SECRETION OF PLASMINOGEN ACTIVATOR BY HUMAN POLYMORPHONUCLEAR LEUKOCYTES*

Modulation by Glucocorticoids and Other Effectors

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Several lines of evidence show that suitably stimulated mononuclear phagocytes synthesize and secrete an activator of the serum proenzyme plasminogen (1). The production of plasminogen activator by these cells appears to be correlated with their involvement in inflammatory reactions: macrophages obtained from inflammatory exudates, or exposed in vitro to inflammatory agents such as asbestos fibers, phorbol myristate acetate, or concanavalin A, secrete substantial amounts of this enzyme, whereas cells obtained from noninflamed peritoneal cavities secrete little or none (1–4). Furthermore, low concentrations of anti-inflammatory steroids, or the mitotic inhibitors colchicine and vinblastine, block the production of this enzyme (5). Based on these and other observations, it has been suggested that plasminogen activator is involved in the migration of mononuclear phagocytes (5).

Polymorphonuclear leukocytes (PMNs)[1] have many properties in common with mononuclear phagocytes: both cell types are migratory, phagocytic, and capable of digesting endocytosed materials and microorganisms. Like monocytes, PMNs are recruited from the blood stream and migrate into sites of inflammation. In contrast to mononuclear phagocytes, PMNs are relatively short lived, and they are considered to be terminally differentiated cells, with little if any biosynthetic capacity. The proteolytic potential of PMNs was recognized by Opie as early as 1905 (6). Human PMNs contain elastase (7), a chymotryptic activity (8), and collagenase (9). In addition, histochemical observations have shown that PMNs can lyse plasminogen-rich, but not plasminogen-poor fibrin, suggesting that these cells contain a plasminogen activator (10).

We report here the identification and partial characterization of plasminogen activator from human PMNs and describe some factors that govern its secretion. We have found that secretion can be modulated during short-term cell culture by some of the same agents that modulate enzyme production by mouse macrophages.

Materials and Methods

Materials. Reagents were obtained as follows: heparin (Bio-Heprin, Ries Biologicals, Inc.); Dextran T500 and Ficoll 400 (AB, Pharmacia Fine Chemicals, Piscataway, N. J.); Hypaque

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[1] *Abbreviations used in this paper*: AT-FBS, acid-treated fetal bovine serum; CM, conditioned medium; Con A, Concanavalin A; DFP, diisopropylfluorophosphate; FBS, fetal bovine serum; MEM, minimal essential medium; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMNs, polymorphonuclear leukocytes; SDS, sodium dodecyl sulfate; TLCK, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, L-1-tosylamide-2-phenylethyl-chloromethyl ketone.

(Winthrop Laboratories, New York); *N*-acetyl-(ala)$_4$-chloromethyl ketone was kindly provided by Dr. J. Powers, Georgia Institute of Technology, Atlanta, Ga.; elastase (purified elastase from swine pancreas, 72.7 U/mg, Worthington Biochemical Corp., Freehold, N. J.); *p*-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, Mo.); Dulbecco's minimal essential medium (MEM) and fetal bovine serum (FBS; GIBCO, Grand Island, N. Y.); acrylamide, bis-acrylamide, N,N, N'N'-tetramethylethylenediamine and ammonium persulfate (Eastman Kodak Co., Rochester, N. Y.); concanavalin A (Con A) crystallized three times (Miles Laboratories Inc., Elkhart, Ind.). Phorbol myristate acetate (PMA) and phorbol were the gifts of Dr. S. Belman, New York University Medical Center, N. Y.; fibrinogen, dibutyryl-cAMP, cGMP, 8-bromo-cGMP, dibutyryl cGMP, dexamethasone, cycloheximide, and elastin were all from the Sigma Chemical Co.; cholera enterotoxin (Schwartz/Mann, Div. Becton, Dickinson & Co., Orangeburg, N. Y.); actinomycin D (Aldrich Chemical Co., Inc., Milwaukee, Wis). All other reagents were of the highest grade available.

Preparation of Cells. Human peripheral blood PMNs were obtained from heparinized (2 U/ml) blood of healthy volunteers and purified by a method derived from that of Böyum (11): 1/5 vol of Dextran T500 (6% wt/vol in 0.9% NaCl) was mixed with the freshly drawn blood, and the red cells allowed to sediment for 45 min at 37°C. The plasma, containing white blood cells and platelets, was layered over a solution of 6.35% wt/vol Ficoll 400 and 10.03% Hypaque, and centrifuged for 30 min at 500 *g* at 4°C. The PMNs and some contaminating erythrocytes were found as a pellet at the bottom of the tube, whereas the lymphocytes, monocytes, and platelets remained at the plasma/Ficoll-Hypaque interface. The pellet was resuspended in phosphate-buffered saline (PBS), washed by centrifugation (300 *g*, 5 min), and the erythrocytes lysed in 0.83% NH_4Cl (pH 7.4). After resuspending and washing in Dulbecco's MEM, the cells were counted in Türk's solution, or stained either with Giemsa, or with trypan blue. This method gave over 99% of PMNs, of which more than 95% were viable, as estimated by their capacity to exclude trypan blue. During the entire preparation procedure, and during culture, the cells were never exposed to glass, and all manipulations were performed using plasticware. All cultures were performed at 37°C, in a 5% CO_2-95% air atmosphere.

Fibrinolytic Activity. The fibrinolytic activity of purified PMNs was determined by culturing the cells in ^{125}I-fibrin-coated Linbro wells (see below). The cells were plated in 1 ml Dulbecco's MEM supplemented with 5% FBS or with 5% plasminogen-depleted FBS (12), in the presence or absence of Con A or PMA. Fibrinolysis was estimated by withdrawing samples of medium and assaying them for solubilized radioactivity.

Preparation of Conditioned Media (CM). Purified PMNs (1–10 × 10^6/ml) were cultured either in Dulbecco's MEM supplemented with 1% plasminogen-depleted acid-treated (1) FBS (AT-FBS) or in Hepes buffered Gey's salt solution (for assay of alkaline phosphatase), in Linbro wells (FB 16-24TC). The cultures were incubated in the presence of the compounds to be assayed as indicated. At the end of the incubation period the CM were collected, the cells sedimented by centrifugation (500 *g*, 5 min), and the pellet either discarded or lysed (see below) and assayed for enzymatic activity. The CM were frozen and stored at −20°C until assayed.

