

# Evidence for an Essential Role of Tissue Factor Dependent Blood Coagulation in the Pathogenesis of the Local Shwartzman Reaction

Ariella Zivelin<sup>1</sup>, L. Vijaya Mohan Rao<sup>2</sup> and Samuel I. Rapaport<sup>3</sup>  
Department of Medicine, University of California, San Diego, La Jolla, CA

(communicated by Samuel I. Rapaport, M.D., 01/04/95)

**ABSTRACT.** Adherence and aggregation of leukocytes within the vessels of a prepared skin site has been shown to be essential for the pathogenesis of the local Shwartzman reaction (LSR)(Argenbright and Barton: *J Clin Invest* 89: 259, 1992). We have now performed experiments in rabbits to evaluate whether coagulation within the vessels of a prepared site is a second requirement for the reaction. Skin sites were prepared by an intradermal injection of endotoxin 24 hours before a provoking intravenous injection of endotoxin. Thirteen control rabbits all developed the LSR. Seven of 12 rabbits given warfarin to achieve anticoagulation approximating that used therapeutically in humans before the provoking injection were protected against the LSR ( $p=0.003$ ). Five of nine rabbits given anti-rabbit factor X IgG before the provoking injection to yield mean values in individual rabbits of between 7% and 18% plasma factor X activity were protected against the LSR ( $p=0.009$ ). Six of 11 rabbits given anti-rabbit factor VII IgG before the provoking injection to yield mean values in individual rabbits of between <0.5% and 2.2% were protected against the LSR ( $p=0.007$ ). Four rabbits failed to develop the LSR at an endotoxin-prepared skin site when an infusion of tissue factor (TF) causing substantial intravascular coagulation was substituted for a provoking injection of endotoxin. It would appear that two events are required for the pathogenesis of the LSR provoked by endotoxin: formation of aggregated masses of WBC in the prepared skin vessels and deposition of fibrin due to TF-initiated coagulation.

## INTRODUCTION

In the classical local Shwartzman reaction (LSR), a rabbit is injected intradermally with a gram-negative endotoxin and 18-24 hrs later is given an intravenous dose of the endotoxin. Within hours after the provoking intravenous injection, an area of hemorrhagic necrosis develops at the

site of the intradermal injection. An understanding of the pathogenesis of the LSR has clinical relevance since the acute infectious purpuras, e.g., the dermal lesions of overwhelming meningococcemia, are thought to represent clinical equivalents of the LSR (1).

Agents other than endotoxin (2), including the

---

<sup>1</sup>Present address: Institute of Thrombosis and Hemostasis, Chaim Sheba Medical Center, Tel Hashomer, 52621, ISRAEL.

<sup>2</sup>Present address: Department of Biochemistry, The University of Texas Health Center at Tyler, Tyler, TX 75710.

<sup>3</sup>Reprint requests to: Samuel I. Rapaport, M.D., UCSD Medical Center, Mail Code 8423, 200 West Arbor Drive, San Diego, CA 92103; phone (619) 543-3552, fax (619) 543-3231.

cytokines interleukin-1 and tumor necrosis factor (3,4), can be injected intradermally in place of endotoxin to prepare a skin site for the LSR. Recently, Argenbright and Barton have presented evidence that agents preparing a skin site for the LSR do so by promoting the expression of the intracellular adhesion molecule (ICAM-1) on vascular endothelium in dermal vessels (5).

Materials other than endotoxin (6), e.g., starch, kaolin powder, glycogen and zymosan, can also be used for the intravenous injection provoking the reaction. Shortly after a provoking injection the capillaries and small veins of a prepared skin site become occluded with masses of platelets and leukocytes (7). In the experiments of Argenbright and Barton, zymosan was injected intravenously to provoke the LSR. The zymosan activated complement with resultant increased expression of CD11/18 on leukocytes. It was found that antibodies against CD18, as well as antibodies against ICAM-1, prevented the LSR (5). This led Argenbright and Barton to propose that a reaction between ICAM-1 expressed on vascular endothelium at a prepared skin site and CD11/CD18 expressed on circulating leukocytes after a provoking injection results in an intravascular adhesion/aggregation reaction of leukocytes that damages vessel walls with the resultant hemorrhagic necrosis of the LSR.

The occluding thrombi of the LSR also contain fibrin (1,5,7). In the 1950's it was reported that heparin, in doses sufficient to prolong blood clotting beyond 1 hr at the time of a provoking intravenous injection, protected rabbits against the LSR (8-10). However, heparin has biologic effects beyond anticoagulation, including the ability to inhibit serum complement. Therefore, it is noteworthy that several investigative groups in the 1950's also carried out experiments in which a vitamin K antagonist was used for anticoagulation (10-13). One investigative group reported that a vitamin K antagonist could protect against the LSR (11,13), a second group reported that it

could not (12), and a third group reported that a vitamin K antagonist partially protected against the LSR (10). Although details of the test methods used to monitor anticoagulation are lacking in these early reports, it appears that in each study rabbits received much more intense anticoagulation than currently recommended for therapy with warfarin in humans.

