Effects of Bacterial Endotoxin on the Fibrinolytic Activity of Normal Human Leukocytes

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Two modifications of Todd's histochemical fibrin slide technique were used to study the effect of bacterial endotoxin on the plasminogen activator activity of intact normal human leukocytes in vitro. Leukocyte suspensions were obtained from heparinized peripheral blood by dextran sedimentation. Plasminogen activator activity was measured as the percentage of individual cells producing zones of fibrinolysis in plasminogen-rich fibrin. Of the cells from normal individuals, 27 ± 2 per cent were fibrinolytically active. The addition of lipopolysaccharide B from Salmonella abortus equi resulted in a marked increase in the percentage of active cells without impairment of cell viability. The stimulation of plasminogen activator release appeared to be dose-related, since at higher endotoxin concentrations the number of active cells gradually approached the granulocyte level. Maximum effect on leukocytes was observed 2 hours after addition of endotoxin, followed by a steady decline in activity. Plasminogen activator activity in the suspending medium became detectable after 2 hours' incubation. Simultaneous assays of beta-glucuronidase revealed increasing amounts of this lysosomal enzyme in the suspending medium during incubation. Addition of endotoxin enhanced the rate of this increase. The parallel appearance of plasminogen activator and beta-glucuronidase activities without concomitant cell destruction from unstimulated and endotoxin-treated leukocytes is consistent with the release of both compounds from lysosomes following noncytoidal stimulation.

Previous studies of the fibrinolytic activity of normal animal and human leukocytes have implicated both nonspecific protease1-7 and an activator of the plasminogen-plasmin system3-7 as initiators of fibrinolysis. Disruption of leukocytes has appeared necessary for the detection of plasminogen activator activity,3-5 indicating a firm enclosure of the active compound within the cells.

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Assaying cellular subfractions, Saba et al. identified plasminogen activator in a lysosomal cationic protein fraction prepared from rabbit and human polymorphonuclear leukocytes. In studies based on the enhancing effect of bacterial endotoxins on the release of hydrolytic enzymes from lysosome preparations, Lack effected the release of plasminogen activator from isolated rabbit polymorphonuclear leukocyte lysosomes by subjecting them to endotoxin and other chemical and physical stresses.

It was the purpose of this study to investigate the influence of bacterial endotoxin on the plasminogen activator activity of intact leukocytes from normal human peripheral blood.

**Materials and Methods**

**Preparation of Leukocyte Suspensions**

Venous blood from normal volunteers was collected in plastic syringes and transferred to siliconized conical glass tubes containing heparin sodium (10 units/ml of blood). Red cells were sedimented with the addition of either 3 volume 5 per cent dextran (mean molecular weight 232,000) (Sigma Chemical Co., St. Louis, Mo.) or 3 per cent gelatin in 0.9 per cent NaCl. After 15 to 30 minutes' sedimentation, the supernatant was removed and centrifuged at 400 x g. for 10 minutes. Residual erythrocytes were removed from the cell pellet by hypotonic lysis and the remaining leukocytes were washed three times with Hank's balanced salt solution (HBSS).

The leukocytes, now essentially free from erythrocyte, platelet, or plasma contamination, were suspended in 5 per cent human serum albumin (Merck, Sharp & Dohme, Philadelphia, Pa.) to a final leukocyte concentration of 15,000 to 25,000/cu. mm. The pH of the final suspension ranged from 7.1 to 7.2, and 98 to 99 per cent of the cells treated in this fashion retained their viability as determined by trypan blue exclusion. Differential counts were performed from Wright's stained smears of the suspensions.

Endotoxin (lipopolysaccharide B from *Salmonella abortus equi*, Difco, Detroit, Mich.), epsilon amino caproic acid (EACA, Sigma Chemical Co.) and Trasylol (Prep. A 128, FBA Medical Research, Division of Metachem, New York, N.Y.) were diluted in HBSS and added to the suspensions either individually or in combination.

**Assay of Leukocyte Fibrinolytic Activity**

Fibrinolytic activity was assayed histochemically utilizing modifications of the fibrin slide technique of Todd. Bovine thrombin was added to either plasminogen-rich or plasminogen-free bovine fibrinogen to prepare thin fibrin films overlying freshly made smears of the leukocyte suspensions. Fibrin films were also prepared utilizing the fibrinogen solutions in which the leukocytes were previously suspended. All slides were incubated for 2 hours at 37°C in a moist chamber, fixed in formalin, and washed prior to staining with hematoxylin. The slides were then examined microscopically (100 x magnification), and all cells observed in seven preselected fields on triplicate slides were counted. The percentage of individual cells which were surrounded by a zone of fibrinolysis was determined. No attempt was made to quantify the diameter or area of each zone of lysis.

