Release of Plasminogen Activator from Viable Leukocytes of Man, Baboon, Dog and Rabbit[1] (36840)

BARBARA WÜNSCHMANN-HENDERSON, DAVID L. HORWITZ,[2] AND TAGE ASTRUP

James F. Mitchell Foundation, Institute for Medical Research, Washington, D. C. 20015; and the Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland 20014

Normal, viable, human neutrophilic granulocytes contain small amounts of plasminogen activator, the release of which parallels that of beta-glucuronidase and is enhanced by bacterial endotoxin suggesting a common lysosomal localization (1). We now report a comparative study of the fibrinolytic response to endotoxin of leukocytes from some animal species (baboon, dog, rabbit) frequently used in studies of endotoxin effects. Some precautions, essential for obtaining reproducible results with leukocyte suspensions, are indicated.

Materials and Methods. Leukocytes. Using plastic tubes, blood was regularly collected in heparin sodium (10 units/ml blood). Citrate (1 ml of 0.11 *M* trisodium citrate/9 ml blood), used occasionally, gave identical results. Blood samples were processed within less than 1 hr after collection. Human leukocytes were obtained from venous blood of healthy donors by dextran (Pharmacia, Ltd.) sedimentation (1, 2). For comparison, separations were also made with 0.4% bovine fibrinogen [either Fraction I (Armour, Kankakee, IL) or a preparation made with ammonium sulfate (3)]. Leukocytes from baboon, dog and rabbit were separated with dextran. Blood from healthy, adult male baboons (*Papio doguera*) was collected through a polyethylene catheter in the femoral vein. Samples from dogs were obtained by femoral venipuncture. Rabbit blood was obtained by heart puncture.

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[2] Current address: Department of Medicine, University of Chicago Hospital, 950 East 59th St., Chicago, IL 60637.

Suspensions containing 15,000 to 25,000 leukocytes/mm^3 were prepared in 5% human serum albumin (Alumisol, Merck, Sharp, and Dohme, Philadelphia, PA). For viability testing, aliquots were added to a solution of 0.2% trypan blue in saline (Chroma, Stuttgart) and the percentage of unstained cells was determined. Only suspensions with more than 95% viable leukocytes were used. Differential counts (200 cells) were made on Wright stained smears. Endotoxin (lipopolysaccharide B from *Salmonella abortus* equi, Difco, Detroit, MI) was dissolved in Hanks' balanced salt solution. Smears were prepared 30 min after addition of the endotoxin, freshly dissolved, to leukocyte suspensions.

For comparison, some suspensions of human leukocytes were frozen three times in dry ice and thawed at 37° in rapid succession. Others (of man, baboon and dog) were centrifuged for 20 min at 1250 rpm, the pellets of closely packed leukocytes were rapidly frozen, and sections were cut in the cryostat at 8 μm and briefly dried in the air.

Fibrinolytic studies. For localization of fibrinolytic activity, the smears and sections were covered with fibrin (4, 5), using plasminogen-rich bovine fibrinogen (3, 6) and bovine thrombin (Leo Pharmaceuticals, Copenhagen). In tests for nonspecific protease, plasminogen-free fibrinogen (6) was used. The fibrinogen–thrombin mixture was spread over an area (2.5 $\times$ 5 cm) slightly larger than before, and the rinsing in tap water of the formaldehyde-fixed slides was extended to 20 hr to make the zones of weak lysis around individual cells more visible. Fibrin slides with smears were incubated for 60 or 120 min at 37°, and the percentage of fibrinolytic cells was determined from counts

