Procoagulant Activity of Lymphocyte-Macrophage Populations in Rabbits: Selective Increases in Marrow, Blood, and Spleen Cells During Shwartzman Reactions

By Henry Rothberger, Francis B. Dove, Tung-Kuang Lee, Maria P. McGee, and Bruce Kardon

The present experiments examine leukocyte procoagulant activity using mononuclear cell populations purified or enriched from rabbit bone marrow, blood, spleen, lymph node, thymus, and pulmonary alveoli. Cells from these six sites, obtained from control and endotoxemic animals and assayed without an intermediate culture step, were found to have procoagulant activity identified as tissue factor. Under control conditions, tissue factor activity was found to be at low levels in marrow and blood populations compared to median activities 3- and 11-fold higher in populations from spleen and lymph node, and 33- and 45-fold higher in thymus and alveolar populations. By contrast to respective controls, significantly increased amounts of tissue factor (35-, 15-, and 12-fold at median levels) were found in marrow, blood, and spleen populations from endotoxemic animals. The types of leukocytes in these latter three populations were morphologically and histochemically indistinguishable from respective controls, indicating that endotoxin induced increases of activity in cells with relatively low amounts under control conditions. Activity did not change significantly in lymph node, thymus, or alveolar populations after endotoxia. These studies show that tissue factor is present in a range of leukocyte populations not previously reported to have procoagulant activity. In addition, the finding of widespread gains of tissue factor in the marrow-blood-spleen pool due to endotoxia provides new evidence supporting the importance of leukocyte procoagulants in Shwartzman-like reactions.

Leukocytes isolated from blood, spleen, liver, and peritoneal exudates produce procoagulant activity in vitro. In view of this ability to produce procoagulants, leukocytes have been implicated in the pathogenesis of disseminated intravascular coagulation and inflammatory reactions. Freshly isolated leukocytes contain relatively little procoagulant activity, but levels increase dramatically after incubation in the presence of endotoxin, allogeneic leukocytes, immune complexes, serum lipoproteins, activated complement components, viruses, gram-positive bacteria, sensitized red cells, or lectins. The procoagulant activity generated during incubation usually has been found to be tissue factor, although prothrombinases and a factor X activator have been described. Procoagulant activity generated by incubated mononuclear leukocyte mixtures appears to be almost exclusively in macrophages and monocytes rather than lymphocytes. However, there is considerable evidence that lymphocytes regulate the increases in procoagulant activity. Experiments with rabbits have shown increases of tissue factor activity in leukocytes obtained from blood and peritoneal exudates after intraarterial and intraperitoneal injection of endotoxin, indicating that such increases occur pathologically in vivo. However, to date, little is known about tissue factor or other possible procoagulant activity in leukocytes residing in the series of organs comprising the reticuloendothelial system, and it is not known whether endotoxia induces generalized increases of this activity.

The experiments described here use a rabbit model to investigate procoagulant activity as present in vivo in several leukocyte populations of the reticuloendothelial system. Procoagulant activity with the molecular specificity of tissue factor is demonstrated in all of the populations studied, suggesting a link between tissue factor, the coagulation system, and defense functions carried out by cells in these populations. It is also shown that during endotoxia and Shwartzman reactions, increases of leukocyte tissue factor activity occur not only in blood, but also in marrow and spleen cells. Tissue factor from all three of these populations may be involved in initiation of thrombosis in Shwartzman reactions.

