The Effect of Leukocyte and Platelet Transfusion on the Activation of Intravascular Coagulation by Endotoxin in Granulocytopenic and Thrombocytopenic Rabbits

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The effect of transfusion of peritoneal leukocytes, platelets, or cell suspension medium on the activation of intravascular coagulation and on the generation of capillary microclots was studied in 51 granulocytopenic and thrombocytopenic rabbits. Granulocytopenia and thrombocytopenia induced by feeding the cytotoxic drug busulfan prevented the activation of intravascular coagulation and the occurrence of renal glomerular microclots after two injections of endotoxin. The transfusion of platelets into busulfan-pretreated rabbits increased the mean platelet count from 2,400 to 205,000 cells/μl, but platelet-transfused rabbits did not exhibit activation of intravascular coagulation after endotoxin injection. If, however, granulocytopenic and thrombocytopenic rabbits were transfused with peritoneal leukocytes (1.0×10^9 cells/kg) before the second injection of endotoxin, activation of intravascular coagulation occurred, and microclot formation in renal glomerular capillaries was observed in a high percentage of animals. Positive reactions to endotoxin were obtained in leukocyte-transfused rabbits even with platelet counts of 1,000 cells/μl before the second injection of endotoxin. Thus platelets do not seem to be essentially involved in the activation of intravascular coagulation by endotoxin, whereas the presence of leukocytes is required for triggering endotoxin-induced generalized intravascular coagulation. (Am J Pathol 84:239–258, 1976).

THE GENERALIZED SHWARTZMAN REACTION (GSR) is a multifactorial entity characterized by the development of renal glomerular capillary microclots.[1,2] The reaction can be induced by two appropriately spaced intravenous injections of bacterial endotoxin. It is now well established that the reaction is mediated by a process of intravascular coagulation, since treatment with heparin, dicoumarol, or streptokinase protects animals against the development of the GSR,[3-6] and since hypofibrinogenemia prevents the reaction.[7] The generation of glomerular microclots is a two-phase event. First, the coagulation system has to be activated to form soluble fibrin complexes (soluble fibrin), and, second, these complexes have to be precipitated by a process independent of the coagulation mechanism.[8,9] Recent studies have shown that the endotoxin-induced precipitation of soluble fibrin is independent of the presence of platelets

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and leukocytes.[10,11] No obvious connection could be seen between the decrease in platelet counts and fibrin deposition in different organs following the second dose of endotoxin,[12] as platelet counts after endotoxin injection drop even in animals in which intravascular coagulation has been inhibited by nitrogen mustard or heparin treatment.[11,13]

The aim of this study was to investigate the role of leukocytes and or platelets in the activation of intravascular coagulation by endotoxin. The importance of platelets in triggering intravascular coagulation by endotoxin has been questioned, as Platelet Factor 3 (the most potent procoagulant constituent of platelets) did not induce the generalized Shwartzman reaction if intravenously infused into rabbits or rats prepared for the GSR by pregnancy, ε-aminocaproic acid, Thorotrast, or endotoxin.[14,15] Furthermore, intravascular coagulation and the generalized Shwartzman reaction could be evoked in C6-deficient rabbits,[16] although the platelets of these animals were not capable of releasing Platelet Factor 3 after endotoxin administration because of the defect in the complement system.[17]

The granulocytes have first been implicated in the pathogenesis of the GSR by Thomas and Good.[18] They demonstrated that severe neutropenia produced by nitrogen mustard treatment prevented the development of renal glomerular microclots. This experimental setup has been repeated several times, confirming the original data of Thomas and Good.[19-21] Based on their experiments, Horn and Collins [20] suggested that a clot-promoting factor originates in granulocytes and is operative in the activation of intravascular coagulation. As nitrogen mustard used for the induction of neutropenia also causes moderate thrombocytopenia, it has remained open to question whether platelets are equivalently involved in the activation of intravascular coagulation.

In the present study the generalized Shwartzman reaction was prevented by treating the rabbits with busulfan. This cytotoxic drug causes thrombocytopenia as well as granulocytopenia with extremely low platelet counts.[22] The present investigation indicates that transfusion of peritoneal leukocytes into thrombocytopenic as well as granulocytopenic rabbits restores the susceptibility to activation of intravascular coagulation by endotoxin. The transfusion of platelets, however, did not render the rabbits susceptible to develop intravascular coagulation after endotoxin injection.

Materials and Methods

Endotoxin

Salmonella enteritidis endotoxin, Lipopolysaccharide B (Difco Laboratories, Detroit, Mich.) was dissolved in sterile, pyrogen-free isotonic saline so that the desired amount of

endotoxin was contained in 1 ml. To induce intravascular coagulation and the generalized Shwartzman reaction, the rabbits received 25 μg of endotoxin as a first intravenous injection, and 150 μg as a second injection 22 hours later.

Busulfan

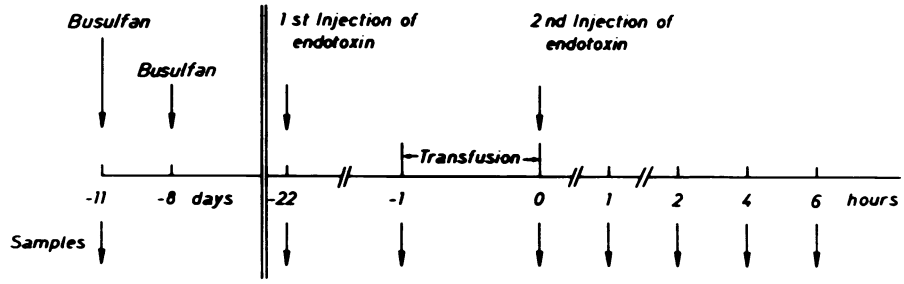
The alkylating agent was obtained from Burroughs Wellcome and Co., London, and dissolved in polyethylene glycol 400 (Roth, Karlsruhe, Germany) to a concentration of 5 mg/ml. To induce thrombocytopenia and leukopenia, the solution was given to the animals by means of a stomach tube as previously described.[10] Two doses of 25 mg busulfan/kg body weight each were given 72 hours apart (Text-figure 1).

