Disseminated Intravascular Coagulation Induced with Leukocyte Procoagulant

Gary J. Kociba, DVM, PhD and Richard A. Griesemer, DVM, PhD

The procoagulant activity of rabbit peritoneal leukocytes significantly increased when the leukocytes were incubated in suspension cultures at 37 C for 24 hours. Intravenous infusions of lysates of $232 \times 10^6$ rabbit leukocytes which had been incubated in cultures at 37 C for 24 hours produced disseminated intravascular coagulation and vasculitis involving the pulmonary arteries in normal rabbits. Intravenous infusions of lysates of $230 \times 10^6$ similarly incubated leukocytes produced renal thrombosis and renal cortical necrosis in normal rabbits. These observations suggest that the procoagulant of granulocytic leukocytes could play a role in the generalized Shwartzman reaction and other syndromes of disseminated intravascular coagulation (Am J Pathol 69:407-420, 1972).

**Disseminated Intravascular Coagulation** is recognized as a pathologic phenomenon secondary to a wide variety of disease processes. The generalized Shwartzman reaction is an experimental model for disseminated intravascular coagulation. This experimental disease of rabbits is characterized by disseminated intravascular coagulation, with capillary thrombosis in many organs and renal cortical necrosis. The pathogenetic mechanism of the generalized Shwartzman reaction is not known, although several studies indicate that leukocytes play a fundamental role in its development. McKay et al. proposed that damaged leukocytes release a thromboplastic substance which causes disseminated intravascular coagulation and glomerular capillary thrombosis. Their attempt to produce the generalized Shwartzman reaction using infusions of lysates of rabbit heterophils was unsuccessful. Previous experiments have revealed only weak thromboplastic activity in rabbit leukocytes. Horn and Collins suggested that thromboplastic activity may be a constituent of granulocytes and successfully produced the lesions of the generalized Shwartzman reaction using a combination of endotoxin and leukocytes or endotoxin and leukocyte fractions.

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We have found that canine granulocytic leukocytes and rabbit leukocytes develop increased procoagulant activity when incubated in cultures at 37 C for 24 hours. The leukocyte procoagulant has characteristics similar to tissue thromboplastin. The purpose of this report is to describe the development of procoagulant activity in cultured rabbit heterophils and the induction of disseminated intravascular coagulation in rabbits using intravenous infusions of lysed rabbit heterophils.

**Materials and Methods**

Male New Zealand albino rabbits weighing 1.8 to 2.4 kg were used for the infusion studies. Adult New Zealand albino rabbits of either sex were used as leukocyte donors.

Peritoneal exudates containing approximately 98% granulocytic leukocytes were collected from rabbits 4 hours after intraperitoneal injection of 0.1% glycogen in physiologic saline. The leukocytes were washed with Eagle's Minimum Essential Medium and cultured as stationary suspensions at a concentration of $6.0 \times 10^6$ leukocytes ml, with 20 ml 4 oz bottle. The culture medium consisted of Eagle’s Minimum Essential Medium with penicillin (25.0 units ml), streptomycin (0.1 mg ml) and 20% normal rabbit serum (Flow Laboratories, Rockville, Md). The leukocytes in their medium were frozen at $-20$ C without incubation (0-hours) or after 24 hours incubation at 37 C.

Leukocyte lysates were prepared by centrifugation of the previously frozen leukocyte cultures at 16,000g for 15 minutes, resuspension of the sediment in 30 ml of physiologic saline and ultrasonic treatment (Branson Sonifier, Model S125, Branson Instruments, Danbury, Conn) at 7.5 amp for 30 seconds. The intravenous infusions were performed in unanesthetized rabbits through a cannula (Intrufusor, No. V-5919, McGaw Laboratories, Inc, Milledgeville, Ga) inserted in the central ear vein. Twenty-nine ml of a lysate of $232 \times 10^6$ leukocytes in physiologic saline were infused at a constant rate over a 2-hour period using a Technicon roller pump. Intraaortic infusions were performed in rabbits anesthetized with sodium pentobarbital through a cannula passed down the ligated left carotid artery to the approximate level of the aortic arch. Twenty-four ml of lysate of $230 \times 10^6$ leukocytes in physiologic saline were infused at a constant rate over a 4-hour period.