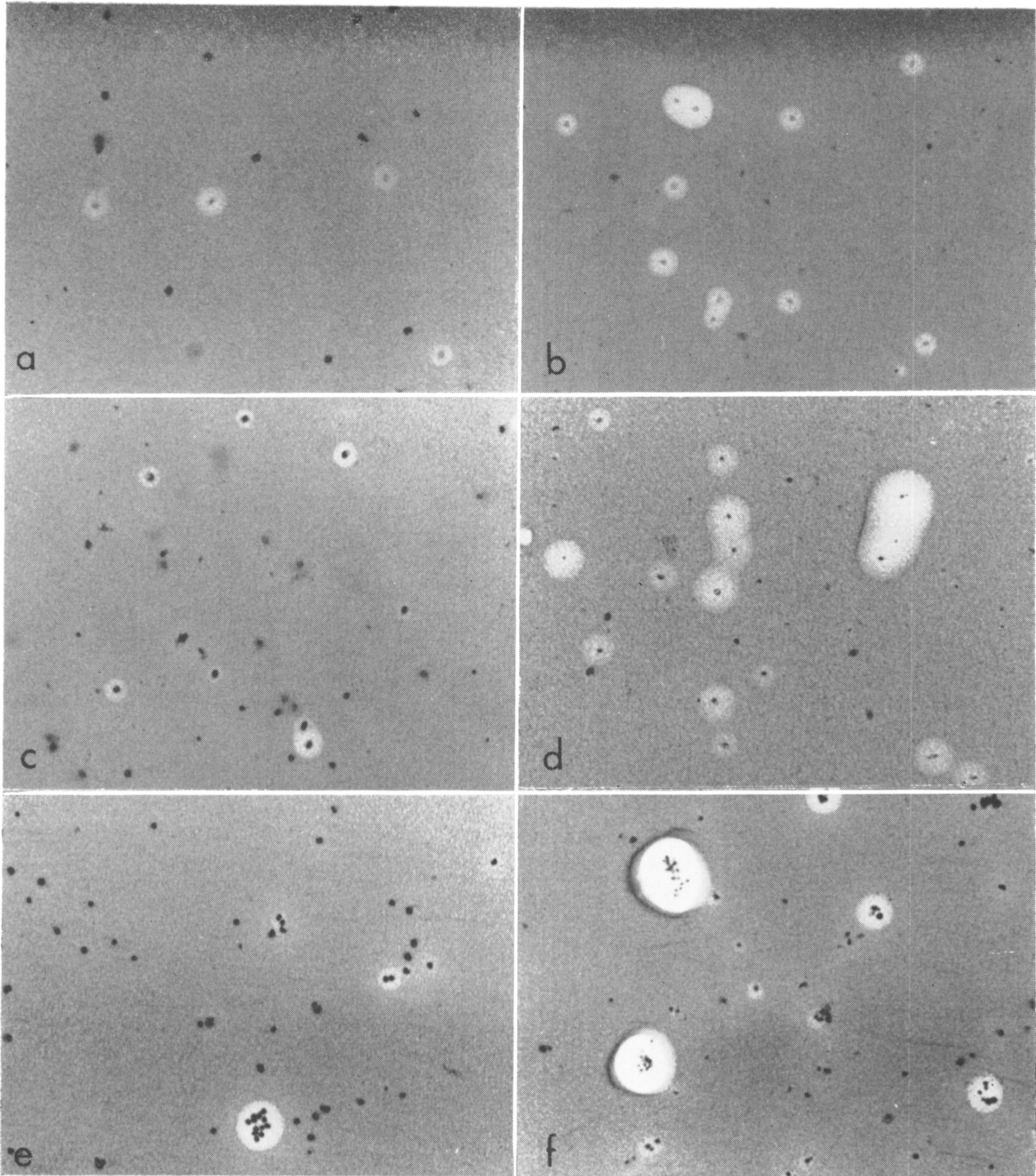

FIG. 1a–f. Fibrinolysis in smears of dextran separated leukocytes from peripheral blood of baboon (a, b), dog (c, d), and rabbit (e, f). Untreated (a, c, e) or after *in vitro* exposure to 100 μg/ml endotoxin (b, d, f). Fibrin slide technique, plasminogen-rich fibrin, incubated 120 min, reduced from $\times$ 154. Lysis indicated by clear zones in the stained fibrin.

in seven preselected fields on triplicate slides. Fibrin slides with frozen sections were incubated from 15 to 120 min.

Results and Discussion. Smears covered with plasminogen-rich fibrin showed small, demarcated zones of lysis around individual leukocytes from baboon and dog after incubation for 60 to 120 min (Fig. 1a and c). The percentage of fibrinolytic leukocytes was lower in baboon and dog than in man, in part reflecting the lower percentage of neutrophilic granulocytes (Table I) (7). Lysis was absent on slides with plasminogen-free fibrin. In smears from rabbit, leukocytes

TABLE I. Influence of Endotoxin on the Number of Fibrinolytic Cells Among Leukocytes from the Peripheral Blood of Man, Baboon and Dog.

Endotoxin (μg/ml)	Human: Neutrophilic granulocytes (% ± SE)	Human: Lytic cells (% ± SE)	Human: Subjects	Baboon: Neutrophilic granulocytes (% ± SE)	Baboon: Lytic cells (% ± SE)	Baboon: Animals	Dog: Neutrophilic granulocytes (% ± SE)	Dog: Lytic cells (% ± SE)	Dog: Animals
0	70 ± 10	34 ± 8	5	42 ± 13	15 ± 7	7	51 ± 10	11 ± 6	7
50	70 ± 10	56 ± 10	5	42 ± 13	40 ± 6	5	51 ± 10	21 ± 11	7
100	70 ± 10	62 ± 10	5	42 ± 13	36 ± 11	7	51 ± 10	34 ± 14	7

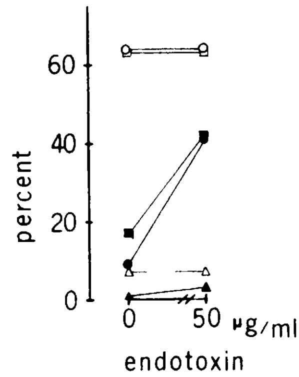

FIG. 2. Correlation between percentage of fibrinolytic cells and neutrophil yield using different leukocyte separation procedures. Human peripheral blood.