Animals

Male and nonpregnant female rabbits of mixed New Zealand breeds were fed rabbit pellet food and water *ad libitum*. Rabbits used for the induction of intravascular coagulation weighed between 2.1 and 2.6 kg. Before feeding busulfan, blood was drawn from an ear vein. Immediately before injecting the first dose of endotoxin (Text-figure 1), the second blood sample was drawn from an ear vein. On the next day, one-half to 1 hour before starting the experiment, a plastic catheter was inserted into the marginal vein of an ear for infusing agents. Under local anesthesia, a second catheter was inserted into an ear artery for blood sampling. This catheter was continuously perfused by pyrogen-free sterile isotonic saline by means of an infusion pump to prevent occlusion of the catheter by clotting (speed of infusion, 0.9 ml/hr). This method only slightly affected the animals' good health and assured blood sampling over a longer period of time without general anesthesia. Immediately before starting the transfusion, a blood sample was drawn from the arterial catheter. Thereafter, leukocytes, platelets, or the cell suspension medium were intravenously administered over a period of 1 hour, and subsequently, another blood sample was obtained. Afterwards, the second dose of endotoxin was intravenously injected. The interval between the first and the second dose of endotoxin varied between 21 and 23 hours. Further blood samples were drawn as indicated in Text-figure 1. All rabbits were treated in the same manner with respect to operation, timing, and blood sampling. Furthermore, all rabbits received the same total volume of fluid.

Intravenous Infusions

Test materials and cells were dissolved and/or diluted for intravenous infusions or injections with sterile, pyrogen-free isotonic saline. Solutions were infused into unanesthetized rabbits with infusion pumps (model Perfusor from B. Braun, Melsungen, Germany).



TEXT-FIGURE 1—Experimental design showing time relationship of various injections and infusions into rabbits.

Preparation of Peritoneal Leukocytes for Transfusion

Suspensions of rabbit leukocytes were prepared from peritoneal exudates by a modification of the method of Hirsch.[23] Rabbits of the same strain as above, but weighing between 3 and 4 kg, were anesthetized with sodium pentobarbital and ether and intraperitoneally infused with 400 ml of isotonic saline containing 0.1 mg glycogen/ml. This procedure of priming was repeated once a week for at least 4 weeks to obtain a good yield of cells. Primed rabbits were again infused with 400 ml of glycogen-saline solution, and 6 to 8 hours later, rabbits were once again anesthetized, and exudates were obtained through inside-needle catheters (from C. R. Bard, England). The exudates were collected in chilled siliconized glass cylinders containing sodium citrate for anticoagulation. One part of 0.13 M trisodium citrate was mixed with nine parts of exudate. The yield obtained with 1 rabbit usually amounted to 200 to 300 ml of exudate composing 3000 to 4000 white blood cells (WBCs)/μl. Differential counts revealed 90 to 95% granulocytes.

Immediately after collection the exudates were filtered through four layers of gauze and centrifuged at 150g for 10 minutes, the supernatant was discarded, the packed cells resuspended and washed in a chilled solution containing eight parts isotonic saline, one part 0.13 M trisodium citrate, and one part Tris buffer (0.1 M, pH 7.4). Centrifugation, resuspension, and washing were once repeated. After the second washing, the cells were separated by centrifugation and resuspended in a solution containing six parts citrated rabbit plasma, nine parts washing solution, and five parts citrated saline. Total leukocyte counts were determined in a standardized volume, and the cell suspension transferred to a plastic syringe and further diluted so that 1.0×10^9 cells/kg body weight were contained in 20 ml solution. This amount of leukocytes was transfused over a period of 1 hour by slowly tilting the syringe in order to keep the cells in homogeneous suspension.

Preparation of Platelets for Transfusion

Blood samples for the preparation of platelets were obtained from rabbits of the same strain as above but weighing between 2.8 and 3.0 kg. With the rabbit under sodium pentobarbital anesthesia, a siliconized polyvinyl tubing with an inner diameter of 1.5 mm was inserted into a carotid artery, and the blood was collected into plastic tubes containing one part of an anticoagulant solution (0.07 M citric acid, 0.21 M sodium citrate) for nine parts of blood. The anticoagulant solution reduced the pH of the anticoagulated blood to 6.9 to 7.0. The blood was centrifuged at 100g for 10 to 30 minutes to obtain platelet-rich plasma. Platelets were concentrated by centrifugation at 1,300g for 30 minutes and resuspended in plasma saline solution (one part of plasma and one part of citrated saline).

Preparation of Rabbit Plasma for Transfusion

Rabbit blood was collected in one-tenth of its volume of anticoagulant (0.13 M sodium citrate), centrifuged at 1,300g for 30 minutes, and the platelet-poor plasma separated and recentrifuged at 10,000g for 5 minutes. Subsequently the plasma was filtered sterile through a Millex filter (0.22 μ; Millipore Co., Bedford, Mass.) and frozen to −22 C for storage. This plasma was used for preparing the cell suspension medium.

Experimental Protocol

All rabbits reported in this study were fed two doses of busulfan of 25 mg/kg each. The first dose was given 11 days and the second dose 8 days before the provoking second injection of endotoxin (Text-figure 1). Furthermore, all rabbits were intravenously injected with 25 μg of endotoxin for preparing and 21 to 23 hours later with 150 μg of endotoxin for provoking the generalized Shwartzman reaction. Only those animals are listed here and included in the statistical evaluation which lived for 6 hours after the second dose of endotoxin. The rabbits were divided into three groups as follows.

Group A

Twelve rabbits received busulfan and endotoxin and were transfused with leukocytes (1.0×10^9 cells/kg) over 1 hour before the second injection of endotoxin. Animals selected for this group had a mean WBC count of 1340 cells/μl and a mean platelet count of 9260 cells/μl before leukocyte transfusion. Rabbits exhibiting renal glomerular microclots were classified as Group A_1, and those without glomerular microclots as Group A_2.

Group B

Eleven rabbits received busulfan and endotoxin as in Group A but were transfused with platelets (1.5 to 3.0×10^{10} cells/kg) instead of leukocytes. Animals selected for this group exhibited a mean WBC count of 1400 cells/μl and a mean platelet count of 3650 cells μl before platelet transfusion.

Group C

Twenty-eight rabbits were given busulfan and endotoxin and were transfused with the cell suspension medium used for leukocyte suspension. Mean WBC count before transfusion amounted to 1400 cells/μl and the mean platelet count to 4900 cells/μl.

Blood (4.5 ml) was collected immediately before the first dose of busulfan; before the first and second injection of endotoxin; before starting the transfusion; and 1, 2, 4, and 6 hours after the second injection of endotoxin (Text-figure 1).