The experimental groups of rabbits and their treatments are presented in Table 1. The rabbits of Groups A and B were pretreated with intramuscular injections of 25 mg of hydrocortisone acetate (Merck, Sharp and Dohme, West Point, Pa) for 3

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rabbits</th>
<th>Route of infusion</th>
<th>Length of incubation at 37 C (hrs)</th>
<th>Pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4</td>
<td>Intravenous</td>
<td>24</td>
<td>Cortisone, heparin</td>
</tr>
<tr>
<td>B</td>
<td>4</td>
<td>Intravenous</td>
<td>24</td>
<td>Cortisone</td>
</tr>
<tr>
<td>C</td>
<td>4</td>
<td>Intravenous</td>
<td>24</td>
<td>None</td>
</tr>
<tr>
<td>D</td>
<td>4</td>
<td>Intravenous</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>E</td>
<td>5</td>
<td>Intraaortic</td>
<td>24</td>
<td>None</td>
</tr>
<tr>
<td>F</td>
<td>4</td>
<td>Intraaortic</td>
<td>0</td>
<td>None</td>
</tr>
</tbody>
</table>
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days prior to infusion. In addition to cortisone pretreatment, Group A received 1500 units kg of heparin (Fellows-Testagar, Detroit, Mich) intravenously at 0 hours and 750 units kg intravenously after the completion of 2-hour intravenous infusions. The heparin was administered to inhibit blood coagulation in the rabbits of Group A, which were considered the controls for comparison of the groups.

Four hours after the start of the infusions, all surviving rabbits were killed with an overdose of sodium pentobarbital and necropsied. Representative sections of tissues were fixed in 10% buffered formalin or Zenker's fixative, sectioned at 6 μ and stained with hematoxylin and eosin. Sections of kidney and other selected tissues were stained with phosphotungstic acid hematoxylin (PTAH) or periodic-acid-Schiff.

Six-ml blood samples were collected in plastic syringes from the external jugular veins of rabbits which received intravenous infusions at 0, 2 and 4 hours and from rabbits which received intraaortic infusions at 0 and 4 hours. Blood for coagulation studies was mixed with one-ninth volume of 0.13 M sodium citrate. All plasma samples were frozen at −20 C until the coagulation tests were performed. Blood for total leukocyte counts was diluted in Unopettes (Becton, Dickinson and Company, Rutherford, NJ) and counted in a hemocytometer. Hematocrits were determined on blood collected in heparinized capillary tubes. Fibrinogen concentration was estimated by a biuret determination of thrombin-clottable protein. Corrections were made for anticoagulant dilution of the plasma according to the hematocrit values. Platelet counts were performed under light microscopy according to the technic of Stefanini and Dameshek. Factor VIII levels were determined by comparing the degree of shortening of the activated-partial thromboplastin time of canine Factor VIII-deficient plasma by a 1:10 dilution of test plasma with that of dilutions of pooled plasma from 10 normal rabbits. To facilitate comparison of the groups of rabbits, the Factor VIII level and plasma fibrinogen concentration in each rabbit at 0 hours was converted to 100% activity. The plasma samples from heparinized rabbits were treated with protamine sulfate prior to the fibrinogen and Factor VIII assays to block the activity of heparin. The lowest concentration of protamine sulfate which achieved maximal shortening of the thrombin times of the plasma samples was used.