Preparation of Cell Lysates. Purified PMNs (1.5 × 10^6) were lysed in 0.5% (vol/vol) Triton X-100 in the presence or absence of *N*-acetyl-(ala)$_4$-chloromethyl ketone (2 mM) and soybean trypsin inhibitor (100 μg/ml), and centrifuged at 500 *g* for 6 min; the supernate was then electrophoresed in a sodium dodecyl sulfate (SDS) 11% polyacrylamide gel, and the gel analyzed for fibrinolytic activity (see below).

Alternatively, Dulbecco's MEM supplemented with 1% plasminogen-depleted AT-FBS, or Hepes buffered Gey's salt solution, containing 5 × 10^6 PMNs/ml, were made 0.5% in Triton X-100, and then frozen and thawed rapidly three times. After clearing by centrifugation (500 *g*, 10 min), these cell lysates were assayed immediately for enzymatic activity.

Detection of Plasminogen Activator Secretion by Single Cells. The proportion of cells secreting plasminogen activator was determined by a casein-agar overlay procedure previously described (4). PMNs (5 × 10^3) were plated in 60-mm tissue culture dishes (Falcon 3002, Falcon Plastics, Oxnard, Calif.) in 1 ml of Dulbecco's MEM supplemented with 1% plasminogen depleted AT-FBS, in the presence or absence of dexamethasone. After 3 h of incubation, Con A or PMA were added where required, and 1 h later the medium was removed and the plates overlaid with a casein-agar mixture containing dexamethasone and/or Con A or PMA as indicated; the overlay mixture was prepared either with or without purified human plasminogen (20 μg/ml). The

cultures were fixed and stained 5 h later. The proportion of cells surrounded by lytic zones was determined microscopically in triplicate cultures by scoring 200 cells from each culture.

Preparation of ^{125}I-Fibrin-Coated Wells. ^{125}I-Fibrin-coated wells of Linbro disposo trays (FB16-24TC) were prepared as described (5). Each well contained approximately 20 μg ^{125}I-fibrin (5,000 cpm/μg). The amount of ^{125}I-fibrin solubilized is expressed as the percentage of the total radioactivity that can be released by an excess of trypsin.

Enzyme Assays. Plasminogen activator (5), lysozyme (13, 14), and β-glucuronidase (15) were assayed as described. Alkaline phosphatase activity was tested using *p*-nitrophenyl phosphate as a substrate (16). Elastinolytic activity was measured as described by Rifkin et al. (17), using Sepharose-bound ^{125}I-elastin as substrate.

Polyacrylamide Gel Electrophoresis. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli (18). Samples of CM or cell lysates were mixed with double strength sample buffer (0.125 Tris HCl pH 6.8, 5% SDS; 20% glycerol), and applied to a 0.1% SDS-11% polyacrylamide slab gel using a 4% polyacrylamide stacking gel. Electrophoresis was conducted at a constant current of 8 mA for 15 h at room temperature. The gel was then processed and assayed for fibrinolytic activity in the presence and absence of plasminogen, by a method described fully elsewhere.[2]

In brief, the gel was layered onto the surface of a fibrin agar gel which is opaque due to the presence of fibrin. Fibrinolytic activity was monitored visually by dark-field illumination and could be observed as gradually enlarging clear areas developing in the surrounding opaque fibrin-agar layer. After a desired degree of fibrinolysis was observed, the electrophoretic gel was removed and the fibrin-agar layer was fixed and stained with amido-black (0.1% in methanol-acetic acid), and then destained.

Results

Fibrinolysis by Cultured PMNs. The fibrinolytic activity of purified PMNs was measured after plating these cells in ^{125}I-fibrin-coated culture wells. Fibrinolysis progressed linearly with time, and was proportional to cell concentration up to 5×10^5 cells per well (Fig. 1). In the absence of plasminogen, the fibrinolytic activity was always lower, by a factor of 2- to 10-fold, varying from experiment to experiment (Figs. 1–2). This suggested that these cells could promote fibrinolysis by at least two different pathways; one of these required the presence of plasminogen and was therefore presumably due to plasminogen activator(s), and the other was independent of the presence of plasminogen, and hence due to a different enzyme(s).

The fibrinolytic activity of purified mononuclear cells was much smaller than that of PMNs: 1×10^5 mononuclear cells did not promote detectable fibrinolysis during the first 8 h of assay. Inasmuch as the proportion of mononuclear cells in the purified PMN populations did not exceed 1% of the total, this demonstrated that the fibrinolytic activity observed was due to PMNs and not to contaminating mononuclear cells.

The plasminogen-dependent fibrinolytic activity was selectively stimulated by incubation of the cells in the presence either of the lectin Con A (10^{-7} M) or of low concentrations of the tumor promoter and inflammatory agent PMA (8×10^{-10} M) (Fig. 2). The extent of this stimulation was somewhat variable: preparations of PMNs that exhibited a rather high "spontaneous" plasminogen-dependent fibrinolytic activity could not be stimulated as much as preparations that had only very low spontaneous activity; this stimulation varied, therefore,

[2] Granelli-Piperno, A., and E. Reich. Manuscript submitted for publication.