In 1965 it was postulated that endotoxin-induced intravascular coagulation is essential not only for the glomerular capillary thrombosis of the generalized Shwartzman reaction (GSR) but also for the hemorrhagic skin necrosis of the LSR (6). Although strong evidence has since been obtained for an essential role of TF-initiated intravascular coagulation in the pathogenesis of the GSR (14,15), the importance of triggering coagulation in the pathogenesis of the LSR has remained unsettled. In 1967 it was reported that an infusion of thrombin causing substantial disseminated intravascular coagulation (DIC) could not replace endotoxin as a provoking agent for the LSR (16). A recent search of the literature failed to reveal additional relevant data other than a single abstract in which giving rabbits human activated protein C was reported to attenuate the LSR (17).

We report here further experiments to evaluate the role of coagulation in the pathogenesis of the LSR. Our experiments were designed to answer the following questions: (1) can an infusion of purified reconstituted rabbit TF, which unlike thrombin can trigger the full sequence of the tissue factor-dependent coagulation reactions, substitute for a provoking dose of endotoxin; (2) can administration of warfarin to achieve anticoagulation comparable to the clinically recommended therapeutic range for warfarin protect against the LSR; (3) can selective immunodepletion of factor X--which is required to activate prothrombin in both tissue factor-dependent and non-tissue factor-dependent pathways of coagulation--protect against the LSR; and (4) can

selective immunodepletion of factor VII--which is required only for the function of the tissue factor-dependent pathway of coagulation--protect against the LSR?

## MATERIALS AND METHODS

Sodium warfarin for intravenous administration was a gift from DuPont Pharmaceuticals (Wilmington, DE). *E. coli* endotoxin (0111:4B) was from Sigma Chemical Co. (St. Louis, MO). Commercial rabbit brain thromboplastin was from Sigma and aPTT reagent was from Helena Laboratories (Beaumont, TX). Human substrate plasma deficient in factor VIII was from a patient with severe hemophilia A, and human substrate plasma deficient in factor V was prepared from normal plasma by immunoadsorption.

### *Rabbit TF for Infusion*

One microgram of purified rabbit brain TF apoprotein (18) was reconstituted into 1 mg/mL of mixed phospholipid vesicles containing 40% phosphatidylserine and 60% phosphatidylcholine (14) and stored at -75°C.

### *Goat Anti-rabbit Factor VII IgG*

Monospecific polyclonal antibodies to purified rabbit factor VII were raised in a goat. Different bleeds yielded IgG with a specific activity of 10-50 inhibitory units per mg protein. The IgG bound strongly to factor VII on an immunoblot and had no inhibitory activity against rabbit factors IX, X or prothrombin. Details of purification of the factor VII for use as antigen and of separation of the IgG fraction from the antiserum can be found elsewhere (19).

### *Monoclonal Anti-rabbit Factor X IgG*

Monoclonal antibodies were prepared against rabbit factor X as described earlier (19). mAB 409 was used to prepare rabbit factor X-deficient substrate plasma. mAB 541, which was of subclass IgG3 and had a titer of 75 U/mg IgG, was injected into rabbits to immunodeplete plasma factor X activity.

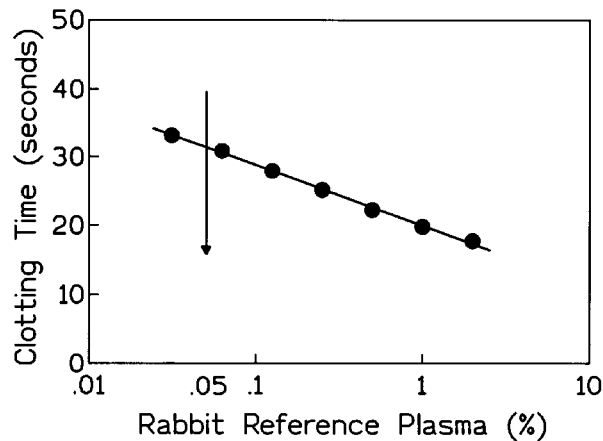
### *Rabbit-specific Deficiency Substrate Plasmas for Clotting Assays*

Rabbit substrate plasmas deficient in factor VII or factor X were prepared by immunoadsorption with specific anti-rabbit factor VII or factor X antibodies. A rabbit substrate deficient in prothrombin was prepared by mixing equal parts of barium-adsorbed rabbit plasma and rabbit serum. Details of preparation of these substrates have been provided earlier (19).

### *Clotting Factor Assays*

Rabbit plasma reference standard. Blood from the central artery of six to eight rabbits was collected in plastic tubes containing 1/10th volume of a balanced citrate anticoagulant consisting of 0.06 mol/L sodium citrate and 0.04 mol/L citric acid. Plasma was separated at 4°C by two successive centrifugations at 10,000 g and was pooled and stored in capped plastic vials at -75°C.