The effects of the leukocyte lysates on normal rabbit plasma were determined by incubating pooled normal rabbit plasma (0.1 ml) with Centrolex suspension (0.1 ml) and leukocyte lysate (0.1 ml) at 37 C for 60 seconds, adding 0.025 M CaCl₂, and timing to clot formation with a fibrometer. The leukocyte lysates were tested at the same concentration as that infused in the intravenous groups of rabbits (8.0 × 10⁶ cells ml⁻¹).

Samples of the leukocyte lysates were collected prior to infusion and incubated in thioglycollate medium at 37 C to eliminate the possibility of bacterial contamination.

Student's t-test was used for determination of the statistical significance of differences between the means of the groups.

Results

Procoagulant Development in Cultured Rabbit Leukocytes

The lysate of rabbit granulocytic leukocytes that had been frozen without incubation at 37 C shortened the clotting time of pooled normal rabbit plasma (Table 2). The procoagulant activity was much greater in the lysate of rabbit granulocytic leukocytes cultured at 37 C.
Table 2—Effects of Leukocyte Lysates on the Clotting Time* of Normal Rabbit Plasma

<table>
<thead>
<tr>
<th>Test agent</th>
<th>No. of samples</th>
<th>Mean clotting time (secs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No incubation at 37 C</td>
<td>12</td>
<td>38.4 ± 4.6</td>
</tr>
<tr>
<td>Incubation for 24 hours at 37 C</td>
<td>5</td>
<td>20.2 ± 3.7</td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>64.6 ± 5.1</td>
</tr>
</tbody>
</table>

* Centrolex suspension added; determined with fibrometer as described in Materials and Methods

for 24 hours, as indicated by the pronounced shortening of the clotting time of normal rabbit plasma.

Effects of Intravenous Infusions of Leukocyte Lysate

Multiple changes in the levels of blood coagulation factors occurred in the rabbits receiving intravenous infusions of lysed leukocyte suspensions. The effects of the infusions on fibrinogen levels are presented in Text-figure 1. The heparin-treated group (A) served as controls for comparison of the changes in fibrinogen concentration. A decrease in mean plasma fibrinogen concentration occurred in all groups. The mean fibrinogen concentrations in Group D at 88.4% (P < 0.10), Group C at 82.9% (P < 0.05) and Group B at 76.0% (P < 0.05) were significantly decreased from that of the heparinized group (A) at 98.4% of the preinfusion level. The decrease in fibrinogen in Group C, which received the lysate of leukocytes cultured at 37 C for 24 hours, was not significantly different from that of Group D, which re-

Text-fig 1—Significant decreases in mean plasma fibrinogen concentration occurred in all experimental groups (B, C, D) which received 2-hour intravenous infusion of lysed leukocytes, with the exception of the heparinized group (A).
received lysed leukocytes that had been frozen without incubation at 37 C. The mean platelet concentration decreased in all experimental groups (Text-figure 2). The small decrease in the group (D) that received lysate from noncultured leukocytes was not statistically different ($P > 0.10$) from that of the heparinized group (A), while significant decreases occurred in groups B ($P < 0.05$) and C ($P < 0.10$), which received cultured leukocytes. In all groups of rabbits the mean Factor VIII activity in a one-stage assay of the plasma decreased to a level between 80 and 90% of normal. Wide variations occurred within the groups, and the differences between the groups were not significant at the $P = 0.10$ level.

Leukocytosis developed over the 4-hour period of the infusion experiments. Although the initial heterophil counts were higher in cortisone-treated groups, the increases in heterophil counts were not significantly different ($P > 0.10$) between the intravenous groups (A, B, C and D) and were considered together. The absolute heterophil counts increased from a mean of $3234 \pm 2536$/cu mm at the start of the infusion to a mean of $7395 \pm 2719$/cu mm 4 hours later.