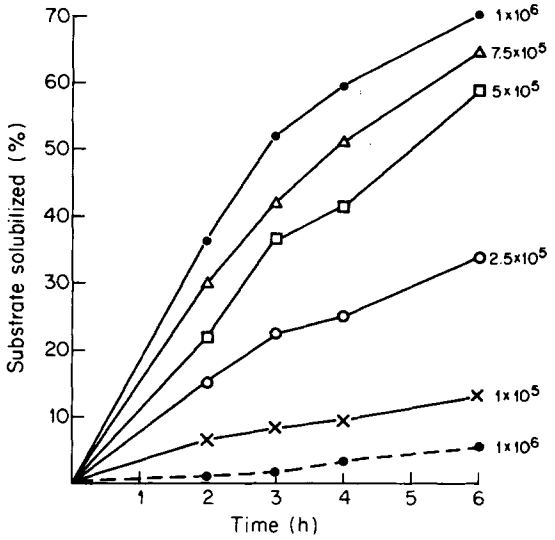

FIG. 1. Fibrinolysis by purified human PMNs. Serial dilutions of cells were cultured in ^{125}I-fibrin coated wells in 1 ml Dulbecco's MEM supplemented with 5% AT-FBS (——) or 5% plasminogen-depleted AT-FBS (- - -). Fibrinolysis was estimated by withdrawing aliquots of medium at various times, and assaying them for solubilized radioactivity, as described in Materials and Methods. ●——●, 1×10^6 cells/ml; △——△, 7.5×10^5 cells/ml; □——□, 5×10^5 cells/ml; ○——○, 2.5×10^5 cells/ml; ×——×, 1×10^5 cells/ml; ●– –●, 1×10^6.

from experiment to experiment between 2- and 10-fold.[3] In contrast, the plasminogen-independent fibrinolytic activity was always totally unaffected under these conditions.

Higher concentrations of PMA, but not of Con A (up to 10^{-6} M), caused an increase in plasminogen-independent fibrinolysis: in the presence of 1.6×10^{-9} M PMA an early increase in plasminogen-dependent fibrinolysis was followed after 2–3 h by a marked increase in plasminogen-independent lysis. Even higher concentrations of PMA (1.6×10^{-8} M) caused a still more rapid and short-lived increase in plasminogen-independent fibrinolysis; this could be detected within 30 min after addition of PMA, and was practically terminated by 2 h (Fig. 2).

Phorbol, an analogue of PMA that is inactive both as a tumor promoter and as an inflammatory agent, did not affect fibrinolysis by PMNs.

The viability of PMNs cultured under these conditions was estimated by their ability to exclude trypan blue. Most of the cells cultured in the absence or presence of Con A (10^{-7} M) or low concentrations of PMA (8×10^{-10} M) were viable throughout all of the experiments: after 8 h over 95% of these cells excluded trypan blue. In contrast, higher concentrations of PMA (1.6×10^{-8} M) dramatically reduced the viability of the cells: whereas after 2 h of incubation

[3] The reasons for this variability are not clear, but it should be recalled that the purification of PMNs involved exposure of the cells to Dextran and Ficoll, compounds known to activate the complement system, and hence perhaps capable of generating yet undefined mediators affecting plasminogen activator secretion.

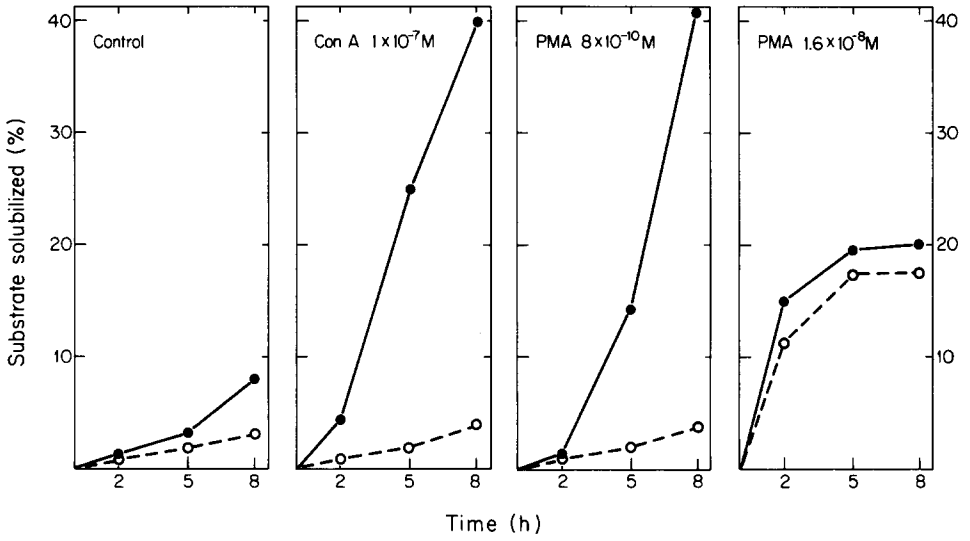

FIG. 2. Fibrinolysis by PMNs; effects of Con A and PMA. Purified PMNs (5×10^5) were plated in ^{125}I-fibrin-coated wells in 1 ml Dulbecco's MEM supplemented with 5% of FBS (●——●) or 5% of plasminogen-depleted FBS (O- - -O). Con A and PMA were present at the indicated concentration throughout the period of fibrinolysis measurements. Fibrinolysis was estimated by assaying aliquots of the medium for solubilized radioactivity at the indicated times.

over 90% were viable, by 5 h only 10% still excluded trypan blue. Hence stimuli that evoked plasminogen-dependent fibrinolysis gave no evidence of cell damage for many hours; in contrast, conditions giving rise to a plasminogen-independent fibrinolysis produced cell death within a few hours. On the other hand, the slow loss of viability that occurs during prolonged incubation of untreated PMNs did not lead to enzyme release because there was no detectable increase either in plasminogen-independent or plasminogen-dependent fibrinolysis under these conditions.

Secretion of Plasminogen Activator. CM from cultures of purified PMNs contained plasminogen activator, as shown by the capacity to catalyze plasminogen-dependent fibrin degradation. The amount of enzyme secreted increased with time, and secretion was stimulated by Con A (10^{-7} M) or low concentrations of PMA (8×10^{-10} M) (Fig. 3). Both the spontaneous and the Con A- or PMA-stimulated production of plasminogen activator were decreased by addition of actinomycin D or cycloheximide (Table I), suggesting that maintenance of RNA and protein synthesis were required for initiation of enzyme secretion.