General clotting assay conditions. Automated instruments yielding a photo-optical end point were used. Test plasmas were diluted for assay in TBS/BSA (50 mmol/L Tris, 0.15 mol/L NaCl, 1 mg/mL bovine serum albumin, pH 7.5) and tested in duplicate. Rabbit plasma reference standard, arbitrarily assigned a value of 100% (1 U/mL activity), was used for dilution curves for all assays except the measurement of fibrinogen.



**Figure 1:** An example of a reference curve used to measure rabbit plasma factor VII activity to illustrate the sensitivity of the assay in the range of 2-0.34% of the rabbit plasma reference standard. A test sample diluted 1/10 that gave a clotting time longer than the clotting time delineated on the reference curve by the vertical arrow was reported as containing <0.5% plasma factor VII activity. Duplicate test samples yielded either identical clotting times or times that differed by only a fraction of a second. A blank time with buffer was approximately 5 seconds longer than the clotting time of the last data point shown.

#### Prothrombin and proconvertin (P&P) test (20).

In the test as modified for this study, 100  $\mu$ L of rabbit plasma diluted 1:5 with barium sulfate adsorbed rabbit plasma were incubated for 3 min at 37°C with 100  $\mu$ L rabbit brain TF (Sigma). Clotting was triggered by the addition of 100  $\mu$ L of 25 mmol/L  $\text{CaCl}_2$  warmed to 37°C. A reference curve to convert clotting times to percent normal rabbit plasma activity was prepared from the clotting times of increasing dilutions of rabbit plasma reference standard made in a barium sulfate adsorbed rabbit plasma. The clotting time of the 1:5 dilution was designated as 100% activity. The lower level of sensitivity of the test was 5%.

Rabbit factor VII assay. Factor VII activity was measured in a one-stage assay in which 100  $\mu$ L of an equal part mixture of rabbit immunodepleted factor VII-deficient plasma and barium adsorbed rabbit plasma, 100  $\mu$ L of a dilution of test plasma, and 100  $\mu$ L of rabbit brain TF were incubated together in a plastic cuvette for 3 min at

37°C. Clotting was triggered by the addition of 100  $\mu$ L of 25 mmol/L  $\text{CaCl}_2$ . This assay, in which both a rabbit factor VII-deficient substrate and rabbit TF are used, is very sensitive to rabbit factor VII activity. As shown in Figure 1, a reference curve for converting clotting times to percent factor VII activity is made with dilutions of rabbit plasma reference standard from 1:50 (2%) to 1:3200 (0.034%). The latter gave a clotting time approximately 5 seconds shorter than a blank time with buffer. When a test sample was diluted 1:10, this sensitive assay could be used to measure accurately plasma factor VII levels as low as 0.5%.

Rabbit factor X assay. Rabbit factor X was measured essentially as for factor VII except that rabbit factor X-deficient plasma was used as the substrate and the standard curve for the assay was made with from 1:20 to 1:640 dilutions of rabbit reference plasma. When rabbit test plasma was diluted 1:10, a factor X activity level as low as 1.5% could be measured accurately.

Other specific clotting factor assays. Fibrinogen concentration was determined from the clotting time of rabbit plasma after addition of a high concentration of bovine thrombin essentially as described by Clauss (21). Factor V was measured in a one-stage assay with a human factor V-deficient substrate and rabbit tissue factor as the activator. Factor VIII was measured in a one-stage assay with a human factor VIII-deficient substrate and an aPTT reagent as the activator. Prothrombin was measured in a one-stage assay with an equal part mixture of rabbit serum reagent and barium-adsorbed rabbit plasma as the prothrombin-deficient substrate and rabbit tissue factor as the activator. Details of these assays are described

elsewhere (19).

### *Hematologic Tests*

WBC and platelet counts were determined with a Coulter ST counter (Coulter Electronics, Inc., Hialeah, FL) in the hematology laboratory of the UCSD Medical Center.

### *Animal Experimental Procedures*

Animal protocols. Female New Zealand rabbits, 2.0-2.4 kg, were used in protocols approved by the Animal Subjects Committee of the University of California, San Diego.

Blood sampling. Blood samples, 1.5-2.0 mL, were obtained by inserting a 23-gauge needle into a marginal ear vein and collecting 9 parts of blood into 1 part of balanced citrate anticoagulant in an Eppendorf tube. Platelet-poor plasma was obtained by double centrifugation at 4°C at 10,000 g and stored in capped plastic vials at -75°C. Blood for WBC and platelet counts was collected into EDTA-coated Becton-Dickinson microcontainers (Fisher Scientific, Pittsburgh, PA).