The incidences of selected lesions in the experimental rabbit groups are summarized in Table 3. Arteritis involving large and small branches of the pulmonary arteries (Figure 1) was detected in 50% of the rabbits in the intravenous groups. The arteritis was characterized by a subendothelial accumulation of heterophils (Figure 2) with variable degrees of invasion of the muscular arterial wall. Some affected vessels...
had periarterial accumulations of heterophils. Hemorrhages were noted in the lungs (Figure 3), liver or kidneys of all rabbits in the intravenous groups. The hemorrhages ranged in severity from microscopic to diffuse ecchymoses.

Vasculitis and massive thrombosis of the external jugular vein extending from the posterior facial vein to the thoracic inlet and sometimes into the anterior vena cava was observed on the cannulated side of all rabbits that received the lysate of cultured leukocytes without heparin pretreatment (Figure 4) and in 2 of 4 rabbits that received lysates of leukocytes that were frozen without incubation at 37 C (Group D). The remaining 2 rabbits from Group D and the 4 heparinized rabbits (Group A) had vasculitis and only microscopic evidence of thrombus formation in the jugular veins through which the lysate was infused.

Fibrinous thrombi were detected in the pulmonary arteries of all rabbits in the cortisone-pretreated group and in 3 of 4 rabbits that received the lysate of cultured leukocytes without pretreatment (Group C). Two of 4 rabbits that received the lysate of leukocytes frozen without incubation at 37 C (Group D) also had fibrinous pulmonary thrombi. Thrombosis of an interlobar artery of the kidney was detected in 1 rabbit in Group B.

Effects of Intraaortic Infusions of Leukocyte Lysates

To bypass the pulmonary vascular bed, two groups of rabbits were infused with leukocyte lysates through an intraaortic cannula positioned at the level of the aortic arch. Thrombosis of renal arteries was observed in 4 of 5 rabbits which received the lysate of leukocytes that had been cultured at 37 C for 24 hours (Figure 5). The renal thrombi were associated with lesions ranging from solitary infarcts to extensive renal cortical necrosis. No large fibrin deposits were detected in glo-
merular capillaries. Figure 6 illustrates the renal cortical necrosis of a rabbit that received lysate of leukocytes that had been cultured at 37°C for 24 hours (Group E), while Figure 7 illustrates the absence of necrosis in the kidney of a second rabbit which received lysate of an equal number of cells from the same peritoneal exudate, except that they were frozen without prior incubation at 37°C (Group F). Two rabbits in Group E had fibrinous thrombi in pulmonary arteries. Two rabbits that died during the intraaortic infusions with massive clot formation involving the entire length of the aorta were not included in the experimental group. The intraaortic cannula in one of the above rabbits extended to the level of the aortic valve, resulting in thrombosis of the coronary arteries.

In contrast, none of the rabbits that received the lysate of leukocytes which were frozen without incubation at 37°C developed renal cortical necrosis, nor were renal thrombi detected microscopically.

Discussion

The decrease in fibrinogen levels and thrombocytopenia suggests that disseminated intravascular clotting occurred in the rabbits following infusion of leukocyte lysate. Further evidence for intravascular coagulation was provided by the microscopic observation of thrombi in various organs and the blocking of the changes with intravenous heparin. Large quantities of rabbit brain thromboplastin can be intravenously administered in the jugular vein of rabbits and the majority of the thromboplastin is removed by the lungs. We, therefore, used intraaortic infusions in two groups of rabbits to determine if renal thrombosis could be induced by leukocyte lysates. To prevent lethal clot formation in the aorta, it was necessary to infuse the leukocyte lysate at a rate slower than that used for the intravenous groups.

