sustained, severe hypofibrinogenemia.
Fig. 6. Effect of administration of Arvin 18 hr following Thorotrast without subsequent endotoxin (model 5). Arvin was readministered every 12 hr throughout experiment. Each point represents mean of five animals. Time in hours after administration of Thorotrast is shown. Fibrinogen levels increased and platelet counts decreased following administration of Thorotrast, before Arvin was administered.

Table 1. Production of the Generalized Shwartzman Reaction

<table>
<thead>
<tr>
<th>Model</th>
<th>Positive Shwartzman/Total</th>
<th>p†</th>
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<tbody>
<tr>
<td>1. Thorotrast <strong>18 hr</strong> endotoxin</td>
<td>5/6 (83%)</td>
<td>—</td>
</tr>
<tr>
<td>2. Thorotrast <strong>18 hr</strong> Arvin <strong>3 hr</strong> endotoxin</td>
<td>3/5 (60%)</td>
<td>NS</td>
</tr>
<tr>
<td>3. Arvin <strong>3 hr</strong> Thorotrast <strong>18 hr</strong> endotoxin</td>
<td>3/7 (42%)</td>
<td>NS</td>
</tr>
<tr>
<td>4. Arvin <strong>30 hr</strong> Thorotrast <strong>18 hr</strong> endotoxin</td>
<td>3/18 (16%)</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>5. Thorotrast <strong>18 hr</strong> Arvin</td>
<td>0/5 (0%)</td>
<td>&lt;0.01</td>
</tr>
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* Fractions indicate number of rabbits that developed the GSR/total number of experiments. All animals survived 12 hr or more following injection of endotoxin.
† Compares each experimental model with model 1 (positive controls). NS, not significant.
was increased suggested that a prolonged period of severe hypofibrinogenemia, prior to the preparatory injection of Thorotrast, might inhibit the production of the GSR. The initial injection of Arvin was given 30 hr before Thorotrast. Only three of 18 animals (16%) developed the GSR. Two of three positive animals did not have sustained, severe hypofibrinogenemia (Fig. 5). These three animals had the typical lesions of the GSR, including PTAH-positive material in their renal glomeruli. The platelet counts and fibrinogen levels were similar in the positive and negative animals. Prior to death, greater concentrations of FDP were present in the circulation of animals that did not develop the GSR (mean = 1:2000) than in the three positive animals (mean = 1:85).

**Effect of Hypofibrinogenemia Following Blockade of Reticuloendothelial System**

To determine whether the induction of hypofibrinogenemia following blockade of the RES by Thorotrast would produce the GSR, Arvin was administered as the provocative injection 18 hr after the preparatory injection of Thorotrast (model 5). None of the animals developed the GSR, despite changes in platelet counts and fibrinogen levels similar to those seen previously (Fig. 6). Neither hyaline thrombi nor PTAH-positive material was detected in the glomerular capillaries. The mean levels of FDP produced in these animals (1:30,000) were the highest observed.

The results produced by the five experimental models are summarized in Table 1. Levels of FDP bore no relationship to concomitant levels of circulating fibrinogen. In many animals, significant elevations of FDP persisted many hours after fibrinogen no longer was detectable in the circulation. The adrenal glands were grossly and histologically normal in all experimental models. In all instances, there was good correlation between the gross appearance of the kidneys and the adrenals, and the histologic findings.

**DISCUSSION**

The generalized Shwartzman reaction apparently results from the production of intravascular coagulation in rabbits in which the RES has been blocked. Under these experimental conditions, the clearance of products resulting from intravascular coagulation is decreased. The deposition of fibrin with occlusion of glomerular capillaries produces renal cortical ischemia with subsequent hemorrhage and necrosis. Lee has demonstrated the importance of diminished RES function in the production of the GSR. Activation of the fibrinolytic system inhibits the production of the GSR. Inhibition of the GSR by anticoagulation or thrombocytopenia also provides evidence for the role of platelets and blood coagulation in the production of this phenomenon.