Glucocorticoids inhibit the production of plasminogen activator by various cell types in culture, including hepatoma cell lines (19), human embryo lung fibroblasts (20), rat tongue organ culture (21), and, in particular, mouse peritoneal macrophages (5). The same was true for enzyme secretion by human PMNs (Table I). The data in Fig. 4 show a dose-response curve for the dexamethasone effect on enzyme secretion by unstimulated and by Con A-treated PMNs: significant inhibition was detected at drug concentrations as low as 10^{-10} M, and 50% inhibition was observed at approximately 10^{-9} M.

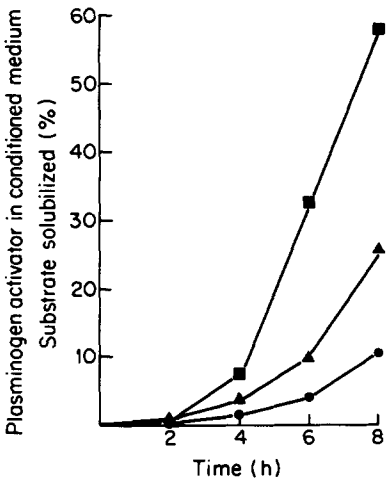

FIG. 3. Time-course of the effect of Con A and PMA on plasminogen activator secretion by PMNs. PMNs (1×10^7) were cultured in 1 ml Dulbecco's medium supplemented with 1% plasminogen-depleted AT-FBS, in the presence or absence of Con A or PMA. Aliquots of CM were collected at the indicated times and 10 μl assayed for plasminogen activator activity, as described in Materials and Methods. Control, ●——●; Con A, (10^{-7} M), ■——■; PMA (8×10^{-10} M) ▲——▲.

TABLE I

*Effect of Inhibitors on the Secretion of Plasminogen Activator and Elastase by PMNs**

Inducer added:	–		Con A, 10^{-7} M		PMA, 8×10^{-10} M		PMA, 1.6×10^{-8} M	
Plasminogen present:	+	–	+	–	+	–	+	–
–	6.1	0.2	62.8	0.2	12.6	0.1	42	40
Actinomycin D, 5 μg/ml	3.2	0.6	9.8	0.3	4.2	0.2	40	39
Cycloheximide, 10 μg/ml	2.5	0.4	17.4	0.2	5.5	0.3	39	40
Dibutyryl cAMP, 10^{-3} M	2.3	0.2	4.0	0.2	3.0	0.1	46	44
Theophylline, 10^{-3} M	5.4	0.3	7.4	0.3	3.8	0.2	41	36
Cholera toxin, 10^{-9} M	2.0	0.2	4.5	0.2	6.0	0.1	44	38
Dexamethasone, 10^{-7} M	2.6	0.2	12.8	0.2	4.2	0.2	40	38

Purified PMNs (1×10^7) were incubated in 1 ml Dulbecco's MEM supplemented with 1% plasminogen-depleted AT-FBS in the presence or absence of drugs. After 3 h Con A or PMA were added. 6 h later CM was collected and 10 μl of the samples were assayed for plasminogen-dependent and independent activity on ^{125}I-fibrin plates (4 h assay).

* Substrate solubilized, %.

Other steroids were also tested, and the effective concentrations for 50% inhibition of plasminogen activator secretion were: hydrocortisone, 10^{-8} M; deoxycorticosterone, progesterone, $>10^{-6}$ M; β-estradiol $>10^{-5}$ M. The inhibitory effect of dexamethasone occurred rapidly: it could be detected as early as 1 h after drug addition and persisted for at least 10 h.

Cyclic AMP and related compounds also affect the production of plasminogen activator: cAMP stimulates enzyme production by ovarian granulosa cells (22), and inhibits enzyme production by macrophages (5). Enzyme secretion by PMNs, whether or not stimulated by Con A or PMA, was inhibited by dibutyryl cAMP, by theophylline, and by cholera toxin, an adenyl-cyclase stimulant that increases cAMP levels in many cell types, including PMNs (23; Table I). In contrast, neither cGMP, dibutyryl cGMP, nor 8-bromo-cGMP affected enzyme production under our conditions.

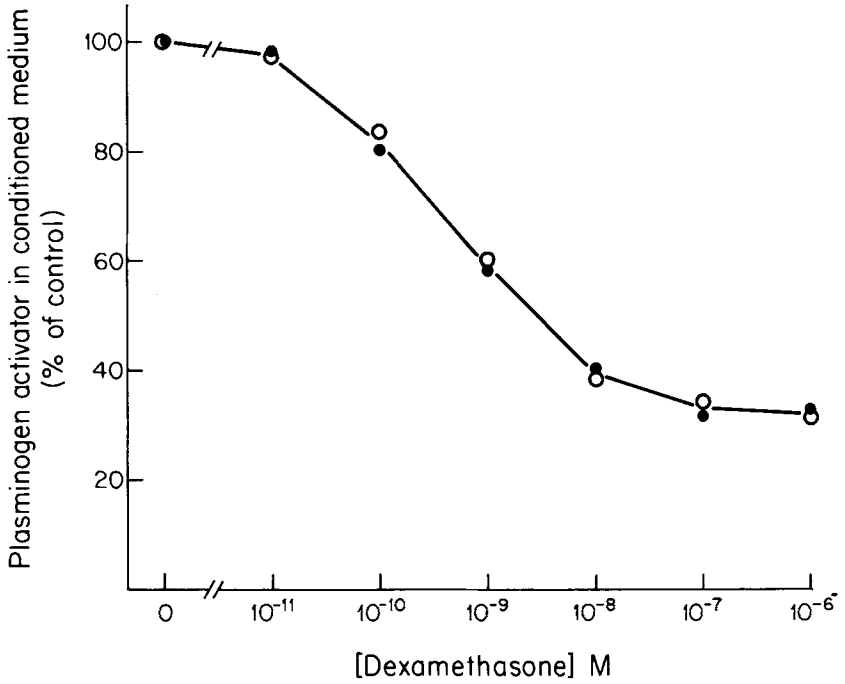

FIG. 4. Secretion of plasminogen activator by PMNs; effect of dexamethasone. Purified PMNs (5×10^6) were cultured in 1 ml Dulbecco's MEM supplemented with 1% plasminogen-depleted AT-FBS, in the presence or absence of various concentrations of dexamethasone. After 2 h, some of the cultures received Con A (10^{-7} M): the CM was collected from all cultures 6 h later, and assayed for plasminogen activator as described in Methods. The results are expressed as percentages of the plasminogen activator activity present in CM from dexamethasone-free cultures. Control, O——O; Con A, ●——●.