Administration of warfarin. Rabbits were injected intravenously daily with 0.5 mg/kg of a 1-mg/mL stock solution of sodium warfarin in isotonic sterile saline. Since warfarin prolonged the normal rabbit prothrombin time of 8-9 seconds to only 10-11 seconds, the modified P&P test described above was used as a more sensitive test to monitor warfarin treatment. With one exception, the rabbits used in experiments had a P&P test result on day 4 of warfarin between 5% and 30% of the activity of normal rabbit plasma.

Infusion of TF. Rabbits were infused through a marginal ear vein with 1 µg/kg of reconstituted

purified rabbit tissue factor in 100 mL of saline at a rate of 25 mL per hour. Intravascular coagulation was monitored at 2 and 4 hrs by measurement of fibrinogen, factor V and factor VIII.

### *Production of the LSR*

Skin sites were prepared by the intradermal injection of 200 µg of *E. coli* endotoxin dissolved in 0.5 mL of isotonic saline into the shaved skin of the lateral abdominal wall. Twenty-four hours later a provoking dose of 100 µg/kg of endotoxin was injected intravenously into a marginal ear vein. WBC and platelet counts and rectal temperatures were measured after both the preparatory and provoking injections. The rabbits were examined 4 hrs after the provoking injection. When an area of hemorrhagic necrosis, regardless of size, was seen at a prepared skin site, the site was considered as positive for the LSR (Figure 2). When no abnormality or only erythema was seen at a prepared skin site, the site was considered to be negative for the LSR.

In early experiments involving 17 rabbits, only a single skin site of a rabbit was prepared for the LSR. In later experiments involving 32 rabbits, two opposite sites of the shaved skin of the lateral abdominal wall were prepared for the reaction. In 27 of the animals, results were consistent in that both skin sites were either positive or negative for the LSR. In one control animal, two animals given warfarin, and two animals given anti-factor VII IgG, one skin site was positive and the other negative. These five animals have been counted as positive even if only a minimal area of hemorrhagic necrosis was seen at the positive site in order to eliminate any tilt of the data favoring the hypothesis that coagulation is a requirement for the LSR.

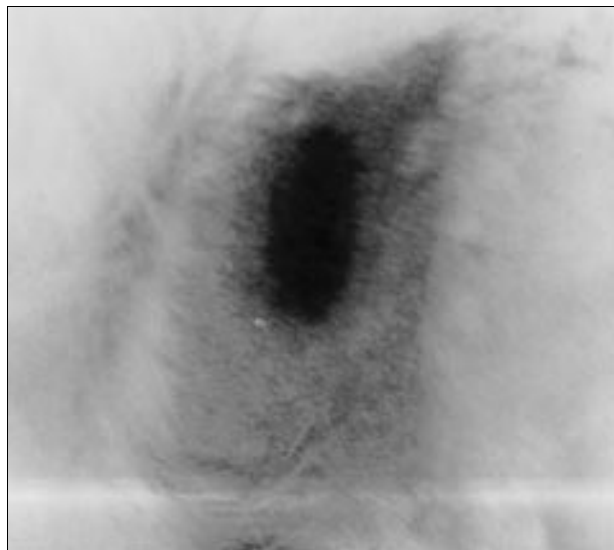


Figure 2: The appearance of a positive LSR in the shaved skin of the lateral abdominal wall of a control rabbit given a provoking injection of endotoxin 4 hrs earlier.

### Statistical Methods

When the frequency of the LSR in two groups of rabbits was compared, Fisher's exact test was used to evaluate statistical significance.

## RESULTS

### Control Animals

Thirteen control rabbits received a preparatory intradermal dose of endotoxin, an intravenous dose of endotoxin 24 hrs later, and no experimental agent. All animals had a rise of body temperature of 2-3°F and a substantial fall in WBC count of about 70% after both the preparatory and provoking injection of endotoxin. The platelet count, which was measured in five animals, fell to a mean value of 41% ± 17% of the initial value 4 hrs after the intravenous injection of endotoxin. Fibrinogen, factor V and factor VIII levels, also measured in these five animals, fell

after the intravenous injection of endotoxin (Table 1). All 13 animals developed a LSR.

### *The Failure of an Infusion of Tissue Factor to Provoke the LSR*

Twenty-four hours after the skin was prepared with an intradermal injection of endotoxin, four rabbits of 2 kg body weight were infused over a 4-hr period with 100 mL of saline containing 2 µg of purified rabbit TF apoprotein reconstituted into mixed phosphatidylcholine/phosphatidylserine vesicles. The TF infusion had no effect upon body temperature or upon the WBC count (mean WBC count before the infusion,  $8.6 \times 10^3$  per µL; mean WBC count at the end of the TF infusion  $7.9 \times 10^3$  per µL). In contrast, the mean platelet count fell to 63% ± 24% of the initial value by the end of the TF infusion. Fibrinogen, factor V and factor VIII levels fell substantially over the course of the TF infusion (Table 1). Despite initiating intravascular coagulation extensive enough to reduce the mean fibrinogen level to 35% of its initial plasma level, the infusion of TF failed to provoke the LSR in these four rabbits.

