NEUTRAL PROTEASE SECRETION BY HUMAN MONOCYTES

Effect of Surface-Bound Immune Complexes*

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Monocytes and macrophages contain, synthesize, and secrete a variety of enzymes active at acid or neutral pH (1). The enzyme content and secretory activity of mononuclear phagocytes are related to the degree of cellular differentiation and to the effects of exogenous stimuli. Resident peritoneal macrophages continuously secrete lysosomal hydrolases (2) and neutral proteases at low levels (3–6). Macrophages obtained from inflammatory exudates or stimulated by lymphokines or complement products contain and secrete increased amounts of lysosomal hydrolases (2, 7, 8). Elicited or activated macrophages also synthesize greatly increased amounts of neutral proteases with secretion occurring after appropriate stimulation (3–6, 9–11).

Properties of macrophages are also modified by alterations in the surface to which they adhere. Macrophages cultured on surfaces coated with immune complexes exhibit enhanced spreading (12) and reduced phagocytosis of sensitized erythrocytes (13). The effects of surface-bound immune complexes on enzyme secretion by monocytes or macrophages have not been examined. Immune complexes are present in articular and periarticular tissues of patients with rheumatoid arthritis and in the glomeruli of patients with immunological renal disease. Mononuclear phagocytes are abundant in the proliferating tissue infiltrates found in rheumatoid arthritis (14) and may contribute to immune-mediated glomerular injury (15). Macrophage-derived enzymes are capable of degrading structural elements of articular tissues (16). An analysis of the effects of adherent immune complexes on human monocytes and macrophages may yield information relative to pathophysiological events in human diseases.

In this paper we confirm that human peripheral monocytes release plasminogen activator during in vitro culture. When exposed to surface-bound immune complexes, however, the monocytes exhibit a burst of plasminogen-independent enzyme activity, and no plasminogen activator secretion occurs. Studies with enzyme inhibitors reveal that plasminogen-independent fibrinolysis is due primarily to the release of elastase-like enzymes from the human monocytes. In addition, deiodinating enzyme activity is observed when human monocytes interact with surface-bound immune complexes.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DME),[1] Hanks' balanced salt solution (HBSS), and fetal calf serum (FCS) were purchased from Grand Island Biological Co., Grand Island, N. Y. Ficoll-Hypaque, Sephadex G-200 and G-100 were obtained from Pharmacia Fine Chemicals, Piscataway, N. J. Plasminogen-free bovine fibrinogen, human serum albumin (HSA), and rabbit albumin were purchased from Miles Laboratories Inc., Elkhart, Ind. Lactalbumin hydrolysate (LH) was obtained from ICN Nutritional Biochemicals Div., Cleveland, Ohio, and trypsin and soybean trypsin inhibitor (STI) from Worthington Biochemical Corp., Freehold, N. J. Epsilon-amino caproic acid (EACA), diaminobenzoic acid (DABA), alpha-chymotrypsin, pork pancreatic elastase, urokinase, pepstatin A, casein, and elastin Congored were obtained from Sigma Chemical Co., St. Louis, Mo. Streptokinase-streptodornase (SK-SD) was purchased from Lederle Laboratories, Pearl River, N. Y. Dr. John Powers of the Georgia Institute of Technology kindly provided Ac-Ala-Ala-Pro-ValCH$_2$Cl, MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl, and Z-Gly-Leu-PheCH$_2$Cl. Dr. John D. Lonsdale-Eccles and Dr. Kenneth Walsh of the University of Washington generously supplied us with Tos-PheCH$_2$Cl (TPCK), Tos-LysCH$_2$Cl (TLCK), and di-isopropylfluorophosphate (DFP). Through them we also obtained elastatinal and chymostatin from Dr. Walter Troll of New York University Medical Center, New York.

Preparation of Cells. Mononuclear leukocytes (MNL) were obtained by Ficoll-Hypaque centrifugation of whole blood anticoagulated with EDTA. The MNL were washed once in saline buffered with 0.05 M phosphate (PBS) containing 1 mM EDTA, then twice in HBSS. The cells were counted and diluted in DME with 0.05% lactalbumin hydrolysate (DME-LH) to contain 2×10^6 MNL/ml. 15–20% of these cells contained peroxidase granules (17) and ingested latex particles (18). About 1% were polymorphonuclear neutrophils (PMN) as determined by examination of Wright's stained smears. More than 95% of the MNL were viable as determined by exclusion of trypan blue dye. These MNL preparations were applied to ^{125}I-fibrin substrates as described below.