Materials and Methods

Method of Collection of Tissues and Cells From Experimental Animals

Female New Zealand white rabbits (2-3 kg) were sacrificed with an overdose of sodium pentobarbital anesthetic (3-4 ml of 1 g/ml solution intravenously). Tissues and cells were immediately collected from the following 6 organs: To obtain cardiac blood, a pneumothorax was created by puncturing the right and left hemidiaphragms through a thoracoabdominal incision, and the right ventricle was entered before cardiac standstill with a 16-gauge needle. Seventy milliliters of blood was aspirated into 10 ml of 0.1 M citrate anticoagulant at pH 5.0. The citrated blood was immediately dispensed into 6-8 15-mI tubes, centrifuged (700 g for 10 min at
22°C), and theuffy coat layer was aspirated and placed in media: Hanks balanced salt solution, pH 7.0–7.4 with 6 g/liter HEPES buffer (Calbiochem Corp., La Jolla, Calif.), 1% bovine serum albumin, and 223 mg/ml disodium methylenediaminetetraacetate (EDTA). The blood remaining afterbuffy coat removal was mixed, centrifuged as above, and asecond set ofbuffy coats was collected and pooled with the first set. The spleen and thymus were removed and placed in Petri dishes containing media at 4°C. Pulmonary alveolar macrophages were obtained as previously described by removing skin and reflecting muscles anterior to the trachea. A 14-gauge intercath was inserted between the 2 superior trachial rings. The metal cannula was withdrawn and the catheter was advanced to the trachial bifurcation. Using a 50-ml syringe, the lungs were filled slowly with 25 ml of media. This fluid containing macrophages was withdrawn from the lungs. The process was repeated twice using 40 ml of media and the 3 washings were pooled. A lymph node was obtained from the posterior aspect of each ear and from the popliteal spaces of the 2 hind limbs. The 4 lymph nodes were placed together in media in a Petri dish. Bone marrow was obtained by exposing the distal femurs and proximal tibias with bone cutters. Using a 16-gauge needle and a 50-ml syringe containing 25 ml of media, bone marrow cavities were injured by alternate injections and aspirations of media and the contents were pooled. The entire leukocyte collection process took < 20 min. Immediately following these procedures, cells from bone marrow, spleen, lymph node, and thymus were freed from surrounding tissue by teasing, repeated suspension in a pipette, and flushing through a screen mesh with chilled media. These cells were washed by centrifugation (200 g at 4°C). Cell pellets obtained from each of the 6 anatomic sites as described above were suspended in 40 ml of media (0.5–1 ml pellet/40 ml media), and each 40 ml of suspension was layered with 10 ml of Ficoll-Hypaque gradient (specific gravity 1.072) in 50-ml tubes. Gradients were centrifuged (700 g for 30 min at 22°C), and interface layers containing mononuclear cells were collected and washed twice in media without EDTA (200 g for 10 min at 4°C). The final cell pellets were suspended at 4°C in 1 ml of the media without EDTA. Leukocytes were counted using both a hemocytometer and Coulter Counter (Coulter Electronics, Hialeah, Fla.) and adjusted to 10^6/ml. Viability was determined immediately after cell isolation by trypan blue exclusion and cells were then frozen at –20°C in preparation for a one-stage assay. Nonspecific esterase stains using an a-naphyl acetate substrate and Wright stains of cytocentrifuged smears were used to identify the types of cells isolated. Collectively, monocytes and macrophages as identified by this stains are referred to as macrophages.