Hematologic Studies

Platelets were counted directly by phase contrast microscopy using procaine hydrochloride (3.5 g/100 ml). Leukocytes were counted directly by conventional method. Differential counts were obtained with few exceptions on 100 leukocytes in blood films stained with May-Gruenwald-Giemsa stain. In a few cases, leukocyte counts were extremely low, so that only 50 leukocytes were viewed for differentiation. Fibrinogen was determined by a slight modification of the method of Jacobsson [24] as described elsewhere.[25] Factor V activity was assayed according to Schultze and Schwick [26] using commercially available reagents (Behring-Werke, Marburg, Germany). Factor VII activity was determined in a one-stage test system [27] using congenital Factor VII–deficient plasma (Factor VII activity less than 1%). The partial thromboplastin time was performed according to Procter and Rapaport [28] using the reagents of Behring-Werke, Marburg. The one-stage prothrombin time activity was measured by the method of Quick [29] using the reagents of Merz and Dade, Bern, Switzerland. The ethanol gelation test was performed as described by Godal and Abildgaard.[30] Microhematocrits were determined using standard heparinized tubes (Clay Adams, New York, N.Y.). All the concentrations were corrected for anticoagulant dilution as described elsewhere.[25]

Histology

Six hours after the second dose of endotoxin, the surviving animals were sacrificed by an overdose of sodium pentobarbital, and necropsies were performed immediately. Organs were fixed in neutral 5% formalin. Sections were stained with hematoxylin and eosin, and fibrin was identified by the dimethylaminobenzaldehyde (DMAB)–nitrite reaction for the histochemical demonstration of tryptophane according to the method of Adams.[31]

Statistical Evaluation

The results were subjected to the analysis of variance (partially hierarchical situation with three factors) and the Scheffé test [32] using the statistical tables as described by Sachs.[33] Chi-square tests were applied to the histologic results to evaluate the significance of

differences between groups. The levels of significance are listed under Results; a value of less than $P = 0.05$ was considered significant.

Results

Pathologic Alterations

All rabbits were pretreated with busulfan to cause thrombocytopenia and leukopenia and injected with two doses of endotoxin in order to induce intravascular coagulation and the generalized Shwartzman reaction. Gross examination revealed congestion and hyperemia of the abdominal and thoracic viscera of the animals. Most of the thrombocytopenic animals of Groups A and C showed hemorrhages of different sizes in lungs and abdominal viscera. These latter changes were less frequently seen in rabbits transfused with platelets.

Microscopic examinations of the organs revealed striking differences between the groups. In the control group (Table 1, Group C) 3 of 28 leukopenic and thrombocytopenic rabbits exhibited microclots in the renal glomerular capillaries typical of the generalized Shwartzman reaction. If, however, leukopenic and thrombocytopenic rabbits were transfused with 1.0×10^9 leukocytes/kg before the second injection of endotoxin (Table 1, Group A) 8 of 12 animals demonstrated renal glomerular microclots 6 hours after the second injection of endotoxin. In Group B, the transfusion of platelets instead of leukocytes into thrombocytopenic and leukopenic rabbits did not restore the susceptibility to developing the generalized Shwartzman reaction: only 1 of 11 rabbits generated renal glomerular microclots after endotoxin injection. The incidence of microclot formation in the kidneys differed significantly between leukocyte-transfused and platelet-transfused rabbits as well as between leukocyte-transfused and control rabbits (Table 1). The incidence of microclot formation in liver, spleen, and lungs is summarized in Table 1. Similar to the findings in the kidneys, microclots were most frequently found in lungs of animals transfused with leukocytes. The incidence of microclot formation in liver and spleen, however, did not significantly differ between groups.

Hematologic Studies

Text-figures 2–8 show the mean levels of the individual measurements. The values for all the parameters determined and for all groups were not corrected for hemodilution, since the drop in packed cell volume (PCV) was uniform in all groups ($F_{interaction} = 0.85$; $\nu_1 = 21$; $\nu_2 = 301$; $P > 0.1$). In Groups B and C, the values of only those animals without renal glomerular microclots were reproduced, whereas the animals of Group A were

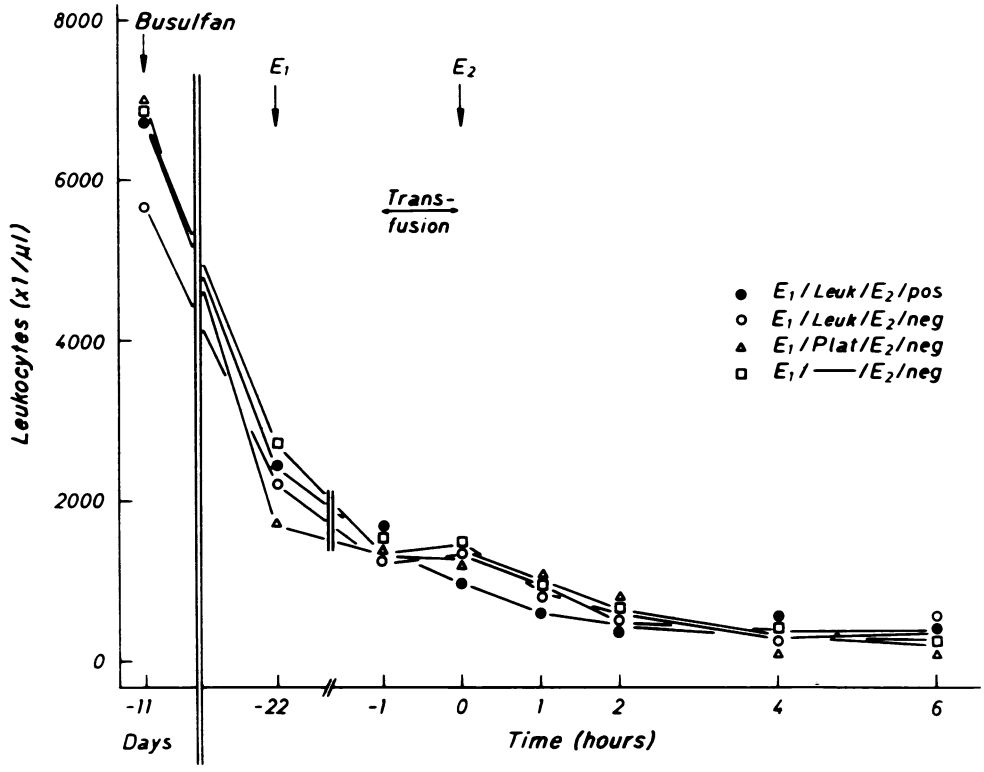
Table 1—Incidence of Microclot Formation in Renal Glomerular Capillaries, Lungs, Liver, and Spleen After Two Intravenous Injections of Endotoxin (E_1, E_2) in Rabbits