For some experiments, MNL isolated from 70–150 ml blood were cultured overnight in siliconized glass culture vessels. The medium was then decanted, and the vessel was incubated for 10 min in ice-cold PBS containing 10 mM EDTA to induce resuspension of adherent cells. The PBS and medium were pooled and centrifuged again on Ficoll-Hypaque gradients. The MNL were washed as outlined above, and their concentration was adjusted to contain 300,000 peroxidase-positive cells/ml. 8–12% of these MNL contained peroxidase granules, and >90% excluded trypan blue.

Purified PMN were obtained by Ficoll-Hypaque centrifugation and dextran sedimentation (19). These preparations contained >95% PMN, and the cells were >98% viable. Purified lymphocytes were obtained after nylon wool filtration of MNL (20).

Preparation of ^{125}I-Fibrin Substrates. ^{125}I-Fibrin plates were prepared by the method of Gordon et al. (21). Plasminogen-free bovine fibrinogen (500–1,000 cpm/μg) was dissolved in PBS and diluted with distilled water to 333 μg/ml. For the formation of immune complexes, HSA was added as antigen to some of the fibrinogen so that its final concentration was 33 μg/ml. Aliquots of fibrinogen or fibrinogen-HSA (0.3 ml) were dispensed into 35-mm plastic culture dishes, spread with a sterile glass rod, and dried at 45°C for 2 d. Before use, the fibrinogen was converted to fibrin by a 2-h incubation with HBSS containing 10% FCS, and the plates were washed twice with HBSS. About 90% of the counts applied were adherent after this incubation, and 1–2% were solubilized in the absence of cells over 3–6 h. Trypsin digestion removed >98% of the counts from the plates.

For preparation of ^{125}I-fibrin plates containing surface-bound immune complexes, 1 ml of

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[1] *Abbreviations used in this paper:* DABA, diaminobenzoic acid; DFP, di-isopropylfluorophosphate; DME, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EACA, epsilon-aminocaproic acid; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; HSA, human serum albumin; IC$_{50}$, 50% inhibitory concentration; LH, lactalbumin hydrolysate; MNL, mononuclear leukocyte(s); PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; PMN, polymorphonuclear neutrophil(s); SK-SD, streptokinase-streptodornase; STI, soybean trypsin inhibitor; TLCK, Tos-LysCH$_2$Cl; TPCK, Tos-PheCH$_2$Cl.

rabbit IgG anti-HSA at 100 μg/ml was added to each plate to be sensitized for 15 min at room temperature. The plates were then washed twice with HBSS. Studies with labeled HSA showed that about 2 μg of HSA was adherent after the 2-h incubation with serum, and 75% remained adherent over a 9-h incubation in DME. About 2 μg of anti-HSA adhered to the plates containing HSA, and <0.2 μg adhered to plain fibrin substrates. The presence of HSA and immune complexes did not affect the adherence or stability of fibrin. The incorporation of immune complexes into the substrate did not affect SK-activated plasminogen-dependent fibrinolysis.

Plasminogen was isolated from outdated human plasma by the method of Deutsch and Mertz (22). Anti-HSA was purified by affinity chromatography from rabbit antisera, and monomeric IgG antibodies were obtained by gel filtration on Sephadex G-200 (23).

Assay of Fibrinolysis. 1-ml vol of MNL were added in triplicate to 35-mm culture dishes coated with ^{125}I-fibrin alone or with ^{125}I-fibrin incorporating surface-bound immune complexes. Human plasminogen (8–10 μg/ml) was added to some cultures. Enzyme inhibitors were added in some experiments as well, and when needed, 1% dimethylsulfoxide (DMSO) was used to enhance the solubility of the inhibitors. DMSO did not affect the observed fibrinolysis. Fibrinolysis was measured for 3 h as the cells settled onto the substrates. At the end of this incubation period, the medium was collected and counted along with a 1-ml wash. The plates were then washed four times with warm HBSS to remove loosely adherent cells. Less than 1% of the counts was removed by these washes. The remaining cells and substrate were digested with trypsin overnight at 37°C. The trypsin digest and a 1-ml wash were counted to determine the amount of residual radioactivity. The ratio of ^{125}I-fibrin solubilized to the total counts initially adherent was calculated and expressed as the percent fibrinolysis. Correction was made for the amount of radioactivity solubilized in the absence of the cells.