**Assay for Leukocyte Procoagulant Activity**

 Assays were performed using leukocytes lysed by freezing and thawing. The assay was initiated by adding 10^6 leukocytes in 0.1 ml of media to a siliconized test tube, followed by 0.1 ml 0.025 M calcium chloride and 0.1 ml of platelet-poor citrated rabbit plasma. The clotting time was determined in duplicate by the manual tilt method and the average of the 2 times was used. Duplicate times with cells differed by less than 6%. Buffor blank times were ≥ 550 sec. Exogenous lipid had no effect on clotting times with cells or buffer blanks, as shown in preliminary studies using rabbit brain cephalin (Sigma, St. Louis, Mo.) in the assay mixture as recommended by the manufacturers. To characterize procoagulants as tissue factor, leukocytes and rabbit brain were assayed using plastic tubes with normal human plasma or human plasma severely deficient in a single clotting factor (obtained from George King Biomedical, Overland Park, Kans., Dade Diagnostics, Miami, Fl., and the North Carolina Memorial Hospital Clinical Coagulation Laboratory, Chapel Hill, N.C.). Clotting times were converted to tissue factor units using a calibration curve made with serial dilutions of cell suspension or rabbit brain thromboplastin standard made in our laboratory. The amount of standard yielding a clotting time of 50 sec was considered to have 1000 U of activity. In this assay, 3.6 mg of commercially available dried rabbit brain yielded a time of 50 sec (Thromboplastin C, Dade Diagnostics, Inc., Miami, Fla.). In some experiments, type III phospholipase-C from Bacillus cereus (Sigma) and concanavalin-A-agarose (Sigma) were incubated with 10^6 leukocytes before assay, using respectively, 0.01 mg and 0.09 mg of protein. In experiments assaying membrane fractions and cytosol separately, 0.2 ml portions of cell lysates were centrifuged at 165,000 g for 5 min. The resultant membrane-enchriched pellet was reconstituted in the original volume of media and clotting times were compared to the supernatant containing cytosol and the initial unfractionated sample.

**Induction of Shwartzman Reactions**

 Intravenous infusion of saline was started using a ¼-inch 23-gauge butterfly needle inserted into an ear vein and connected to a pediatric infusion set (McGaw Laboratories, Sabana Grande, Puerto Rico). E. coli 026B6 endotoxin (Difco Laboratories, Detroit, Mich.) was added (0.4 mg) to 100 ml sterile saline, and this volume was administered over a 6–8-hr period. Alternatively, 40 ml of endotoxin solution was infused over 15 min, and the remaining 60 ml was infused by intravenous drip over a 1-hr period prior to sacrificing the animal. Control rabbits received saline without endotoxin. Fifteen hours after starting infusions, animals were sacrificed by anesthesia as described above. Shwartzman reactions were verified in endotoxemic animals by noting obtundation and shock and by examining kidneys for characteristic macroscopic and microscopic changes.24

**Random Design and Statistical Methods**

 As appropriate for multiple comparisons, analysis of variance (ANOVA) was used as an initial test for differences in results obtained with 22 consecutive rabbits randomly allocated to control and Shwartzman groups. (In our series, 3 additional rabbits died during endotoxin infusion and therefore could not be studied.) Further tests for differences were made using the Scheffe method of contrasts. A significance level at p = 0.05 was chosen. Correlation coefficients were calculated by the Pearson product-moment method.

**RESULTS**

**Characterization of the Classes of Cells Investigated**

To identify cell types in the six leukocyte populations studied for procoagulant activity, cytocentrifuge smears were examined using Wright's and nonspecific esterase stains. Control blood, spleen, lymph node, and thymic populations were found to be mixtures composed almost exclusively of lymphocytes and macrophages (Table 1). On the other hand, the control alveolar population contained macrophages without lymphocytes or other cells. Control bone marrow populations contained approximately 50% lymphocytes-macrophages, and the remainder of the cells were myeloid precursors. The range of cell viability was from 86% to 98%. There were no changes detectable by morphological and cytochemical examination in the
types of cells isolated or in viability in any of the six populations from endotoxemic animals compared to controls (Table 1).

**Measurements of Procoagulant Activity in Leukocyte Populations From Control Animals**

The procoagulant activity of leukocytes isolated from bone marrow, blood, spleen, lymph node, thymus, and pulmonary alveoli was measured as described in Materials and Methods. Procoagulant activity was found to be present in all six of these cell populations obtained from the 11 control animals. However, amounts of activity in the six populations differed significantly \((p < 0.001, \text{ANOVA, see Fig. 1})\). Control marrow and blood cells showed significantly lower levels of activity than spleen and lymph node cells \((p < 0.05, \text{Scheffe test})\). Further, as compared to these latter two populations, significantly greater amounts of procoagulant activity were present in control leukocytes from thymus and pulmonary alveoli \((p < 0.05, \text{Scheffe test})\).