(○, □, △) neutrophilic granulocytes; (●, ■, ▲) fibrinolytic cells; (○, ●) dextran sedimentation; (□, ■) fibrinogen sedimentation (Armour, Fraction I); (△, ▲) fibrinogen sedimentation (Armour, Fraction I) with spurious fibrin formation.

tended to clump. Lytic activity appeared after 120 min in relation to clusters of cells (Fig. 1e). The results show that viable neutrophilic granulocytes of baboon, dog and rabbit contain low concentrations of plasminogen activator. Rabbit leukocytes were the least active, an observation correlating well with the low overall fibrinolytic activity of this species (8).

To determine the influence of different separation techniques on the yield of neutrophils and the percentage of fibrinolytic cells, human leukocytes were simultaneously separated with dextran and fibrinogen. In six experiments no difference was observed in the mean percentage of fibrinolytic cells. A slightly lower yield (13%) of neutrophils in the fibrinogen sedimented samples was not statistically significant. In two additional fibrinogen experiments, spurious formation of fibrin occurring in the final leukocyte suspension markedly decreased the number of neutrophils and fibrinolytic cells. Representative experiments are presented in Fig. 2. Carefully applied dextran sedimentation produces suspensions containing more than 95% viable cells, with 1/3 to 1/2 of the potentially active neutrophilic granulocytes showing fibrinolytic activity (Table I). Furthermore,

for accurate comparisons, suspensions should be assayed without delay because prolonged storage produces a transient increase in number of fibrinolytic cells followed by a decrease to lower than starting levels, with a concomitant appearance of plasminogen activator and beta-glucuronidase in the aqueous phase despite retainment of cellular viability (1). Smears prepared from frozen and thawed suspensions showed no fibrinolytic cells on either plasminogen-rich or plasminogen-free fibrin.

Despite the low fibrinolytic activity of individual leukocytes, considerable activity was exhibited by accumulations of cells. Thus, in sections of pellets large zones of lysis appeared after 15 to 30 min incubation of human or baboon leukocytes, and after 60 min with dog leukocytes. On plasminogen-free fibrin, sections with human and baboon leukocytes showed weak activity after 120 min incubation while sections with dog leukocytes were inactive. We found the leukoprotease activity of individual cells too low to show up by the slide technique.

The data indicate that complete disruption of suspended cells leaves the stroma devoid of plasminogen activator, an observation reminiscent of the decrease in number of fibrinolytic cells occurring during prolonged storage of the suspensions. Some discrepancies in the literature (9–12) may find their explanation by this observation. Thus, fibrinolytic activity in stroma-rich fractions from disrupted leukocytes from pig (11) or man (12) was reported to be resistant to epsilon-aminocaproic acid (EACA). However, the fibrinolytic activity in freshly prepared human leukocytes was inhibited by EACA (1). Since EACA is a potent inhibitor of plasminogen activation, the reported activity may have been caused by the leukoprotease remaining after disruption.

Results of the experiments with endotoxin showed a fibrinolytic response of baboon and dog leukocytes qualitatively similar to that of human leukocytes (Fig. 1b, d, Table I). The fibrinolyic cells increased in number with increasing concentrations of endotoxin, and at maximum stimulation approached the percentage of neutrophils. The number of active leukocytes also increased in the rabbit

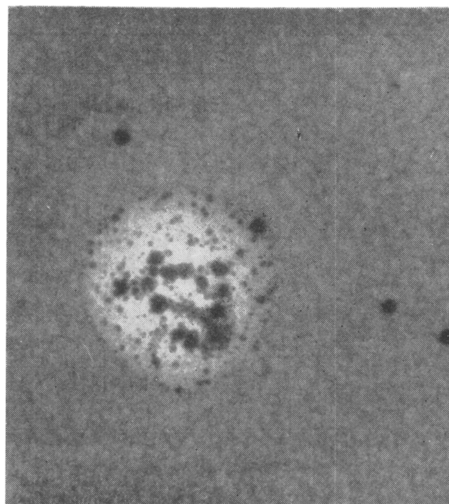

FIG. 3. Smear prepared from dextran separated rabbit leukocytes following *in vitro* exposure for 30 min to 100 μg/ml endotoxin. Fibrin slide technique. Plasminogen-rich fibrin. Incubated 120 min, × 282. Numerous granules are seen distributed over an area of fibrinolysis produced by a cluster of cells.

(Fig. 1f), but clumping prevented an accurate assessment. Endotoxin, even at 100 μg/ml solution, did not decrease the percentage of viable cells, but the cells became degranulated, most prominently in the rabbit where large granules were seen in the fibrin film adjacent to clusters of leukocytes (Fig. 3). It is of interest to mention that Lack (13) used endotoxin to free plasminogen activator from the lysosomal fractions of rabbit polymorphs. The ability of endotoxin to enhance plasminogen activator release may have pathogenic significance in states of endotoxemia associated with increased blood fibrinolysis (14). This possibility should be kept in mind when endotoxin effects are studied in animals of the species used in this report.

Summary. A comparative histochemical study of the endotoxin induced fibrinolytic activity in suspensions of leukocytes from man, baboon, dog and rabbit is reported. Apart from quantitative differences, leukocytes from the animal species behaved as human leukocytes. The number of fibrinolytic cells normally present increased following exposure to endotoxin and approached that of the neutrophils. Cell viability was not af-

fected. Attention is drawn to certain critical aspects of methodology.

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