In some experiments, the cells were not removed after 3 h, but DME-LH was again added, with or without plasminogen, and the fibrinolytic assay was continued for another 6–18 h. In these experiments, 50-μl aliquots of the medium were withdrawn at intervals to study the time-course of fibrinolysis. After the extended fibrinolytic period, the medium and a 1-ml wash were aspirated and counted. The plates were then digested with trypsin as outlined above.

For correlation of fibrinolysis with cell number, the DNA content of the trypsin digests was determined by the method of Kissane and Robins (24). The trypsin aspirate was made 0.4% in rabbit albumin, and the total protein was precipitated by adding 1 ml of cold 20% trichloroacetic acid. In the extended experiments, the medium collected at the end of the experiment was treated in the same fashion as the trypsin digest. Lipids were extracted with 0.2-ml vol of alcoholic potassium acetate and ethanol. Fluorescence was developed with 0.18 ml of 2 N DABA in HCl. The reaction product was diluted to 1 ml with 0.6 N perchloric acid, and fluorescence was read in a Perkin-Elmer spectrofluorometer (Perkin-Elmer Corp., Norwalk, Conn.) at 510 nm with excitation set at 419 nm against a salmon DNA standard. This assay recovered ≅85% of 2 × 10^5 MNL (1 μg) digested in the culture dishes along with the fibrin substrate. Under standard assay conditions, 0.5–2.2 μg of cellular DNA was adherent at 3 h. About 10% of the cellular DNA was lost over the subsequent 6 h and a further 15% was lost over 6–21 h. Cell loss was similar from monolayers on plain fibrin or on fibrin-containing immune complexes.

When fibrinolytic activity was correlated with DNA content, it was expressed as fibrinolytic units (percent fibrinolysis per microgram DNA). Plasminogen-independent fibrinolytic units were calculated by dividing mean percent fibrinolysis by mean monolayer DNA content. Plasminogen-dependent fibrinolytic units were calculated in the same way after subtracting plasminogen-independent activity.

In experiments with enzyme inhibitors, inhibition of plasminogen-independent fibrinolysis was determined with the following formula:

$$\text{Percent inhibition} = \left(1 - \frac{\text{mean percent fibrinolysis with inhibitor}}{\text{mean percent fibrinolysis without inhibitor}} \right) \times 100.$$

Inhibition of plasminogen-dependent fibrinolysis was calculated in the same way after correction for plasminogen-independent fibrinolysis.

Chromatographic Analysis of Supernates.   Supernates for chromatographic analysis were obtained under assay conditions described above, except that the ^{125}I-fibrinogen used in the preparation of plates contained 25,000 cpm/μg. Cells were obtained from three separate donors for assay under each set of conditions. The supernates were analyzed for the presence of free iodide by the method of Klebanoff and Green (25). Duplicate 25-μl aliquots of the supernates were applied to 1-in. wide strips of Whatman no. 1 filter paper, and ascending paper chromatography was performed for 18 h with a butanol acetic acid solvent system. A control strip with ^{125}I-Na was run with each set of test samples. The strips were dried, cut into 0.5-in sections starting at the origin, and counted. The radioactivity present in each strip that migrated with the mobility of free iodide was calculated. The amount of free iodide in the supernate was expressed as a percentage both of the total counts per minute in the supernate and of the total radioactivity present in the system (i.e., in the supernate and remaining on the plate).

Determination of Activity of Enzyme Inhibitors in the Absence of Cells.   Chymostatin was assayed by the method of Umezawa and Aoyagi (26). The 50% inhibitory concentration (IC_{50}) obtained with bovine alpha-chymotrypsin and casein as substrate was 0.24 μg/ml, indicating that our preparation was of comparable potency to that described. Elastatinal was assayed by the method of Umezawa and Aoyagi (26) with porcine pancreatic elastase and elastin Congo-red. The IC_{50} obtained for this inhibitor was 17.8 μg/ml, a potency about 10% of that described.

Inhibition of urokinase by chymostatin was determined with a cell-free ^{125}I-fibrin plate assay. Urokinase, 0.1 or 0.5 U/ml in 0.1 M Tris HCl, pH 8, was incubated with chymostatin in amounts ranging from 0 to 240 μg/ml for 30 min at room temperature. The urokinase-chymostatin mixtures (0.5 ml) were then added to 16-mm plastic wells coated with ^{125}I-fibrin (20 μg ^{125}I-fibrinogen/well and 2,500 cpm/μg). 2 μg plasminogen was added to each well, and the wells were incubated at 37°C for 1 h. Percent fibrinolysis and inhibition of fibrinolysis were determined as described for assays with cells. In the presence of plasminogen and in the absence of inhibitors, 0.1 U of urokinase led to solubilization of 5% of the counts in 1 h and 0.5 U solubilized 20%.