Group	Treatment	No. of rabbits	Incidence of microclots in			
			Renal glomerular capillaries	Lungs	Liver	Spleen
A_1	E_1/Leuk/E_2/pos	8	8/8	7/8	2/8	7/8
A_2	E_1/Leuk/E_2/neg	4	0/4	2/4	0/4	2/4
$A_1 + A_2$	E_1/Leuk/E_2	12	8/12	9/12	2/12	9/12
B_1	E_1/Plat/E_2/pos	1	1/1	1/1	1/1	1/1
B_2	E_1/Plat/E_2/neg	10	0/10	4/10	1/10	6/10
$B_1 + B_2$	E_1/Plat/E_2	11	1/11 $P < 0.01$*	5/11 NS*	2/11 NS*	7/11 NS*
C_1	E_1/—/E_2/pos	3	3/3	2/3	0/3	3/3
C_2	E_1/—/E_2/neg	25	0/25	8/25	3/25	10/25
$C_1 + C_2$	E_1/—/E_2	28	3/28 $P < 0.001$†	10/28 $P < 0.05$†	3/28 NS†	13/28 NS†

The animals of Group A were transfused with leukocytes (*Leuk*), animals of Group B were transfused with platelets (*Plat*), and animals of Group C were transfused with the cell suspension medium before the second injection of endotoxin. The groups are subdivided according to the presence (*pos*) or absence (*neg*) of microclots in the kidneys.

* P between Groups A and B.

† P between Groups A and C.

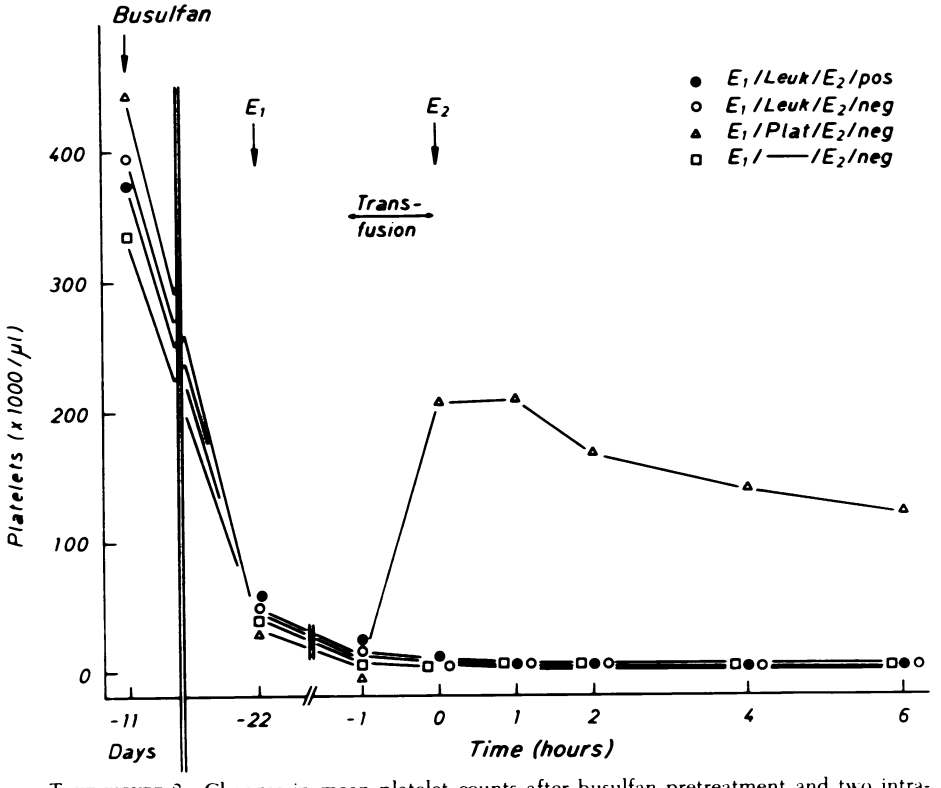


TEXT-FIGURE 2—Changes in mean leukocyte counts after busulfan pretreatment and two intravenous injections of endotoxin (E_1 and E_2) into leukocyte-transfused (*Leuk*), platelet-transfused (*Plat*), and control rabbits. Animals with renal glomerular microclots, *pos*; animals without renal glomerular microclots, *neg*. Group A_1 (N = 8), *solid circles*; Group A_2 (N = 4), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 25), *open squares*. The curves do not significantly differ from each other ($P > 0.05$).

subdivided into Groups A_1 and A_2 according to the presence or absence of renal glomerular microclots. This latter procedure could not be employed on the animals of Groups B and C because of the small numbers of positive results. All animals pretreated with busulfan demonstrated a decrease in leukocyte as well as in platelet counts (Text-Figures 2 and 3). After the first injection of endotoxin, leukocyte counts dropped furthermore and did not show any increase before the second injection of endotoxin as it is observed in normal untreated rabbits. The mean leukocyte count of all 51 busulfan-pretreated rabbits decreased from 6900 WBC/µl to 2500 WBC/µl (37% of the initial values) before the first injection of endotoxin and to 1300 WBC/µl (20% of the initial values) before starting the transfusions. The mean relative granulocyte count

decreased from 2600 cells/μl ± 1700 (mean ± SD) before busulfan to 76 cells/μl ± 92 before the second injection of endotoxin. Leukocyte transfusion did not measurably increase the numbers of WBCs. After the second injection of endotoxin, the WBC count only slightly but not significantly decreased (Text-figure 2). This drop in the WBC count parallels the decrease in PCV. Significant differences between the groups could not be computed (Table 2).

Busulfan pretreatment affected peripheral platelet counts, since 10 days after this treatment, platelet counts were decreased to 11% of the initial values. After the first injection of endotoxin, platelet counts dropped similarly to a mean value of 5500 platelets/μl (1.5% of the initial values). Platelet transfusion (Group B) increased the mean platelet count from 2,400 to 205,000 cells/μl. If endotoxin was injected into these animals, platelet counts decreased in the following 6 hours to a mean of