TABLE II
Plasminogen Activator Production by Individual PMNs

	Cells surrounded by lytic zone	
	% of total	
	–	Dexamethasone 10^{-7} M
Control	21	9
Con A, 10^{-7} M	66	17
PMA, 8×10^{-10} M	55	14

PMNs (5×10^3) were plated in 60-mm tissue culture dishes. After incubation with drugs (see Materials and Methods), the cultures were washed and overlaid with a casein-agar mixture. Cultures were fixed and stained 5 h later. The results are expressed as the percent of cells surrounded by a lytic zone. 200 cells were scored in each of triplicate cultures.

The proportion of cells that secreted plasminogen activator in different experiments was determined by using a casein-agar overlay procedure that permits detection of proteolysis by individual cells. The results in Table II demonstrate that the proportion of cells surrounded by lytic zones was increased by Con A and PMA (8×10^{-10} M), and decreased by dexamethasone; in

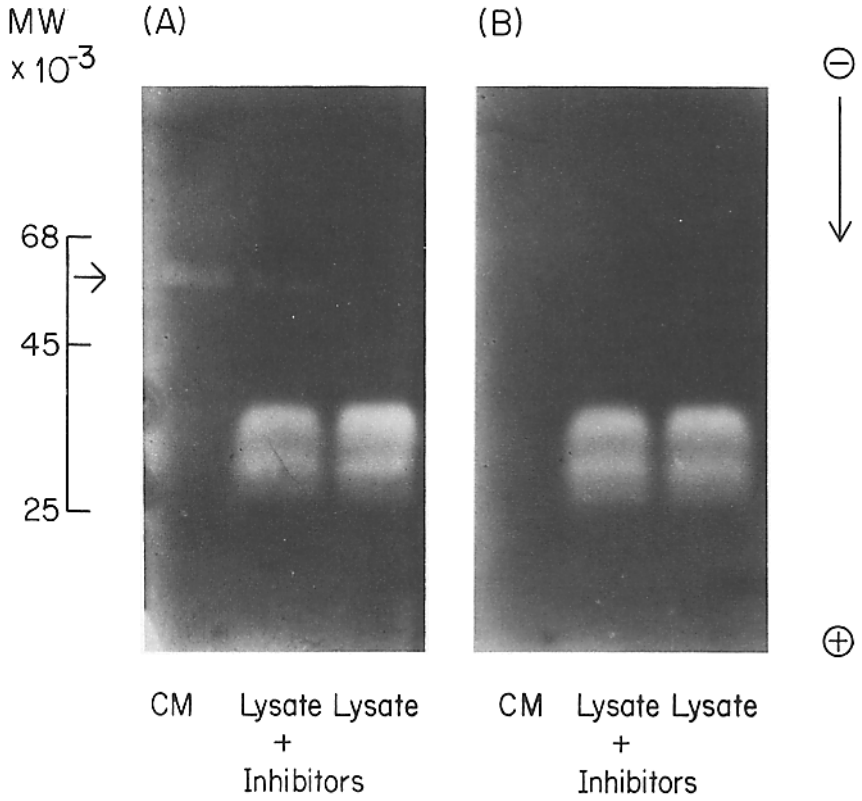

FIG. 5. Identification of plasminogen activator and elastase after SDS-polyacrylamide gel electrophoresis of CM and cell lysate from PMNs. CM from 1×10^6 purified PMNs collected after 8 h of incubation, and cell lysate from 1.5×10^6 cells lysed in the presence or absence of soybean trypsin inhibitor and N-acetyl-(ala)$_4$ chloromethyl ketone, were electrophoresed in an SDS-11% polyacrylamide gel, and the gel processed for detection of fibrinolytic activity either in the presence (A) or in the absence (B) of plasminogen, as described in Materials and Methods. The figure is a photograph of the amido-black stained fibrin-agar layer. The clear areas are due to lysis of fibrin in the agar gel by proteases. The arrow indicates plasminogen activator.

addition to their effect on the fraction of enzyme-producing cells, these agents also influenced the size of the individual lytic zones, whose diameter was increased by Con A or PMA and decreased in the presence of dexamethasone. No lytic zones were ever observed in the absence of plasminogen, even when the cells were treated with high concentrations of PMA (1.6×10^{-8} M), showing both that the digestion of casein was due to the secretion of plasminogen activator, and that the assay performed under these conditions did not detect the plasminogen-independent proteolytic activity. Plasminogen activator production was not restricted to a subpopulation of PMNs because at least 66% of these cells secreted the enzyme.

The plasminogen activator present in CM could be identified after SDS-polyacrylamide gel electrophoresis (Fig. 5). The apparent molecular weight of the enzyme—approximately 60,000—was comparable to that of plasminogen activators secreted by other human cells, such as human embryo lung fibroblasts, or human osteosarcoma and rhabdomyosarcoma cell lines (A. Granelli-

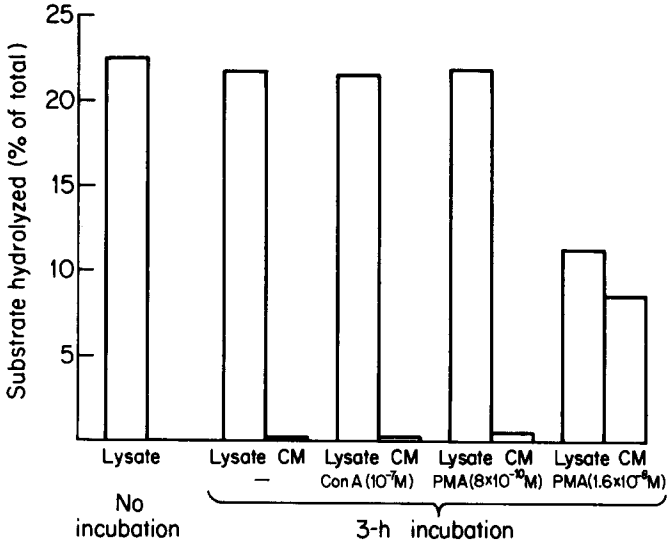

FIG. 6. Elastolytic activity of CM and cell lysates from PMNs. PMNs (1.3×10^6) were plated in 1 ml Dulbecco's MEM supplemented with 1% plasminogen-depleted AT-FBS. After 3 h of incubation in the presence or absence of Con A or PMA, CM was collected and the cells resuspended and lysed in presence of 0.5% Triton X-100. 100-μl samples were assayed for 6 h for elastolytic activity using Sepharose-bound ^{125}I-elastase as described in Materials and Methods.