### *The Effect of Anticoagulation with Warfarin upon the LSR*

Twelve rabbits were injected intravenously with 0.5 mg/kg sodium warfarin daily to achieve a mean P&P test result after 4 days of warfarin of 15% ± 9% (range <5%-31%). In 8 of the 12 rabbits, the degree of anticoagulation fell within the 10%-30% P&P range recommended for oral anticoagulation therapy in patients (22). A preparatory dose of endotoxin was injected into one or both sides of the lower abdomen of experimental rabbits on the morning of the third

day of warfarin, and the intravenous provoking dose of endotoxin was given 24 hrs later. As described above for the control rabbits, all animals had a rise in temperature and fall in WBC count after both the preparatory and provoking injection of endotoxin. Seven of the 12 animals failed to develop a LSR after the provoking injection of endotoxin. This difference from the incidence in the control animals was significant ( $p=0.003$ ). It was not possible to relate the presence or absence of a LSR to an individual rabbit's P&P test result nor to its plasma level of prothrombin, factor VII or factor X activity as measured in assays that used only rabbit reagents (Table 2).

Table 1. Mean falls in plasma levels of factor V, factor VIII, and fibrinogen during an infusion of TF in experimental animals and after a provoking intravenous injection of endotoxin in control animals			
Clotting Factor		Percent of Initial Mean Value	
		TF-Infused (n=4)	Endotoxin Controls (n=5)
Factor V	2 hr	51 ± 21	69 ± 24
	4 hr	25 ± 16	44 ± 25
Factor VIII	2 hr	45 ± 30	78 ± 31
	4 hr	24 ± 11	56 ± 26
Fibrinogen	2 hr	56 ± 30	96 ± 11
	4 hr	35 ± 25	79 ± 25

\*For both groups, the mean initial fibrinogen level was approximately 200 mg/dL before the preparatory dose of endotoxin and was approximately 480 mg/dL 24 hours later before the intravenous administration of TF or endotoxin

### *The Effect of Selective Immunodepletion of Plasma Factor X Activity upon the LSR*

In an earlier study of the contribution of depression of individual procoagulant vitamin K-dependent clotting factors to warfarin's ability to prevent TF-induced intravascular coagulation (19),

we found that selective immunodepletion to 10-20% of normal plasma activity of factor X

Table 2. A tabulation of the intensity of anticoagulation with warfarin obtained in 12 rabbits on the day of a provoking injection of endotoxin, as measured by the P&P test and the activity of factors II, VII and X, and the presence or absence of a resultant LSR.					
	P&P (%)	FII (%)	FVII (%)	FX (%)	LSR
1	<5	4.5	1	2	+
2	5	11	6	7	-
3	7	2.5	3	4.5	+
4	7	7	2	3.5	-
5	11	4.5	16	11	+
6	13	4	2.5	10	-
7	15	12	10	14	+
8	17	7	30	17	-
9	20	ND	ND	ND	-
10	25	15	50	25	-
11	26	54	18	19	-
12	31	22	35	25	+

protected otherwise normal rabbits against the TF-induced intravascular coagulation. Therefore, it was decided to determine whether a similar selective immunodepletion of factor X activity to 10-20% would protect against the LSR. Nine rabbits were prepared with an injection of endotoxin into the skin of both sides of the abdomen. Twenty-four hours later they were given a bolus injection of 300 units of monoclonal anti-rabbit factor X IgG followed by an infusion over 4 hrs of 100 ml of saline containing an additional 200 units of the antibody. Mean cell counts were: before the injection of the IgG, WBC  $7.0 \pm 2.4 \times 10^3$  per  $\mu\text{L}$ , platelets  $453 \pm 140 \times 10^3$  per  $\mu\text{L}$ ; 30 min after the injection of the IgG, WBC  $6.6 \pm 3.0 \times 10^3$  per  $\mu\text{L}$ , platelets  $444 \pm 118 \times 10^3$  per  $\mu\text{L}$ . At

30 min after the injection of the IgG, at which time the plasma factor X activity was between 8% and 23% of normal, the provoking dose of endotoxin was injected into the infusion tubing. Body temperature rose and WBC fell in all animals after the injection of endotoxin. Mean cell counts 3-½ hrs after the injection of endotoxin were: WBC,  $2.2 \pm 0.9 \times 10^3$  per  $\mu\text{L}$ ; platelets,  $249 \pm 81 \times 10^3$  per  $\mu\text{L}$ . Mean values for five measurements of plasma factor X activity in each animal over the 4 hrs of IgG administration varied from 7%-18%. At the end of the IgG infusion, prepared skin sites were negative for the LSR in five animals and positive for the LSR in four rabbits (Table 3). This difference from the incidence in the control animals was significant ( $p=0.009$ )

	FX (%)		LSR
	Before Endotoxin*	Mean <sup>#</sup>	
1	8	7 (6-8)	-
2	8	8 (6-10)	-
3	10	9 (8-10)	-
4	11	9 (6-11)	-
5	15	11 (6-15)	+
6	21	16 (9-21)	+
7	17	17 (15-20)	-
8	21	18 (14-21)	+
9	23	18 (11-23)	+

\*30 min after the initial injection of anti-FX IgG.