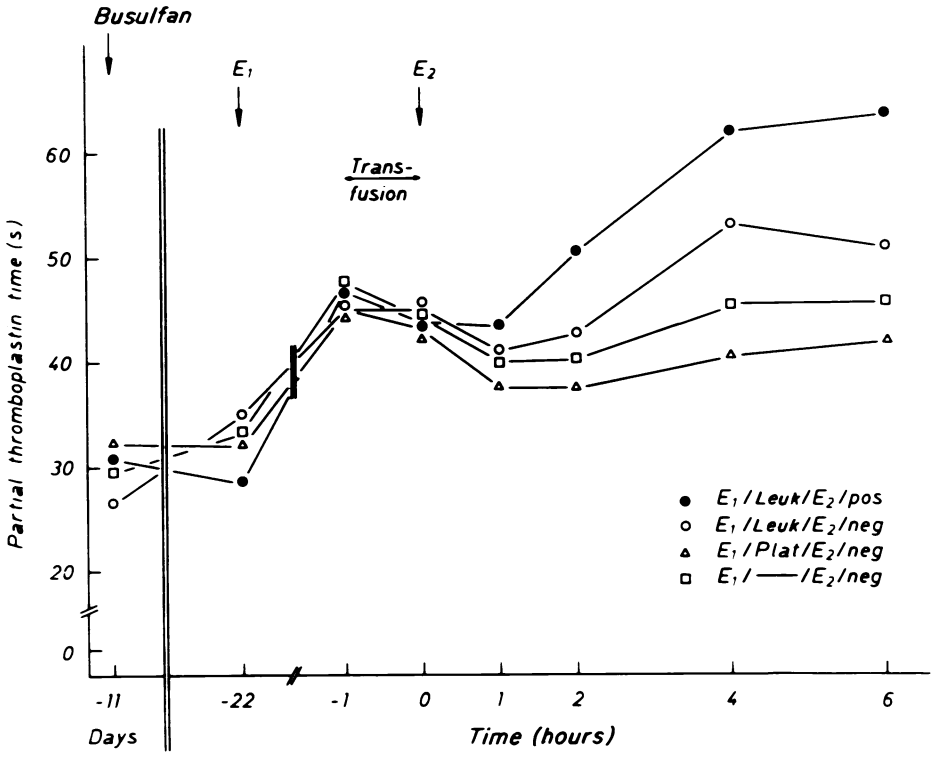
TEXT-FIGURE 3—Changes in mean platelet counts after busulfan pretreatment and two intravenous injections of endotoxin into leukocyte-transfused, platelet-transfused, and control rabbits. Group A_1 (N = 8), *solid circles*; Group A_2 (N = 4), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 25), *open squares*. The curve of Group B_2 differs significantly from those of the other groups ($P < 0.001$; for statistics see Table 2). See Text-figure 2 for abbreviations.

Table 2—Confidence Limits at Different Levels of Significance and Differences Between Means of Group A_1 and Those of Group B_2 at Different Times Before and After the Second Injection of Endotoxin

Confidence limits*	WBC/μl	Platelet counts (× 1000/μl)	Partial thromboplastin time (sec)	Prothrombin time activity (%)	Factor V activity (%)	Factor VII activity (%)	Fibrinogen (mg/100 ml)
$D_{0.05}$	1422	71	8.6	11.1	24.8	18.0	60.3
$D_{0.01}$	1695	84	10.4	13.2	30.1	21.8	71.9
$D_{0.001}$	2022	100	12.6	15.8	36.4	26.4	85.8

Differences between means of Group A_1 and Group B_2†		P		P		P		P		P		P		P
At −11 days	146	NS	68.9	NS	1.5	NS							2.7	NS
At −22 hrs	746	NS	15.2	NS	3.6	NS							35.6	NS
At 1 hr	673	NS	8.4	NS	1.6	NS							18.5	NS
At 0 hr	375	NS	197.0	<0.001	0.8	NS	2.3	NS	2.1	NS	1.9	NS	13.2	NS
At 1 hr	351	NS	200.2	<0.001	6.1	NS	8.0	NS	3.5	NS	11.8	NS	11.4	NS
At 2 hrs	133	NS	160.0	<0.001	13.1	<0.01	10.3	<0.001	38.1	<0.001	21.0	<0.05	8.8	NS
At 4 hrs	85	NS	135.2	<0.001	21.6	<0.001	21.5	<0.001	54.8	<0.001	42.6	<0.001	40.8	NS
At 6 hrs	199	NS	118.6	<0.001	21.8	<0.001	17.0	<0.05	58.6	<0.001	51.3	<0.001	67.8	<0.05

* D = confidence limits, at $P < 0.05$, $P < 0.01$, and $P < 0.001$.
† Group A_1 (E_1/Leuk/E_2/pos), Group B_2 (E_1/Plat/E_2/neg).



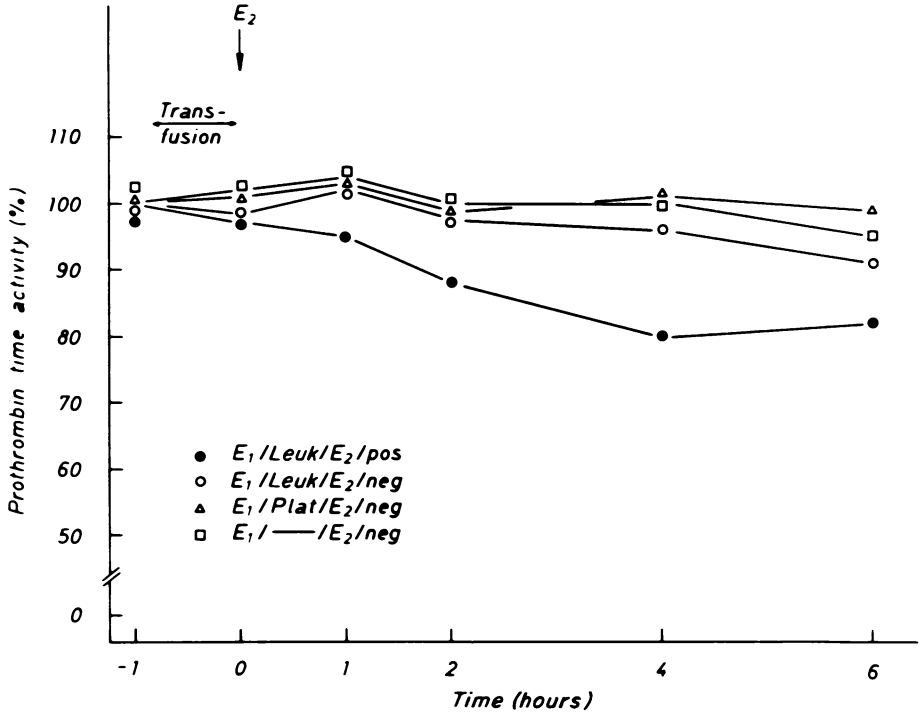
TEXT-FIGURE 4—Changes in mean values of the partial thromboplastin time after busulfan pretreatment and two intravenous injections of endotoxin into leukocyte-transfused, platelet-transfused, and control rabbits. Group A_1 (N = 7), *solid circles*; Group A_2 (N = 4), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 20), *open squares*. The curve of Group A_1 differs significantly from those of Groups B_2 and C_2 at 2, 4, and 6 hours. The curves of Groups A_1 and A_2 differ significantly from each other only at 6 hours (for statistics see Table 2). See Text-figure 2 for abbreviations.