Piperno, unpublished observations). The enzyme appeared to be the same in CM from unstimulated, or Con A- or PMA-treated cultures.

Release of Elastase. As noted above, CM from control cultures of PMNs or from those incubated in the presence of Con A (10^{-7} M) or low concentrations of PMA (8×10^{-10} M) did not promote plasminogen-independent proteolysis; however, higher concentrations of PMA (1.6×10^{-8} M) caused the appearance of such an activity (Table I). This plasminogen-independent component was characterized by its susceptibility to a spectrum of protease inhibitors: diisopropylfluorophosphate (DFP; 1 mM), soybean trypsin inhibitor (100 μg/ml), and a specific inhibitor of elastaselike enzymes, *N*-acetyl-(ala)$_4$-chloromethyl ketone (5 mM), all inhibited plasminogen-independent fibrinolysis by over 85%; in contrast, *N*-α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK; 10 mM), L-1-tosylamide-2-phenylethyl-chloromethyl ketone (TPCK; 10 mM), and Trasylol (10 Kallikrein inactivator units/ml) were without effect. On this basis, we tentatively assigned the plasminogen-independent fibrinolytic activity to elastase, a known human leukocyte enzyme that is reported to be present in the azurophil granules (24).

In addition, using a sensitive and specific assay for elastase (20), we confirmed that the elastolytic activity of CM and cell lysates was unaffected by Con A or low concentrations of PMA (8×10^{-10} M), whereas exposure to higher concentrations of PMA (1.6×10^{-8} M) caused the treated cells to release 40% of the total elastase within 3 h (Fig. 6). By comparing the elastolytic activity of PMN lysates with known amounts of commercial pancreatic elastase, we estimated that 1×10^6 human PMNs contain the equivalent of 0.02 U of pancreatic elastase.

In contrast to the secretion of plasminogen activator, the release of elastase induced by PMA (1.6×10^{-8} M) was completely insensitive to dibutyryl cAMP, theophylline, cholera toxin, or dexamethasone, as well as to actinomycin D or cycloheximide, suggesting that this enzyme was not being actively synthesized by the cells (Table I).

Proteolytic Activity of Cell Lysates. Both plasminogen activator and elastase could be identified after electrophoretic analysis of cell lysates in SDS-polyacrylamide gels (Fig. 5). Plasminogen activator could be detected only if the cell lysates were prepared in the presence of elastase inhibitors, presumably because it was otherwise exposed to proteolytic degradation by elastase. Plasminogen activator in cell extract migrated as a single band of the same apparent molecular weight as enzyme from CM (60,000), whereas elastase migrated as three bands with apparent molecular weights ranging from 33,000 to 28,000; these corresponded well to the molecular weights reported for three elastase isozymes isolated from granules of human myeloid leukemia cells (7). That the three plasminogen-independent bands of proteolysis were due to elastase was inferred from their absence when the gels were developed for fibrinolytic activity in the presence of *N*-acetyl-$(ala)_4$-chloromethyl ketone, a specific inhibitor of elastase.

Other Enzymes. Alkaline phosphatase, β-glucuronidase, and lysozyme were assayed in CM and cell lysates from cultures of purified PMNs. Neither Con A (10^{-7} M) nor low concentrations of PMA (8×10^{-10} M) caused the release of any of these enzymes, in contrast to their effect on plasminogen activator secretion. Higher concentrations of PMA (1.6×10^{-8} M; 2-h incubation) caused the release of over 50% of the lysozyme present in the cells, reduced the alkaline phosphatase content of cell lysates by more than 50%, but did not stimulate the release of significant amounts of β-glucuronidase.

Discussion

Human PMNs are rich in proteolytic enzymes whose maximal activity is expressed at neutral pH; they contain collagenase (9), elastase (7), and chymotrypsinlike enzymes (8). Our data establish the formation of yet another neutral protease by these cells, plasminogen activator. The demonstration of PMN plasminogen activator was achieved by experimental approaches that avoided the problem of degradation associated with the high levels of nonspecific proteases in these cells: (*a*) selective secretion of plasminogen activator could be stimulated in culture either by Con A or by very low concentrations of PMA, without concomitant release of elastase or any other detectable protease, and (*b*) plasminogen activator could be identified in lysates of PMNs provided that the cell extracts were prepared with protective concentrations of elastase inhibitors.

The synthesis and secretion of plasminogen activator by PMNs should be considered both in relation to the other proteases and to the biological properties of these cells. Several of the results obtained so far suggest that plasminogen activator may occupy a unique position among the proteases of PMNs:

(*a*) The stimulation of plasminogen activator production was the most sensitive of the PMN responses to Con A and PMA. Although both of these agents increase glucose oxidation and respiratory metabolism (25, 26), inhibit

phagocytosis (27, 28), and cause microtubule assembly, labilization of specific granules, and lysozyme release (29–31), all of these effects require concentrations of Con A and PMA that are 10- to 1000-fold higher than those yielding optimal stimulation of plasminogen activator secretion. Furthermore, maximal plasminogen activator secretion occurred without degranulation either of the collagenase-containing specific granules or of the azurophil granules in which elastase and other neutral proteases are located. It is also noteworthy that concentrations of PMA that caused release of elastase were associated with prompt and extensive loss of cell viability, whereas those that enhanced plasminogen activator production were not. All of these facts indicate that the storage and release of plasminogen activator are controlled differently than they are for the other proteases.