<sup>#</sup>Of 5 values: the measurement before endotoxin and 4 measurements over 3-1/2 hrs after the injection of endotoxin. Values within parentheses are ranges.

### *The Effect of Selective Immunodepletion of Plasma Factor VII Activity upon the LSR*

Because endotoxin-induced coagulation is TF-dependent (14-15), and TF cannot initiate coagulation in the absence of factor VII, it was thought important to determine the effect of immunodepletion of plasma factor VII activity upon the ability of an intravenous injection of endotoxin to provoke the LSR. Eleven rabbits were prepared for the LSR with an injection of endotoxin into the skin of both sides of the abdomen. Twenty-four hours later they were injected intravenously with 500 units of goat anti-rabbit factor VII IgG followed by an infusion over the next 4 hrs of 100 ml of saline containing an additional 250-500 units of antibody. Mean cell counts were: before the injection of the IgG, WBC  $7.6 \pm 2.8 \times 10^3$  per  $\mu\text{L}$ , platelets  $430 \pm 76 \times 10^3$  per  $\mu\text{L}$ ; 30 min after the initial injection of the IgG, WBC  $8.8 \pm 2.0 \times 10^3$  per  $\mu\text{L}$ , platelets  $362 \pm 87 \times 10^3$  per  $\mu\text{L}$ . At 30 min after the initial injection of the IgG, plasma factor VII activity in all rabbits was <1%, and the provoking dose of endotoxin was injected into the infusion tubing. Body temperature rose after the injection of endotoxin in all animals and WBC counts fell in all animals except one. Mean cell counts measured 3-1/2 hours after the injection of the endotoxin were: WBC  $2.2 \pm 1.2 \times 10^3$  per  $\mu\text{L}$ ; platelets  $252 \pm 71 \times 10^3$  per  $\mu\text{L}$ . Mean values for five measurements of plasma factor VII activity over the 4 hrs of IgG administration varied between <0.5% and 2.2%.

Four hours after the endotoxin injection, six of the rabbits were negative for the LSR at both skin sites. Five rabbits were counted as positive for the LSR, although in two animals one skin site was negative and only minimal hemorrhagic necrosis was visible at the opposite skin site. The difference

in incidence of the LSR from that observed in the control animals was significant ( $p=0.007$ ). Although only one of five animals with a mean plasma factor VII activity of 0.5% or less had a positive LSR (Table 4), the mean plasma factor VII activity of the animals negative for the LSR did not differ from the mean plasma factor VII activity of the animals positive for the LSR ( $p=0.065$ ).

Table 4. A tabulation of the degree of immunodepletion of plasma factor VII activity in 11 rabbits and the presence or absence of the LSR			
	FVII (%)		LSR
	Before Endotoxin*	Mean#	
1	<0.5	<0.5	-
2	<0.5	<0.5	-
3	<0.5	<0.5	+
4	<0.5	0.5	-
5	<0.5	0.5	-
6	0.6	0.6	-
7	0.7	0.6	+
8	0.9	0.7	-
9	0.9	0.9	+
10	<0.5	1.2	+
11	0.5	2.2	+

\*30 min after the initial injection of anti-FVII IgG.

#Of 5 values: the measurement before endotoxin and 4 measurements over 3-1/2 hrs after endotoxin.

## DISCUSSION

The occluding thrombi of the capillaries and small veins of a prepared skin site that give rise to the hemorrhagic necrosis of the LSR contain masses of platelets and leukocytes and also fibrin (1,5,7). Formation of occlusive masses of leukocytes in the vessels of a prepared skin site--in

a reaction between ICAM-1 expressed on vascular endothelium and CD11/CD18 expressed on circulating leukocytes--now appears established as essential for the pathogenesis of the LSR (5). In contrast, limited and somewhat conflicting early data (8-13,16,17) had failed to establish whether the triggering of blood coagulation is a second essential event in the pathogenesis of the LSR.

The experiments here provide substantial evidence that impeding TF-initiated blood coagulation after a provoking injection of endotoxin can protect against the LSR. Whereas all 13 control rabbits developed the LSR after a provoking injection of endotoxin:

(1) Seven of 12 rabbits treated with warfarin to yield a mean P&P test value of  $15\% \pm 9\%$  at the time of the provoking endotoxin injection were protected against the LSR ( $p=0.003$ ).