Inhibition of plasmin by chymostatin was determined in a similar assay except that plasminogen (2.0 μg/ml) was activated with SK-SD (1 U/ml) for 30 min at 37°C in plastic tubes. Chymostatin at 240 μg/ml was added, and the tubes were incubated at room temperature for 30 min. Fibrinolysis was then measured with ^{125}I-fibrin-coated 16-mm wells with an assay period of 30 min. 60% of the counts were solubilized by SK-activated plasminogen during this time.

Results

Cell Populations and Morphology.   Approximately 1% of the MNL obtained by Ficoll-Hypaque centrifugation were PMN and 15–20% of the MNL contained peroxidase granules. 90% of the cells that were bound to a fibrin substrate after 3 h of adherence and washing contained peroxidase granules and took up latex particles. Up to 2% of the adherent cells were PMN by morphological criteria. Examination of the monocytes adherent to a plain fibrin substrate showed most of the cells to be rounded in appearance.

The population of cells bound to a fibrin substrate containing immune complexes differed. Up to 2% of the cells were PMN, but only 65% appeared to be monocytes by the criteria of peroxidase positivity and latex particle uptake. At 3 h, many of the cells incubated on surface-bound immune complexes were spread and had long cytoplasmic extensions. The DNA contents of monolayers incubated on the two substrates were equivalent.

Only 3% of MNL recovered after passage through a nylon wool column contained peroxidase granules and took up latex particles.

Fibrinolysis by MNL on Plain Fibrin.   MNL plated onto a ^{125}I-fibrin substrate exhibited a continuous pattern of fibrinolysis that was largely plasminogen dependent,
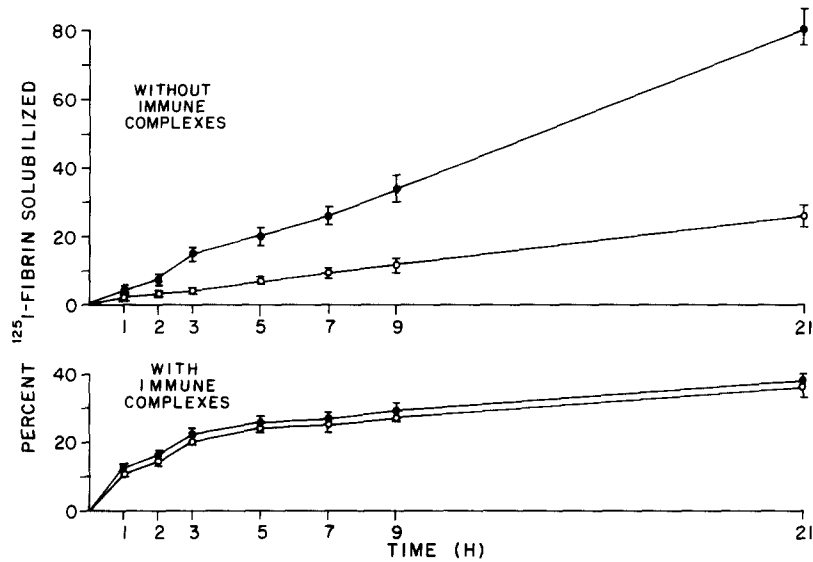
Fig. 1.   Fibrinolysis exhibited by MNL (2 × 10^6) plated onto ^{125}I-fibrin (upper panel) or ^{125}I-fibrin with adherent immune complexes (lower panel). The cumulative percent fibrinolysis is plotted vs. the time of incubation. At 3 h the plates were washed to remove nonadherent cells, and fresh medium was instilled. Experiments with both substrates were performed in the presence (●) or absence (○) of plasminogen. Cells in contact with surface-bound immune complexes show no plasminogen-dependent activity.

reflecting secretion of plasminogen activators (Fig. 1). This fibrinolysis was proportional to MNL cell number (Fig. 2). The same relationship was seen if the percent fibrinolysis was plotted against the DNA content of the adherent cells (data not shown). Similar patterns of fibrinolysis were observed in 33 separate experiments with cells from 14 donors. The amount of plasminogen-dependent fibrinolysis varied from donor to donor by a factor of three to four and from day to day in single donors by a factor of two to three when expressed either in terms of MNL cell number or monolayer DNA content. Results from the 33 experiments showed fibrinolysis in the absence and presence of plasminogen to be 5.6 ± 3.5 and 23.5 ± 14.0% (mean ± 1 SD), respectively, when measured 3 h after plating of cells.