(*b*) Synthesis and secretion of PMN plasminogen activator were inhibited by actinomycin and by cycloheximide and puromycin (unpublished results). This indicates that both RNA and protein synthesis are needed to sustain continuing production of plasminogen activator, and it is again in contrast to the metabolism of the other granule-associated proteases. The release of elastase, when it occurred, was not affected by either actinomycin or cycloheximide; and it is known that the synthesis of the granule-associated components takes place in the bone marrow and is completed before PMNs enter the circulation, namely, at the promyelocyte and myelocyte stages, respectively, for the azurophil and specific granules (32). Hence, plasminogen activator differs from the other known PMN proteases also in its pattern of synthesis.

(*c*) A third property that sets plasminogen activator apart from the other leukocyte proteinases is the response to glucocorticoids. The action of glucocorticoids on PMN biology in vitro has been the subject of many studies, and some effects have been reported (33); however, with the exception of the inhibition of murine granulopoiesis in culture (34), all of these effects have required steroid concentrations 10^3 to 10^4-fold higher than those that inhibit plasminogen activator production. It is therefore significant (*1*) that plasminogen activator secretion is inhibited at very low steroid concentrations, comparable to those present in the body fluids under physiological conditions; (*2*) that the release of elastase and other enzymes, when it occurred, was not influenced by steroids; and (*3*) that the relative potencies of the steroids tested reflected their known anti-inflammatory potencies in vivo (35).

All of the above observations suggest that plasminogen activator synthesis is probably relevant both to PMN function in inflammation and to the anti-inflammatory effects of glucocorticoids. Although we do not yet know the significance of plasminogen activator for PMN physiology, the following working hypothesis appears to provide a reasonable focus for further tests.[4] Because

[4] This hypothesis requires a modification of the prevailing view that PMNs are terminally differentiated end products devoid of significant genetic transcription and minimally active in translation – the biosynthetic equivalents of nucleated erythrocytes or platelets. We provide the experimental basis for the proposed modification in a manuscript soon to be submitted to this journal; it shows that the majority of human peripheral blood PMNs actively synthesize RNA and protein, a substantial fraction of which is secreted under conditions of short-term cell culture. That cultured PMNs are able to synthesize products destined for secretion has already been suggested by the effects of inhibitors of RNA and protein synthesis on the secretion of pyrogen (36, 37).

release of elastase and other proteases is not linked to genetic transcription or translation, is unaffected by steroids, and is associated with prompt loss of cell viability, we suggest that these enzymes are a static part of the enzymatic equipment of PMNs, and are destined to function only intracellularly in conjunction with endocytosis. In contrast, because plasminogen activator production requires RNA and protein synthesis, and is sensitive to low concentrations of glucocorticoids, we propose (*a*) that the synthesis of this enzyme forms part of the response of PMNs to as yet unidentified extracellular stimuli or mediators, and (*b*) that its normal function is expressed in the local microenvironment after secretion by PMNs. We have previously summarized a body of evidence suggesting that the action of plasmin, locally generated by synthesis and secretion of plasminogen activator, forms an essential part of the mechanism of macrophage migration (5). All of these considerations, and a parallel body of supporting evidence, lead to the inference that the same may hold for PMNs; these cells, like macrophages, are migratory, are recruited to sites of inflammation, and their accumulation at such sites is blocked by glucocorticoids (38). In view of the parallel effects of glucocorticoids on macrophages, these results reinforce the proposal that the anti-inflammatory effects of corticosteroids are mediated, in part, through specific reductions in plasminogen activator secretion by both PMNs and macrophages, with secondary inhibition of their migration. If this hypothesis is correct, plasminogen activator secretion may provide a convenient assay for identifying and isolating the putative mediators responsible for recruiting inflammatory cells (39).

Summary

Purified human PMNs secrete plasminogen activator. This secretion is stimulated by Con A and low concentrations of PMA, and is inhibited by low concentrations of glucocorticoids, and by cAMP, actinomycin D, and cycloheximide. In contrast, the release of granule-bound enzymes, such as elastase, is achieved only at higher concentrations of PMA, and is not affected by any of the inhibitors that block plasminogen activator production.

These results show that the production of plasminogen activator by PMNs is controlled by agents that affect inflammations, and that this control is not shared by other lytic enzymes known to be associated with these cells. This suggests a particular role for plasminogen activator in the response pattern of PMNs and also supports the concept, previously developed for macrophages, that the secretion of this enzyme is correlated with cell migration in vivo.

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References

1. Unkeless, J., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* 139:834.
2. Gordon, S., J. Unkeless, and Z. A. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis: evidence for a 2 stage process. *J. Exp. Med.* 140:995.