Some leukocyte suspensions were centrifuged at 400 x g. for 10 minutes and the fibrinolytic activity of the suspending medium was assayed by pipetting 5 μl. of supernatant onto preformed fibrin slides, which were then treated as described above.

The fibrinolytic activity of various dilutions of a plasminogen activator of known activity (tissue activator prepared from pig heart, 300 Astrup and Albrechtsen units per milliliter) in albumin, dextran, endotoxin solutions, and in saline alone was found to be identical, indicating that these substances had no direct influence on the plasminogen activator-plasminogen-plasmin system.

**Beta-glucuronidase Assay**

Aliquots of leukocyte suspensions, with or without added endotoxin, and after various
Fig. 1.—Leukocyte associated zones of fibrinolysis produced during 120 minutes incubation of normal peripheral blood leukocytes covered by plasminogen-rich fibrin. (A) Untreated peripheral blood cells. (B) Peripheral blood cells exposed to 50 µg./ml. of lipopolysaccharide B from Salmonella abortus equi. × 63.

Intervals of incubation at 25°C, were centrifuged at 1000 × g, for 10 minutes and supernatant beta-glucuronidase activity was measured according to the technique of Fishman et al. Activities were expressed as the percentage of control activity, i.e., the activity of the supernatant of a freshly prepared leukocyte suspension containing no endotoxin.

RESULTS

Zones of fibrinolysis surrounding individual cells were observed on fibrin slides prepared with plasminogen-rich fibrinogen. No lytic activity was detectable with the plasminogen-free substrate, indicating that the fibrinolysis
Table 1.—Leukocyte Fibrinolytic Response to Increasing Endotoxin Concentrations

<table>
<thead>
<tr>
<th>Endotoxin (µg./ml.)</th>
<th>Per Cent Active Cells (Mean ± SE)</th>
<th>Per Cent Neutrophils (Mean ± SE)</th>
<th>Trypan Blue Exclusion (Per Cent)</th>
<th>Number of Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>27.1 ± 2.1</td>
<td>79 ± 0.9</td>
<td>99</td>
<td>48</td>
</tr>
<tr>
<td>1</td>
<td>38.8 ± 2.7</td>
<td>79 ± 2.1</td>
<td>99</td>
<td>20</td>
</tr>
<tr>
<td>5</td>
<td>44.9 ± 4.2</td>
<td>79 ± 2.3</td>
<td>99</td>
<td>5</td>
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<tr>
<td>10</td>
<td>42.0</td>
<td>76</td>
<td>99</td>
<td>3</td>
</tr>
<tr>
<td>25</td>
<td>51.3 ± 3.9</td>
<td>78 ± 2.1</td>
<td>98</td>
<td>13</td>
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<tr>
<td>50</td>
<td>52.9 ± 3.7</td>
<td>80 ± 1.2</td>
<td>99</td>
<td>16</td>
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<tr>
<td>100</td>
<td>62.0</td>
<td>80</td>
<td>98</td>
<td>4</td>
</tr>
<tr>
<td>200</td>
<td>63.8</td>
<td>80</td>
<td>98</td>
<td>3</td>
</tr>
<tr>
<td>400</td>
<td>55.0</td>
<td>82</td>
<td>98</td>
<td>2</td>
</tr>
</tbody>
</table>

was the result of plasminogen activation rather than nonspecific protease activity.

Similar results were observed whether the leukocytes were incorporated initially within the fibrin film or smeared on the glass slides prior to the preparation of the fibrin film. The latter technique, however, permitted easier histologic identification of cell types and more precise quantitation of the percentage of cells revealing fibrinolytic activity.

The mean percentage of the active cells, calculated from the results of assays performed on 48 samples of blood from normal donors, was 27 ± 2 per cent; see Fig. 1A. The activity varied directly with the percentage of neutrophils in the suspension, and all leukocytes within areas of lysis that could be identified histologically were of this cell type. Leukocyte suspensions prepared by gelatin sedimentation, consisting almost entirely of lymphocytes, revealed little or no activity.