121,000 cells/μl, but renal glomerular microclots did not generate in these animals. The rabbits of Group A_1—transfused with leukocytes, but exhibiting a mean platelet count of 9000 cells/μl—however, developed glomerular microclots. Some of these leukocyte-transfused animals demonstrated glomerular microclots although their platelet counts before the second injection of endotoxin were less than 1500 cells μl.

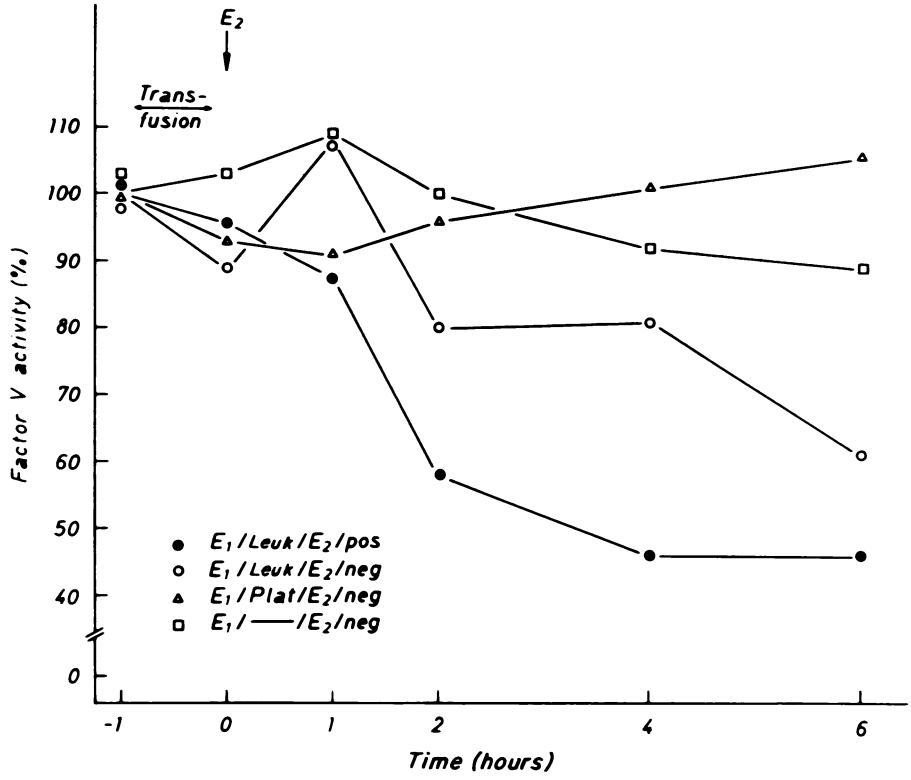
The activation of intravascular coagulation was controlled by measuring individual factor activities and by performing global coagulation assays. Text-figure 4 shows the prolongation of the partial thromboplastin time after the first injection of endotoxin. Before starting the transfusion and before the second injection of endotoxin, no significant differences between the groups could be computed (Table 2). Control rabbits transfused with the cell suspension medium, as well as rabbits transfused with

platelets, did not reveal a significant prolongation of the partial thromboplastin time after the second injection of endotoxin, whereas all rabbits transfused with leukocytes exhibited a prolongation of the partial thromboplastin time. This prolongation was most pronounced in animals demonstrating renal glomerular microclots. In the course of the experiment the prothrombin time activity was prolonged only in those animals which were transfused with leukocytes and injected with endotoxin (Text-figure 5 and Table 2).

In the same manner as the global coagulation assays, individual factor activities demonstrated an activation of intravascular coagulation only in those rabbits transfused with leukocytes. Factor V as well as Factor VII activities continously decreased in the leukocyte-transfused rabbits after the second injection of endotoxin (Text-figures 6 and 7). These coagulation factor activities dropped most pronouncedly in those rabbits demonstrating renal glomerular microclots.

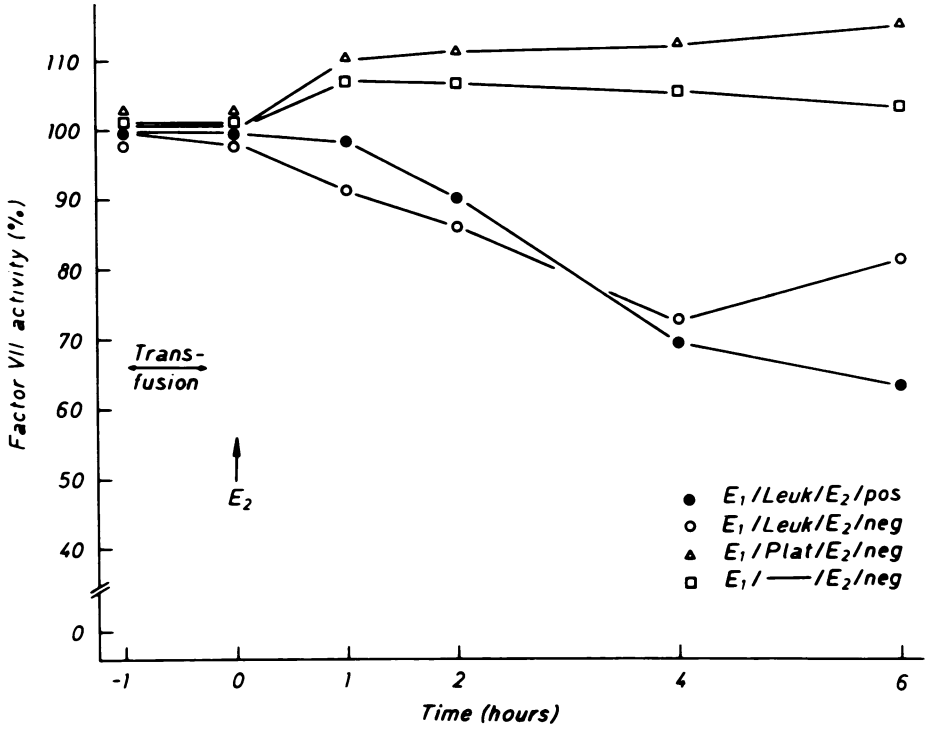


TEXT-FIGURE 5—Changes in mean values of prothrombin time activity after busulfan pretreatment and two intravenous injections of endotoxin into leukocyte-transfused, platelet-transfused, and control rabbits. Group A_1 (N = 8), *solid circles*; Group A_2 (N = 4), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 24), *open squares*. The curve of Group A_1 differs significantly from that of Group B_2 at 2, 4, and 6 hours (for statistics see Table 2). For abbreviations see Text-figure 2.