3. Hamilton, J., J.-D. Vassalli, and E. Reich. 1976. Macrophage plasminogen activator: induction by asbestos is blocked by anti-inflammatory steroids. *J. Exp. Med.* 144: 1689.
4. Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: induction by concanavalin A and phorbol myristate acetate. *Cell.* 11:695.
5. Vassalli, J.-D., J. Hamilton, and E. Reich. 1976. Macrophage plasminogen activator: modulation of enzyme production by anti-inflammatory steroids, mitotic inhibitors, and cyclic nucleotides. *Cell.* 8:271.
6. Opie, E. L. 1905. Enzymes and antienzymes of inflammatory exudates. *J. Exp. Med.* 7:316.
7. Ohlsson, K., and I. Olsson. 1976. The neutral proteases of human granulocytes: isolation and partial characterization of granulocyte elastase. *Eur. J. Biochem.* 42: 519.
8. Rindler-Ludwig, R., and H. Braunsteiner. 1975. Cationic proteins from human neutrophil granulocytes: evidence for their chymotrypsin-like properties. *Biochim. Biophys. Acta.* 379:606.
9. Ohlsson, K., and I. Olsson. 1973. The neutral proteases of human granulocytes: isolation and partial characterization of two granulocyte collagenases. *Eur. J. Biochem.* 36:473.
10. Goldstein, I. M., B. Wünschmann, T. Astrup, and E. S. Henderson. 1971. Effects of bacterial endotoxin on the fibrinolytic activity of normal human leukocytes. *Blood.* 37:447.
11. Bøyum, A. 1976. Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol.* 5(Suppl. 5):9.
12. Ossowski, L., J. Quigley, G. M. Kellerman, and E. Reich. 1973. Fibrinolysis associated with oncogenic transformation: requirement of plasminogen for correlated changes in cellular morphology, colony formation in agar, and cell migration. *J. Exp. Med.* 138:1056.
13. Parry, R. M., Jr., R. C. Chandon, and K. M. Shahani. 1965. A rapid and sensitive assay of muramidase. *Proc. Soc. Exp. Biol. Med.* 119:384.
14. Gordon, S., J. Todd, and Z. A. Cohn. 1974. *In vitro* synthesis and secretion of lysozyme by mononuclear phagocytes. *J. Exp. Med.* 139:1228.
15. Bowers, W. E., J. T. Finkenstaedt, and C. de Duve. 1967. Lysosomes in lymphoid tissue. I. The measurement of hydrolytic activities in whole homogenates. *J. Cell Biol.* 32:325.
16. Garen, A., and C. Levinthal. 1960. A fine-structure genetic and chemical study of the enzyme alkaline phosphatase of *E. coli*. I. Purification and characterization of alkaline phosphatase. *Biochim. Biophys. Acta.* 38:470.
17. Rifkin, D., and R. Crowe. 1977. A sensitive assay for elastase employing radioactive elastin coupled to sepharose. *Anal. Biochem.* 79:268.
18. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.).* 227:680.
19. Wigler, M., J. P. Ford, and I. B. Weinstein. 1975. Glucocorticoid inhibition of the fibrinolytic activity of tumor cells. *In* Proteases in Biological Control. D. Rifkin, E. Reich, and E. Shaw, editors. Cold Spring Harbor Press, Cold Spring Harbor, N. Y. 849.
20. Rifkin, D., and R. Pollack. 1976. Proteolysis and physiological regulation. *Miami Winter Symp.* 11:263.
21. Wünschmann-Henderson, B., and T. Astrup. 1974. Inhibition by hydrocortisone of plasminogen activator production in rat tongue organ cultures. *Lab. Invest.* 30:427.
22. Strickland, S., and W. H. Beers. 1976. Studies on the role of plasminogen activator in ovulation: *In vitro* response of granulosa cells to gonadotropins, cyclic nucleotides, and prostaglandins. *J. Biol. Chem.* 251:5694.

23. Bourne, H. R., R. I. Lehrer, L. M. Lichtenstein, G. Weissman, and R. Zurier. 1973. Effect of cholera enterotoxin on adenosine 3',5'-monophosphate and neutrophil function. *J. Clin. Invest.* 52:698.
24. Dewald, B., R. Rindler-Ludwig, U. Bretz, and M. Baggiolini. 1975. Subcellular localization and heterogeneity of neutral proteases in neutrophilic polymorphonuclear leukocytes. *J. Exp. Med.* 141:709.
25. Romeo, D., G. Zabucchi, and F. Rossi. 1973. Reversible metabolic stimulation of polymorphonuclear leukocytes and macrophages by concanavalin A. *Nat. New Biol.* 243:111.
26. Repine, J. E., J. G. White, C. C. Clawson, and B. M. Holmes. 1974. The influence of phorbol myristate acetate on oxygen consumption by polymorphonuclear leukocytes. *J. Lab. Clin. Med.* 83:911.
27. Berlin, R. D. 1972. Effect of concanavalin A on phagocytosis. *Nat. New Biol.* 235:44.
28. Dechatelet, L. R., P. S. Shirley, and R. B. Johnston, Jr. 1976. Effect of phorbol myristate acetate on the oxidative metabolism of human polymorphonuclear leukocytes. *Blood.* 47:545.
29. Hoffstein, S., R. Soberman, I. Goldstein, and G. Weissman. 1976. Concanavalin A induces microtubule assembly and specific granule discharge in human polymorphonuclear leukocytes. *J. Cell Biol.* 68:781.
30. Estensen, R. D., J. G. White, and B. Holmes. 1974. Specific degranulation of human polymorphonuclear leukocytes. *Nature (Lond.).* 248:347.
31. Goldstein, I. M., S. T. Hoffstein, and G. Weissman. 1975. Mechanisms of lysosomal enzyme release from human polymorphonuclear leukocytes: effects of phorbol myristate acetate. *J. Cell Biol.* 66:647.
32. Bainton, D. F., and M. G. Farquhar. 1968. Differences in enzyme content of azurophil and specific granules of polymorphonuclear leukocytes. I. Histochemical staining of bone marrow smears. *J. Cell Biol.* 39:286.
33. Ward, P. A. 1966. The chemosuppression of chemotaxis. *J. Exp. Med.* 124:209.
34. Golde, D. W., N. Bersch, S. G. Quan, and M. J. Cline. 1976. Inhibition of murine granulopoiesis *in vitro* by dexamethasone. *Am. J. Hematol.* 1:369.
35. Nelson, D. H. 1962. Relative merits of the adrenocortical steroids. *Annu. Rev. Med.* 13:361.
36. Moore, D. M., P. A. Murphy, P. Y. Chesney, and W. B. Wood, Jr. 1973. Synthesis of endogenous pyrogen by rabbit leukocytes. *J. Exp. Med.* 137:1263.
37. Nordlund, Y. Y., R. K. Root, and S. M. Wolff. 1970. Studies on the origin of human leukocyte pyrogen. *J. Exp. Med.* 131:727.
38. Boggs, D. R., J. W. Athens, G. E. Cartwright, and M. M. Wintrobe. 1964. The effect of adrenal glucocorticosteroids upon the cellular composition of inflammatory exudates. *Am. J. Pathol.* 44:763.
39. Vassalli, J-D., and E. Reich. 1977. Macrophage plasminogen activator: induction by products of activated lymphoid cells. *J. Exp. Med.* 145:429.