**Procoagulant Activity in Leukocyte Populations From Endotoxemic Animals**

By contrast to results with the control leukocyte populations, procoagulant activity was significantly increased in marrow, blood, and splenic leukocytes from a group of 11 animals infused with endotoxin, as shown by comparing results for these three populations from both groups \((p < 0.001, \text{ANOVA and Scheffe tests, Fig. 1})\). However, the levels of procoagulant activity in lymph node, thymus, and pulmonary popu-

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**Table 1. Similarities of Composition in Leukocyte Populations Isolated From Control and Shwartzman Groups (Mean Percent ± SEM)**

<table>
<thead>
<tr>
<th>Anatomic Origin</th>
<th>Experimental Group</th>
<th>Bone Marrow</th>
<th>Blood</th>
<th>Spleen</th>
<th>Lymph Node</th>
<th>Thymus</th>
<th>Pulmonary Alveoli</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Mononuclear</td>
<td>Control</td>
<td>56 ± 4.3</td>
<td>99 ± 0.3</td>
<td>99 ± 0.1</td>
<td>100 ± 0.2</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td></td>
<td>Shwartzman</td>
<td>53 ± 1.2</td>
<td>97 ± 0.6</td>
<td>96 ± 0.8</td>
<td>99 ± 0.1</td>
<td>100 ± 0</td>
<td>100 ± 0</td>
</tr>
<tr>
<td>B. Nonspecific</td>
<td>Control</td>
<td>54 ± 3.8</td>
<td>20 ± 5.0</td>
<td>36 ± 2.0</td>
<td>27 ± 1.7</td>
<td>14 ± 1.3</td>
<td>98 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>Shwartzman</td>
<td>56 ± 4.0</td>
<td>15 ± 2.8</td>
<td>39 ± 2.0</td>
<td>29 ± 2.0</td>
<td>8 ± 0.8</td>
<td>99 ± 2.0</td>
</tr>
<tr>
<td>C. Promyelocytes*</td>
<td>Control</td>
<td>9.6 ± 4.0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Shwartzman</td>
<td>10.3 ± 0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D. Myelocytes*</td>
<td>Control</td>
<td>33 ± 3.7</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Shwartzman</td>
<td>36 ± 0.8</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>E. Viability‡</td>
<td>Control</td>
<td>95 ± 2.0</td>
<td>98 ± 0.5</td>
<td>90 ± 1.4</td>
<td>88 ± 1.8</td>
<td>86 ± 2</td>
<td>94 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>Shwartzman</td>
<td>94 ± 0.6</td>
<td>98 ± 1.8</td>
<td>90 ± 2.0</td>
<td>91 ± 1.0</td>
<td>86 ± 6.0</td>
<td>95 ± 1.9</td>
</tr>
</tbody>
</table>

*Enumerated on Wright stained smears and includes lymphocytes + monocytes/macrophages.
†Positive cells are of monocyte/macrophage lineage except in bone marrow, where for rabbits promyelocytes and myelocytes are also positive.
‡Enumerated as percent cells excluding trypan blue after completion of isolation procedures.

For populations other than bone marrow: Percent lymphocytes = (A) – (B). Percent monocytes/macrophages = (B).

For bone marrow: Percent lymphocytes = (A) – (B – C – D). Percent monocytes/macrophages = (B – C – D).

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**Fig. 1.** Tissue factor activity is shown for leukocyte populations from the groups of control and Shwartzman animals. Ranges (vertical bars) and medians (*) are logarithmically transformed to allow use of the same scale throughout, and corresponding clotting times are provided.
controls (p > 0.05, ANOVA and Scheffe tests). As a result of these increases in some of the populations and lack of changes in others, differences in amounts of activity in the six populations from endotoxemic animals were not significant (p > 0.05, ANOVA). Further, it can be seen that only populations with relatively low levels of procoagulant activity under physiologic conditions displayed significantly higher levels of activity after endotoxin infusion (Fig. 1).