(2) Five of nine rabbits given anti-rabbit factor X IgG before the provoking injection of endotoxin to yield mean values (of five measurements of factor X over the course of the experiment) in individual animals of between 7-18% were protected against the LSR ( $p=0.009$ ).

(3) Six of 11 rabbits given anti-rabbit factor VII IgG before the provoking injection of endotoxin to yield mean values (of five measurements of factor VII over the course of the experiment) in individual animals of between <0.5% and 2.2% were protected against the LSR ( $p=0.007$ ).

Overall, 18 of the 32 rabbits (56%) in which blood coagulation was impeded in one way or another were protected against the LSR. In the warfarin experiment we could not relate protection in an individual rabbit either to the level of the P&P test or to the residual activity of factor VII, factor X or prothrombin (Table 2). (For technical reasons it was not possible to measure accurately in rabbits the effect of warfarin upon the activity of the two vitamin K-dependent anticoagulant

factors, protein C and protein S.) However, in the immunodepletion experiments, there appeared to be a relation between the degree of depletion and protection against the LSR. All four rabbits with a mean factor X activity below 10% were protected against the LSR, whereas four of the five rabbits with a mean factor X activity above 10% were not protected against the LSR (Table 3). Four of the five rabbits with a mean factor VII activity of 0.5% or less were protected against the LSR, whereas four of the six rabbits with a mean factor VII activity between 0.6% and 2.2% were not protected against the LSR (Table 4).

The reason for choosing different target levels of immunodepletion of factor X and factor VII deserves mention. In our prior study (19), selective immunodepletion of plasma factor X activity to 10-20% had been found to protect otherwise normal rabbits against intravascular coagulation induced by an infusion of TF. In contrast, selective immunodepletion of plasma factor VII activity to 10%-15% had failed to protect otherwise normal rabbits against intravascular coagulation induced by the TF infusion, whereas depletion of plasma factor VII activity to <1% had protected the rabbits.

Factor VII has no known function in coagulation other than to serve as the enzyme of a factor VIIa/TF complex that triggers TF-initiated coagulation. The protection against the LSR afforded by selective immunodepression of factor VII reported here adds to earlier evidence from this laboratory (14,15) that the coagulation triggered in the rabbit after an intravenous injection of gram-negative endotoxin is TF-dependent.

However, it is important to emphasize that only a very low level of plasma factor VII activity may suffice for effective TF-dependent

coagulation. Clinical experience suggests that only patients with hereditary factor VII deficiency whose plasma factor VII activity is <1% experience severe hemorrhagic manifestations (23). In rabbits, an infusion of TF causing substantial intravascular coagulation failed to consume enough circulating factor VII to reduce its plasma level (14). As mentioned above, in our earlier study immunodepleting plasma factor VII activity to <1% protected rabbits against intravascular coagulation induced by infusing TF, whereas immunodepleting plasma factor VII activity to 10-15% did not protect the animals (19). Finally, although the present data are limited, it appears that selective depletion of plasma factor VII activity to 0.5% or less is required to assure that rabbits are protected against a LSR provoked by endotoxin. When considered together, these observations lead one to believe that only a trace plasma concentration of factor VII may suffice to support the participation of TF-dependent coagulation in the pathogenesis of at least some pathologic states.

Although the present data provide substantial evidence that TF-induced coagulation after a provoking injection of endotoxin is required for the pathogenesis of the LSR, an infusion of purified reconstituted TF, which caused a fall in the platelet count and plasma levels of fibrinogen, factor V and factor VIII (Table 1) but not in the WBC count, was incapable of provoking the LSR. We infer from this negative experiment that none of the products of the tissue factor-dependent blood coagulation reactions are capable of causing leukocytes to aggregate within the small vessels of a prepared site. It is evident that an agent, which, like endotoxin, can trigger tissue factor-induced intravascular coagulation, but which, unlike endotoxin, cannot also cause leukocytes to

accumulate in the vessels of a prepared site, will fail to provoke the hemorrhagic necrosis of the LSR.

Argenbright and Barton have proposed a mechanism for the development of the LSR in which a reaction between ICAM-1 on vascular endothelium and CD18 on leukocytes results in leukocyte-endothelial adhesion and leukocyte-leukocyte aggregation within the vessels of the prepared site, damage to their walls, and resultant hemorrhagic necrosis (5). The evidence presented here leads us to propose that the mechanism for the LSR postulated by Argenbright and Barton should be expanded to include a second event: deposition of fibrin due to TF-initiated coagulation within the vessels of the prepared skin site. It is of interest that whereas both events--formation of adherent, aggregated masses of leukocytes and coagulation with resultant fibrin deposition--are required for the LSR, inhibition by antibodies of either event appears capable of preventing the reaction.