The important role of intravascular coagulation in the production of the GSR and the evidence of deposition of fibrin or fibrinlike material in the renal glomeruli suggested studies to determine whether severe hypofibrinogenemia would inhibit the development of the GSR. The role of circulating
fibrinogen in the development of the GSR was evaluated by the production of severe hypofibrinogenemia with Arvin. This proteolytic enzyme reacts with fibrinogen to produce an abnormal fibrin monomer that is removed primarily by the RES.\textsuperscript{26} This results in progressive depletion of circulating fibrinogen.\textsuperscript{27} Arvin does not cause reduction of other coagulation factors or thrombocytopenia.\textsuperscript{28} The production of hypofibrinogenemia either immediately before the provocative injection of endotoxin or immediately before the preparative injection of Thorotrast slightly decreased the incidence of the GSR but did not prevent its occurrence. However, severe hypofibrinogenemia that was initiated 30 hr before the injection of Thorotrast and was maintained for the duration of the experiment markedly and significantly decreased the incidence of the GSR (Table 1).

The necessity for hypofibrinogenemia of long duration in order to inhibit the development of the GSR suggested that the presence of high concentrations of circulating fibrinogen degradation products might have been sufficient to allow development of the GSR following injection of endotoxin. There was no correlation between levels of circulating fibrinogen and concentrations of fibrinogen degradation products, which often persisted for many hours after detectable fibrinogen had disappeared from the circulation. Serial levels of fibrinogen degradation products 20–24 hr after endotoxin were consistently higher in animals that did not develop the GSR than in otherwise similar animals that did. Our data suggest that the administration of endotoxin to animals with RES blockade and high levels of circulating fibrinogen degradation products seen throughout the study period can result in production of the GSR, with concomitant disappearance of FDP from the circulation. The presence of PTAH-positive material in the renal glomeruli of hypofibrinogennemic animals that developed the GSR indicates that this material had the characteristics associated with fibrinogen or fibrin and further supports the concept that it represented the deposition of fibrinogen breakdown products. In addition, it was demonstrated that production of hypofibrinogenemia and very high levels of fibrinogen degradation products throughout the duration of the experiment, in rabbits in which the reticuloendothelial system had been previously blocked with Thorotrast (model 5), was not sufficient to produce the GSR when endotoxin was not administered. The failure to develop the GSR under similar experimental conditions has been reported recently by Rodriguez-Erdman et al.\textsuperscript{29} There was no significant difference in the levels of circulating fibrinogen and platelets in animals that developed the GSR compared to animals that did not, except in model 4 where two of three animals in which hypofibrinogenemia was not persistent did develop the GSR.

The role of the provocative injection in the production of the GSR is not clear. Amenta and Waters\textsuperscript{30} have demonstrated that large, sulfated polysaccharides can, in a nonionic manner, complex with and precipitate fibrinogen. Endotoxin is a lipopolysaccharide. The observation that thrombi in the glomerular capillaries were both PAS and PTAH positive is compatible with the presence of endotoxin-fibrinogen or endotoxin-fibrinogen breakdown
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product complexes in this material. However, leukocyte or platelet mucopolysaccharides have been suggested as the source of the positive PAS reaction.31,32

The adrenal glands of the animals that developed the Shwartzman reaction did not demonstrate cortical hemorrhage or necrosis. This confirms previous observations that adrenal cortical hemorrhage is not an integral part of the generalized Shwartzman reaction.33,34

The effectiveness of reticuloendothelial blockade by Thorotrast was demonstrated by the very high titers (1:30,000) of FDP in Thorotrast-prepared animals who received Arvin. Other unpublished studies in our laboratory indicate that the administration of similar doses of Arvin to normal rabbits that did not receive Thorotrast resulted in mean FDP titers of 1:16, which often fell to zero after 36-40 hr of Arvin. Arvin does not produce thrombocytopenia or shortened lifespan of platelets in rabbits.35 The administration of Thorotrast can produce thrombocytopenia.34

Our studies confirm the observations of previous investigators36,37 that the administration of endotoxin increases the levels of circulating fibrinogen. Following the injection of endotoxin into markedly hypofibrinogenemic animals, moderate increases in fibrinogen levels often were observed and necessitated larger doses of Arvin.

Our findings are compatible with previous observations that significant interruption of the coagulation mechanism is capable of preventing the GSR. However, our data also indicate that low levels of circulating fibrinogen do not necessarily protect against development of the GSR.

REFERENCES


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