Characterization of Procoagulant Activity as Tissue Factor

Procoagulant activity measured in the six leukocyte populations obtained from the two groups of animals was shown to be tissue factor. Clotting proteins of the classical tissue factor pathway (factors VII, X, and II) were specifically required in substrate plasma for acceleration of clotting by the leukocyte factor, while classical intrinsic pathway proteins (factors XII, XI, IX, and VIII) were not needed (Table 2). Concanavalin-A and phospholipase-C, known inhibitors of brain tissue factor, also inhibited procoagulant activity in the six leukocyte populations (Table 2). Nearly all the procoagulant activity as measured with unfractionated lysates (99% ± 13%) was found to be in membrane fractions pellet by ultracentrifugation. This compared to only 0.9% ± 0.2% of activity as recovered in cytosol fractions (i.e., supernatants) of the same ultracentrifuged preparations. Similar localization of tissue factor in membrane fractions was found with a brain tissue factor control.

Correlations Between Amounts of Tissue Factor Activity and Proportions of Macrophages in Leukocyte Preparations

Since macrophages have been shown to be the predominant source of tissue factor or other procoagulant activity in leukocyte populations examined to date, relationships between amounts of tissue factor activity and macrophage proportions in the leukocyte preparations studied here were investigated. In cell preparations obtained from control animals, tissue factor activity content showed a strong positive correlation with percentages of constituent macrophages (Pearson correlation coefficient for mean percent macrophages/population versus mean TFU/population = +0.88). By contrast, in leukocyte preparations from the endotoxemic group, the content of tissue factor had only a very weak positive correlation with macrophage percentages (Pearson correlation coefficient = +0.05). These findings show that levels of tissue factor activity in cell mixtures from controls were largely dependent on the proportions of macrophages present. On the other hand, following endotoxemia, this dependence was no longer observed.

DISCUSSION

The experiments described here utilize control and endotoxemic rabbits to examine procoagulant activity in lymphocyte-macrophage populations purified or enriched from bone marrow, blood, spleen, lymph node, thymus, and pulmonary alveoli. Procoagulant activity identified as tissue factor was observed to be present in all six populations obtained from the two groups of animals. However, amounts of this activity were variable, depending on the population and group of origin. With cells isolated from the controls, tissue factor was detected in only small amounts in marrow and blood cells compared to greater amounts in cells from spleen and lymph node, and even larger amounts in thymus and alveolar cells. By contrast, in studies using cells isolated from the group of animals infused with endotoxin to produce Shwartzman reactions and disseminated intravascular coagulation, we found that amounts of tissue factor activity did not differ significantly from one population to the other. This was owing to induction of large increases of tissue factor

Table 2. Characterization of Tissue Factor

<table>
<thead>
<tr>
<th>Leukocyte Source</th>
<th>TFU in Normal Plasma Substrates*</th>
<th>TFU in Deficient Plasma Substrates*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NHP</td>
<td>VIII</td>
</tr>
<tr>
<td>Marrow</td>
<td>80</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Blood</td>
<td>96</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Spleen</td>
<td>45</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Lymph node</td>
<td>43</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Thymus</td>
<td>134</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Alveolar</td>
<td>81</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Brain tissue</td>
<td>130</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

*Amounts of procoagulant activity (TFU) of cells obtained after endotoxemia are shown for assays using normal human plasma (NHP) or congenitally monodeficient plasmas. The specificity for factors VII, X, and II indicates that the procoagulant measured is tissue factor. Similar specificity was found with cells from control animals. Further, TFU was reduced to <1 when cell preparations were mixed before assay with phospholipase-C or insolubilized concanavalin-A for 15 min at 37°C.
activity in marrow, blood, and splenic leukocytes relative to respective control populations, and a lack of significant changes in lymph node, thymus, and alveolar cells.