TEXT-FIGURE 6—Changes in mean Factor V activities after busulfan pretreatment and two intravenous injections of endotoxin into leukocyte-transfused, platelet-transfused, and control rabbits. Group A_1 (N = 8), *solid circles*; Group A_2 (N = 10), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 24), *open squares*. The curve of Group A_1 differs significantly from that of Group B_2 at 2, 4, and 6 hours (for statistics see Table 2). See Text-figure 2 for abbreviations.

The mean fibrinogen levels of all groups increased after treatment of the animals with busulfan. The first injection of endotoxin also raised the mean fibrinogen concentration. After the second injection of endotoxin the mean fibrinogen level of the leukocyte-transfused rabbits exhibiting glomerular microclots slightly decreased in comparison to animals not showing glomerular microclots, but this difference was not significant at any time of the experiment. Only at 6 hours after the second injection of endotoxin did the mean fibrinogen concentration of leukocyte-transfused rabbits showing renal glomerular microclots (Group A_1) differ significantly from that of platelet-transfused rabbits not exhibiting renal glomerular microclots (Group B_2). Although the mean fibrinogen level did not significantly differ between the groups studied prior to the 6 hour blood sample, the ethanol gelation test distinctly demonstrated an activation of in-



TEXT-FIGURE 7—Changes in mean Factor VII activities after busulfan pretreatment and two intravenous injections of endotoxin into leukocyte-transfused, platelet-transfused, and control rabbits. Group A_1 (N = 8), *solid circles*; Group A_2 (N = 4), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 24), *open squares*. The curve of Group A_1 differs significantly from that of Group B_2 at 2, 4, and 6 hours (for statistics see Table 2). See Text-figure 2 for abbreviations.

travascular coagulation (Table 3). Before starting the transfusions and before the second injection of endotoxin, the ethanol gelation test was negative with a few exceptions. Gradually, 2, 4, and 6 hours after the second injection of endotoxin, the ethanol gelation test became positive particularly in leukocyte-transfused rabbits. Eleven of 12 leukocyte-transfused rabbits demonstrated positive ethanol gelation tests 6 hours after the second injection of endotoxin, whereas platelet-transfused rabbits as well as control rabbits showed positive test results only in 10 of 25 and 4 of 10 rabbits, respectively. Consequently, the results of the ethanol gelation test obtained in leukocyte-transfused rabbits differed significantly from those obtained in platelet-transfused as well as control animals ($P < 0.01$).

Discussion

It is recognized that the method in the present study used for the production of thrombocytopenia in rabbits is cumbersome, but of the

Table 3—Incidence of Positive Ethanol Gelation Tests After Two Intravenous Injections of Endotoxin Into Rabbits Transfused With Leukocytes, Platelets, or Cell Suspension Medium*

Group	Treatment	Before starting the transfusions	Before E_2	After E_2			
				1 hr	2 hrs	4 hrs	6 hrs
A_1	E_1/Leuk/E_2/pos	0/8	1/8	4/8	7/8	7/8	7/8
A_2	E_1/Leuk/E_2/neg	0/4	1/4	2/4	4/4	4/4	4/4
B_2	E_1/Plat/E_2/neg	0/10	1/10	0/10	1/10	4/10	4/10
C_2	E_1/—/E_2/neg	3/25	3/25	3/25	4/25	7/25	10/25

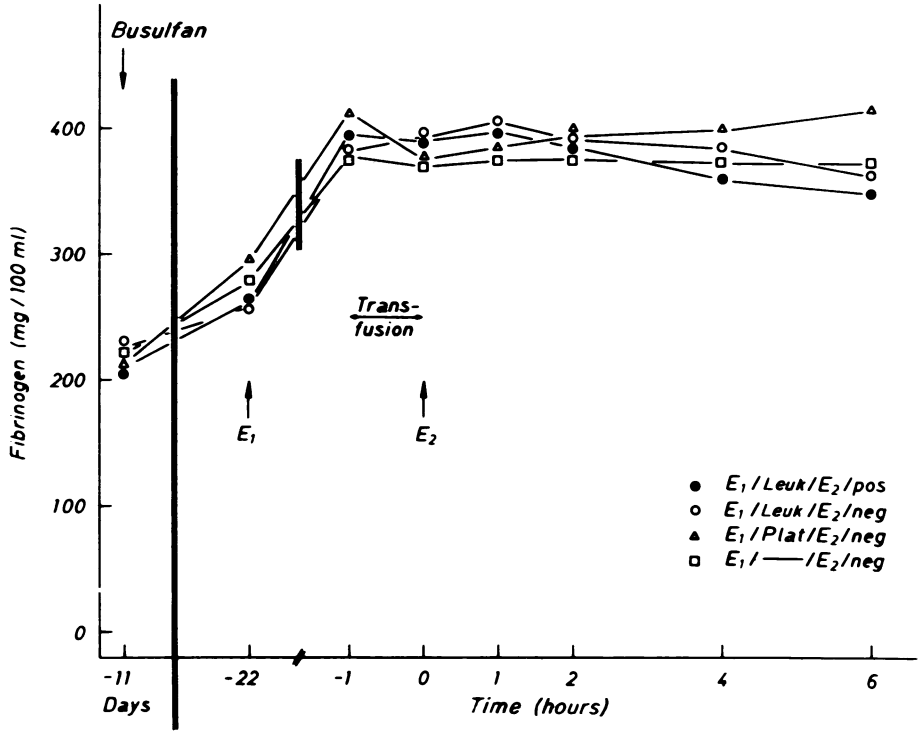
* Expressed as the number of positive tests per total number of rabbits studied. The results obtained at 2, 4, and 6 hours after the second injection of endotoxin differ significantly between Groups A_1 and A_2 and B_2 as well as between Groups A and C_2 ($P < 0.01$). For abbreviations see Table 1.

procedures tried and described in the literature, it was the only one that resulted in thrombocytopenia with extremely low platelet counts.

A reduction in platelet count can be achieved by intravenous administration of neuraminidase.[34-36] Platelet counts after the administration of neuraminidase, however, are not low enough to yield definitive evidence against or for the role of platelets in the induction of intravascular coagulation, as platelet counts in the range between 20,000 and 50,000/μl are observed already after a preparatory injection of Thorotrast.[37,38] In spite of platelet counts between 20,000 and 50,000/μl, intravascular coagulation and the generalized Shwartzman reaction can be induced by the provoking injection of endotoxin.