#### ACKNOWLEDGEMENTS

Supported by grants No. HL 27234 and HL 42813 from the National Heart, Lung and Blood Institute. L.V.M.R. is the recipient of Research Career Development Award HL 02590 from the National Heart, Lung and Blood Institute. We thank Dr. Allen Leir, Professor Emeritus of Reproductive Medicine, UCSD, for advice on the statistical treatment of data.

#### REFERENCES

- Adcock DM, Hicks MJ. Dermatopathology of skin necrosis associated with purpura fulminans. *Semin Thromb Hemost* 16: 283-292, **1990**
- Brozna JP. Shwartzman reaction. *Semin Thromb Hemost* 16: 326-332, **1990**
- Beck G, Habicht GS, Benach JL, Miller F. Interleukin-I: A common endogenous mediator of inflammation and the local Shwartzman reaction. *J Immunol* 136: 3025-3031, **1986**
- Movat HZ, Burrowes CE, Cybulsky MI, Dinarello CA. Acute inflammation and a Shwartzman-like reaction induced by interleukin-1 and tumor necrosis factor. *Am J Pathol* 129: 463-476, **1987**
- Argenbright LW, Barton RS. Interactions of leukocyte integrins with intercellular adhesion molecule 1 in the production of inflammatory vascular injury in vivo. The Shwartzman reaction revisited. *J Clin Invest* 89: 259-272, **1992**
- Hjort PF, Rapaport SI. The Shwartzman reaction: pathogenetic mechanisms and clinical manifestations. *Annu Rev Med* 16: 135-167, **1965**
- Stetson CA Jr. Studies on the mechanism of the Shwartzman phenomenon. Certain factors involved in the production of the local hemorrhagic necrosis. *J Exp Med* 93: 489-504, **1951**
- Good RA, Thomas L. Studies on the generalized Shwartzman reaction IV. Prevention of the local and generalized Shwartzman reactions with heparin. *J Exp Med* 97: 871-888, **1953**
- Cluff LE, Berthrong M. The inhibition of the local Shwartzman reaction by heparin. *Bull of Johns Hopkins Hospital* 92: 353-363, **1953**
- Rall DP, Smith NH, Kelly MG. Effect of anticoagulants on local Shwartzman reaction. *Proc Soc Exp Biol Med* 88: 241-243, **1955**
- Spanoudis S, Eichbaum F, Rosenfeld G. Inhibition of the local Shwartzman reaction by Dicumarol. *J Immunol* 75: 167-170, **1955**
- Rothfeld B, Williams CH, Thaler WJ, Rodas JM. Effect of Dicumarol on localized Shwartzman reaction. *Proc Soc Exp Biol Med* 87: 5-7, **1954**
- Rosenfeld G, Spanoudis S, Nahas L. Influence of coagulation factors on the Shwartzman phenomenon. I: Factor VII. *Anais da Academia Brasileira de Ciencias* 31: 67-75, **1959**
- Warr TA, Rao LVM, Rapaport SI. 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. *Blood* 75: 1481-1489, **1990**
- Sandset PM, Warn-Cramer BJ, Maki SL, Rapaport SI. Immunodepletion of extrinsic pathway inhibitor sensitizes rabbits to endotoxin-induced intravascular coagulation and the generalized Shwartzman reaction. *Blood* 78: 1496-1502, **1991**
- Simmons J, Rapaport SI, Hjort PF. Failure to provoke the local Shwartzman reaction with thrombin. *Proc Soc Exp Biol Med* 124: 742-744, **1967**
- Pichier L, Hruby E, Schwarz HP. Attenuation of the Shwartzman reaction in rabbits by human activated protein C. *Thromb Haemost* 65: 1836a, **1991** (abstr)
- Rao LVM, Hoang AD. Purification and characterization of rabbit brain tissue factor. *Thromb Res* 56: 109-118, **1989**
- Zivelin A, Rao LVM, Rapaport SI. Mechanism of the anticoagulant effect of warfarin as evaluated in rabbits by selective depression of individual procoagulant vitamin K-dependent clotting factors. *J Clin Invest* 92: 2131-2140, **1993**
- Owren PA, Aas K. Control of dicumarol therapy and quantitative determination of prothrombin and proconvertin. *Scand J Clin Lab Invest* 3: 201-208, **1951**
- Clauss A. Gerinnungs physiologische schnell methode zue bestimmung des fibrinogens. *Acta Haematol* 17: 237-246, **1957**
- Le DT, Weibert RT, Sevilla BK, Donnelly KJ, Rapaport SI. The International Normalized Ratio (INR) for monitoring warfarin therapy: reliability and relation to other monitoring methods. *Ann Intern Med* 120: 552-558, **1994**
- Roberts HR, Lefkowitz JB: Inherited disorders of prothrombin conversion. In: Colman RW, Hirsh J, Marder VJ, Salzman EW, eds. *Hemostasis and Thrombolysis: Basic Principles and Clinical Practice*, ed. 3, Philadelphia, JB Lippincott, pp 200-218, **1994**