The demonstration that procoagulant activity measured in the six leukocyte populations under both endotoxemic and control conditions is tissue factor relies in part on identification of the molecular pathway used for coagulation. Clotting factors VII, X, and II of the classical tissue factor pathway were shown to be specifically required. Inhibition of the procoagulant activity by phospholipase-C from *Bacillus cereus* was also shown. This enzyme is known to inhibit human brain tissue factor and tissue factor activity produced in vitro by mononuclear cells isolated from human blood. We also found that the procoagulant activity in all six populations was removed from cell lysates by insolubilized concanavalin-A, a lectin that binds and inhibits human brain tissue factor and tissue factor produced by cultured fibroblasts. Further, using fractionation of cellular components by ultracentrifugation, we demonstrated that procoagulant activity in the six populations is membrane associated. This property is shared by brain tissue factor and is consistent with findings for leukocyte populations studied by others.

Previous work has shown that following incubation or culture, peritoneal exudate macrophages and lymphocyte-macrophage mixtures from blood and spleen produce tissue factor activity. Available evidence from these and other studies using cells cultured in nutrient media at physiologic temperatures show that a lag period of at least 2–3 hr is required before tissue factor activity increases significantly in vitro, even when a potent stimulus such as endotoxin is added. In light of these observations, our results measuring activity in cells frozen immediately after rapid isolation at low temperatures strongly suggest that the tissue factor found in the six leukocyte populations was acquired in vivo. Further, it is well established that endotoxin stimulates leukocyte tissue factor production in vitro, and increases of activity found in the endotoxemic group may have been due to direct cellular stimulation by endotoxin in vivo. Alternatively, due to possible effects of endotoxin on leukocyte migration, any preexisting "high activity" subsets of the cell types identified may have become differentially available in populations showing tissue factor increases. We found that leukocyte preparations isolated from comparable sites in healthy and endotoxemic animals contained classes and proportions of cells that were similar by morphological and histochemical criteria. This similarity provides evidence that the cells with increased amounts of tissue factor activity due to endotoxemia were of the same classes as those present with relatively low activity in populations from control animals.

Macrophages have been shown to be the predominant source of procoagulant activity in mixed mononuclear cell populations. The findings reported here extend previous studies of leukocyte procoagulants by showing that tissue factor activity in leukocytes is more widely distributed in vivo than has been appreciated. Of the six leukocyte populations investigated here, only blood cells were reported in earlier work to have tissue factor activity under conditions of assay without preliminary culture. However, cultured murine spleen cells have been reported to contain a factor-VII-dependent procoagulant that resembles tissue factor. Further, human splenic mononuclear cells that were assayed after culture for several days, but apparently not before culture, were found to contain tissue factor activity. In view of the present observations of tissue factor activity in freshly isolated leukocytes from marrow, spleen, lymph node, thymus, and pulmonary alveoli, it is likely that this procoagulant is available in vivo throughout these anatomically distinct populations of hemopoietic and immunoreactive cells. Therefore, potential for interaction between leukocytes in these populations and the blood coagulation system is suggested. For example, tissue factor in pulmonary alveolar macrophages may contribute to the pathology of interstitial lung diseases characterized by exudation of plasma proteins into the alveolar space (where alveolar macrophages are localized) and fibrin deposition on alveolar membranes. Indeed, Hageman factor activators as found in pulmonary lavage fluid have been similarly postulated as a stimulus for pathologic coagulation in the adult respiratory distress syndrome.
reservoir of tissue factor potentially available for hematogenous circulation. The finding of increases of tissue factor in these two populations and the confirmation of such increases in blood cells provides further support for a mechanism of leukocyte-mediated thrombosis in Shwartzman reactions and coagulopathy for which the Shwartzman reaction serves as a model.

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