In another experimental setup, the effect of endotoxin on the production of the generalized Shwartzman reaction was studied in platelet antiserum–induced thrombocytopenia.[39] The results obtained by these authors may be reinterpreted since the significance of the immune reaction in the production of thrombocytopenia should not be ignored in these investigations. There may be cross reactions with leukocytes or with the complement system.

All the *in vivo* experiments dealing with the role of platelets in the induction of generalized intravascular coagulation by endotoxin are missing the corresponding controls which would indicate that low platelet counts obtained by the different methods that can cause thrombocytopenia no longer influence the coagulation system. Two experimental models could probably overcome this difficulty: a) A dose-response study between platelet count and the incidence of intravascular coagulation would determine whether platelets are essential to the reaction. b) Inhibition of bone marrow cell function and restoring the destroyed cell population by transfusion is a tool that could exclude the cell population not essential to the induction of intravascular coagulation by endotoxin.



TEXT-FIGURE 8—Changes in mean fibrinogen concentrations after busulfan pretreatment and two intravenous injections of endotoxin into leukocyte-transfused, platelet-transfused, and control rabbits. Group A_1 (N = 8), *solid circles*; Group A_2 (N = 4), *open circles*; Group B_2 (N = 10), *open triangles*; Group C_2 (N = 24), *open squares*. The curves do not differ significantly from each other except for the 6-hour determination (for statistics, see Table 2). See Text-figure 2 for abbreviations.

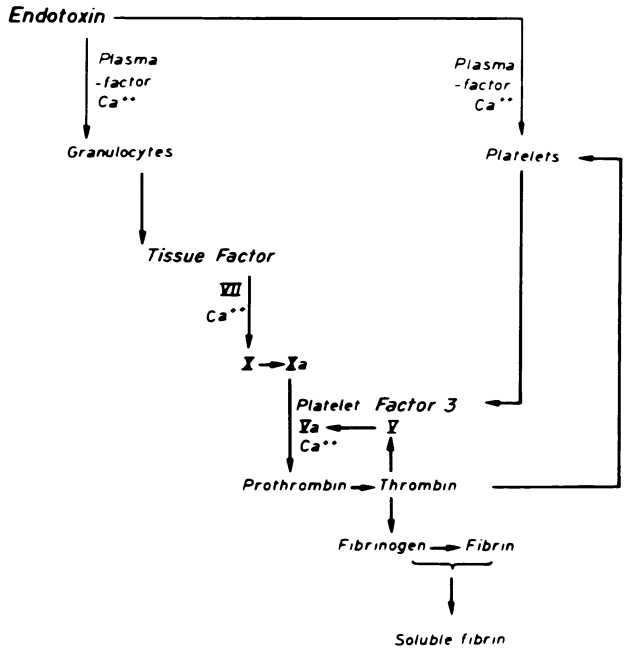
The first model has been studied.[40] A positive dose-response between platelet count before the second provoking injection of endotoxin and the occurrence of renal glomerular microclots after endotoxin injection could not be established if platelet counts were lowered within a range of 50,000 to 500 platelets/μl. Platelet counts before neither the first nor the second injection of endotoxin decisively contributed to the activation of coagulation.

The second model is presented in this study. Thrombocytopenia as well as granulocytopenia were induced in rabbits by feeding the cytotoxic drug busulfan. With a few exceptions, animals treated in this manner did not develop the generalized Shwartzman reaction after two injections of endotoxin. Only 3 of 28 rabbits demonstrated renal glomerular microclots, presumably because these 3 animals exhibited relatively high granulocyte counts before the second injection of endotoxin. If granulocytopenic and

thrombocytopenic rabbits were transfused with platelets so that their cell counts were normal, the succeeding injection of endotoxin did not initiate the generalized Shwartzman reaction. Although the transfused platelets reacted to the injection of endotoxin by disappearing from the circulating blood, intravascular coagulation was not decisively activated. The global coagulation assays as well as Factor V and Factor VII activities did not demonstrate any activation of intravascular coagulation. The ethanol gelation test was positive in 4 of 10 rabbits. This result may indicate some activation of intravascular coagulation, or it may be explained by elevation of the fibrinogen concentration because of an accelerated synthesis of this protein after endotoxin administration.[41] In the present study, platelet-transfused rabbits demonstrated an increase rather than a drop in fibrinogen concentration. All these parameters studied did not significantly differ from those obtained in control rabbits transfused with the cell suspension medium. Therefore, these experiments seem to indicate that platelets are not essential to the activation of intravascular coagulation by endotoxin.

If another group of rabbits with comparable low platelet counts as well as low granulocyte counts were transfused with exudate leukocytes before the second injection of endotoxin, the susceptibility to develop the generalized Shwartzman reaction after endotoxin administration was restored. Rabbits transfused with 1.0×10^9 leukocytes/kg of body weight and injected with two doses of endotoxin displayed the generalized Shwartzman reaction in a high percentage and exhibited a prolongation of the partial thromboplastin time and a decrease in prothrombin time activity as well as in Factor V and Factor VII activities. The ethanol gelation test indicating the presence of soluble fibrin in the circulating blood was positive in 11 of 12 animals.

The trigger mechanism of generalized intravascular coagulation by endotoxin is not completely understood. The present investigation stresses the importance of granulocytes and denies the role of platelets in triggering intravascular coagulation. Stetson and Good,[42] Horn and Collins,[20] and Niemetz and Fani [43] have recognized the importance of leukocytes in activation of intravascular coagulation by endotoxin. The investigations of Lerner *et al.*,[44] Kociba and Griesemer,[45] and Niemetz and Fani [46] render it likely that thromboplastin-like material released from leukocytes represents the trigger of endotoxin-induced intravascular coagulation. The activation sequence of generalized intravascular coagulation as it can be drawn from these studies is represented in Text-figure 9. The main target cell for endotoxin in triggering intravascular coagulation is the granulocyte, whereas the platelet and its constituents may only accelerate



TEXT-FIGURE 9—Activation of intravascular coagulation by endotoxin. Tissue factor (thromboplastin-like material) released from granulocytes activates the extrinsic coagulation system, whereas platelets and their constituents represent non-essential accelerators of coagulation only.

intravascular coagulation. Data recently published by Rivers *et al.*[47] indicate that the thromboplastin-like material released from leukocytes exposed to endotoxin *in vitro* is derived from monocytes and not from granulocytes, as has been assumed up to the present. *In vivo* experiments have to be performed to confirm these results obtained *in vitro*.

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