The addition of endotoxin to the cell suspensions consistently resulted in an increase in the number of active cells detectable on the plasminogen-rich fibrin slides while producing no significant loss of cell viability as determined by trypan blue exclusion; (Fig. 1B). The stimulation of activity appeared to be

Fig. 2.—Plasminogen activator activity of normal human leukocytes as a function of duration of incubation. Results with unstimulated control cells, and cells exposed to 5 µg./ml. and 100 µg./ml. of endotoxin (lipopolysaccharide B from S. abortus equi) are indicated. Values are plotted as the per cent of cells exhibiting fibrinolysis in plasminogen rich fibrin films.
dose related. When endotoxin concentrations were increased, the percentage of active cells approached the percentage of neutrophils in the suspension and in no case exceeded the granulocyte level (Table 1). The lytic activity of individual cells also increased with higher concentrations of endotoxin, as evidenced by increased intensity of the individual zones of lysis.

Furthermore, fibrinolytic activator activity was found to be dependent upon the time interval between addition of the lipopolysaccharide to the leukocyte suspensions and preparation of the smears and fibrin slides. Increases in activity with endotoxin concentrations ranging from 1 to 100 μg./ml. were observed for 2–2½ hours at 25°C with maximal activity after 2 hours, following which there was a steady decline in activity. A similar, though less prominent, pattern was observed without the addition of endotoxin (Fig. 2). Supernatant plasminogen activator activity became detectable after 2 hours of incubation at room temperature, and increased steadily thereafter. This activity was, however, extremely weak and could not be adequately quantified.

EACA (50 mg./ml.) and Trasylol (10 units/ml.) completely inhibited the activity of individual cells, with or without the addition of endotoxin.

Beta-glucuronidase activity was measured in the supernates of the leukocyte suspensions to assess the degree of lysosomal membrane lability produced either by the preparation of the suspensions or by the addition of endotoxin. In absence of endotoxin, supernatant beta-glucuronidase activity was detectable and was found to increase steadily during four hours of incubation at 25°C, indicating some degree of lysosomal lability. When bacterial lipopolysaccharide was added to the suspensions in concentrations ranging from 1 to 100 μg./ml., there was an increase in the rate at which extracellular beta-glucuronidase activity increased. A representative experiment is presented in Fig. 3.
DISCUSSION

The histochemical technique used in this study provided sufficient specificity and sensitivity to enable us to demonstrate the plasminogen activator activity of individual, intact, normal human peripheral blood leukocytes. It did not, however, permit detection of the previously described nonspecific protease activity in these cells. With this system, nonspecific protease activity could be demonstrated only if large numbers of disrupted leukocytes were assayed.

That the plasminogen activator is not merely a constituent of plasma, adsorbed to the surface of the leukocytes and resistant to washing, is supported by the results of the experiments with endotoxin. The time- and dose-dependent increases in activity appear more likely as the result of leakage of activator out of the cells or out of subcellular organelles such as lysosomes. This leakage appears to occur to a minor degree following either spontaneous degeneration or manipulation of the cells during the preparation of the suspensions, but is greatly enhanced in response to endotoxin treatment. We have shown that this occurs parallel to the release of lysosomal beta-glucuronidase, and the known effects of endotoxin on lysosomal membranes support this concept. Degranulation, as has been suggested to occur in response to phagocytosis in general and to phagocytosis of endotoxin in particular, may well have been a contributing factor.

Our results are consistent with leukocyte plasminogen activator being localized within lysosomes. The percentage of active cells that we observed in suspensions with or without endotoxin may therefore be representative of the number of cells having alterations in the integrity of their lysosomal membranes.

It is still impossible to assess the relative contribution made by leukocytes towards the dissolution of fibrin in vivo. Cans3 and Lack6 have suggested that leukocytes may account for a substantial fraction of plasma fibrinolytic activity. Electron and fluorescent microscopic studies of leukocytes migrating into areas of experimentally induced inflammation furthermore revealed the involvement of the specific granules of neutrophils in the digestion of fibrin deposited at the inflammatory site.

Attempts to correlate leukocyte fibrinolytic activity and elevated blood levels of fibrinolytic enzymes following various kinds of stress have remained speculative. Although no evidence for the involvement of leukocytes in the fibrinolytic response to vigorous exercise and injection of adrenalin could be found, they may indeed be the source of the increased fibrinolytic activity in the blood noted following intravenous administration of protein-free bacterial pyrogen.

Stresses like extracorporeal perfusion, hemorrhage, and trauma, which seem to cause increased release of plasminogen activator from liver and kidney lysosomes, may well also effect the leukocytes.

Thus, although the contribution made by leukocytes to the dissolution of fibrin in vivo in most physiologic and pathologic fibrinolytic states remains as yet unknown, it is hoped that techniques such as those used in this study may help to answer some of these questions.
REFERENCES


15. Wünschmann, B., and Goldstein, I.: Unpublished observations.


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