Our data provide evidence for significant quantities of procoagulant activity in rabbit leukocytes. The role played by granulocytic leukocytes in syndromes of disseminated intravascular coagulation is not known although the need for granulocytes for the development of the lesions of the generalized Shwartzman reaction, a syndrome characterized by disseminated intravascular coagulation, is well recognized. Horn and Collins have shown that fragmentation of granulocytes occurs in pulmonary capillaries during the development of the generalized Shwartzman reaction and that the injection of isolated heterophil granules in endotoxin-treated rabbits produces intravascular thrombi and the lesions of the generalized Shwartzman reaction. The lesions of the generalized Shwartzman reaction have been produced using inject-
tions of rabbit brain thromboplastin without endotoxin.\textsuperscript{17} We observed bilateral renal cortical necrosis in a rabbit which received an intra-aortic infusion of the lysate of cultured rabbit leukocytes but could not demonstrate large deposits of fibrin in the glomeruli. The endothelial damage induced by endotoxin\textsuperscript{18} may be an important prerequisite for the development of glomerular thrombosis.

Golub and Spitznagel\textsuperscript{19} have shown that intradermal injection of the granule fraction of polymorphonuclear leukocytes from rabbits causes increased vascular permeability, polymorphonuclear leukocyte infiltration, and vasculitis in venules, capillaries and lymphatics by 4 hours postinjection. Similarly, Janoff and Zwiefach\textsuperscript{20} observed sticking and emigration of leukocytes, stasis of blood flow and petechial hemorrhages in rabbit mesentery exposed to cationic proteins from leukocyte lysosomes. The nature and distribution of the vasculitis in the jugular veins and pulmonary arteries of the intravenously infused rabbits suggests that the high concentration of lysosomal mediators of inflammation were responsible for the vasculitis. The specific relation of the vasculitis to the thrombotic lesions was not determined, although the differences between the incidence of microscopic thrombi and vasculitis suggest that the thrombi were not solely due to vasculitis. The hemorrhages and microscopic jugular thrombi in the heparinized group were considered to be most likely secondary to vasculitis.

The possibility that endotoxin contaminated our leukocyte cultures and was responsible for the observed lesions was considered unlikely, since attempts at bacterial isolation from the lysates were uniformly unsuccessful, and leukopenia did not develop in any of the rabbits during the 4-hour period of the infusion experiments.

The mechanism of procoagulant development in cultured leukocytes has not been determined. Niemetz and Fani\textsuperscript{21} recently reported increased procoagulant activity in peritoneal leukocytes collected from rabbits after two endotoxin injections. The leukocytes from the endotoxin-treated rabbits had significant tissue factor activity \textit{in vitro} and \textit{in vivo}. These observations, coupled with the observation of significant procoagulant activity in cultured rabbit leukocytes and the production of disseminated intravascular coagulation in rabbits using infusions of lysates of rabbit granulocytic leukocytes, suggest that leukocyte procoagulant could play a significant role in the production of the lesions of the generalized Shwartzman reaction and related syndromes of disseminated intravascular coagulation.
References


Fig 1—Heterophils within the intima of a small branch of pulmonary artery of a rabbit 4 hours after intravenous infusion of the lysate of $232 \times 10^4$ leukocytes (H & E, $\times 750$). Fig 2—Subendothelial accumulations of heterophils in the pulmonary artery of a rabbit which received an intravenous infusion of $232 \times 10^4$ lysed rabbit leukocytes (H & E, $\times 1325$).
Fig 3—Hemorrhages in a lung from a rabbit which received an intravenous infusion of the lysate of $232 \times 10^8$ leukocytes.

Fig 4—Thrombosis of jugular vein and facial branches in a nonheparinized rabbit which received an intravenous infusion of the lysate of $232 \times 10^8$ leukocytes that had been incubated at 37 °C for 24 hours.
Fig 5—Thrombosis of an arcuate artery in a kidney from a rabbit which received an intra-aortic infusion of the lysate of $230 \times 10^4$ leukocytes that had been cultured at 37 C for 24 hours (H & E, x 220).

Fig 6—Cortical necrosis in kidney of rabbit which was intraaortically infused with lysate of $230 \times 10^4$ leukocytes that had been incubated at 37 C for 24 hours.

Fig 7—Absence of necrosis in kidney of rabbit which was intraaortically infused with lysate of $230 \times 10^4$ leukocytes that were frozen without incubation at 37 C.