Fibrinolysis by Monocytes on Fibrin with Adherent Immune Complexes.   When MNL were plated onto ^{125}I-fibrin incorporating HSA-anti-HSA immune complexes vigorous plasminogen-independent fibrinolysis was observed. High activity was observed soon after plating of cells, but very little further fibrinolysis occurred after washing of the monolayers at 3 h (Fig. 1). This plasminogen-independent fibrinolysis was elicited by 2 μg/plate of antibody in immune complexes. The same activity was elicited to a lesser degree when large amounts of antigen were dried onto the surface. None of this activity was elicited when plain fibrin plates were sensitized with anti-HSA (Table I). From 32 experiments with cells cultured on surface-bound immune complexes, the plasminogen-independent fibrinolysis averaged 28.5 ± 12.0% (± 1 SD) during the first 3 h.

When high plasminogen-independent activity was elicited, plasminogen-dependent activity was absent, as in the case of MNL incubated on complexes, or diminished, as when cells were in contact with high amounts of antigen (Table I). No return of
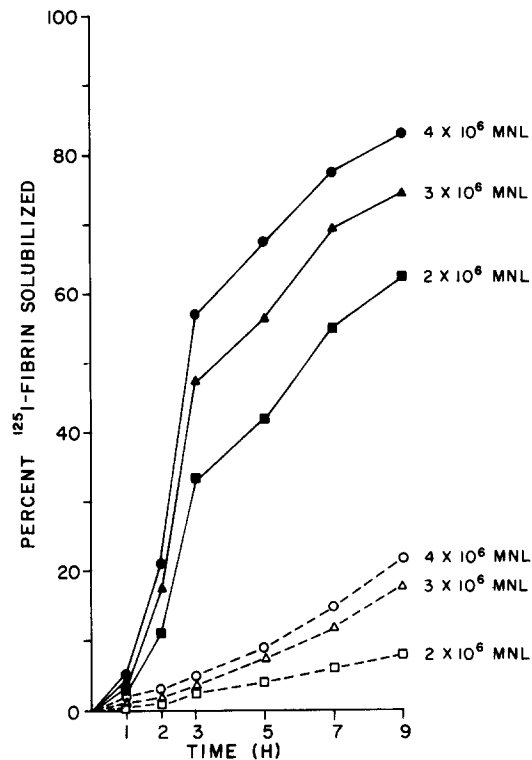
Fig. 2. Effect of MNL cell number on fibrinolysis. The percent cumulative fibrinolysis in the presence (●) or absence (○) of plasminogen is plotted, with serially increasing numbers of MNL, as indicated on the figure.

plasminogen-dependent activity was observed even when cells plated onto complexes were washed and fibrinolysis was allowed to continue for another 18 h (Fig. 1).

Conditions eliciting plasminogen-independent fibrinolysis affected cell viability. Although >95% of MNL incubated on a plain fibrin substrate excluded trypan blue dye at 21 h, viability of MNL incubated on surface-bound complexes averaged 90% at 3 h, 85% at 9 h, and 66% at 21 h.

Fibrinolysis by Purified Lymphocytes and PMN. Because the adherent cell populations on the two substrates differed and the preparations contained a trace contamination with PMN, the fibrinolytic activities of nylon wool-purified lymphocytes and of isolated PMN were measured to determine the possible contribution of these cells to the fibrinolysis observed with the MNL preparations.

The MNL eluted from a nylon wool column contained 97% lymphocytes. A portion of these cells adhered to surface-bound immune complexes but did not produce any detectable fibrinolysis (Table II).

Purified PMN produced an amount of plasminogen-dependent fibrinolysis equal to that seen with MNL when enzyme activity was correlated with the DNA content of the adherent cells (Table III). However, far more plasminogen-independent activity was elicited from PMN than from MNL incubated on surface-bound complexes. When fibrinolytic activity was correlated with monolayer DNA content, PMN were three to four times more active than adherent MNL (Table III). This experiment,

TABLE I

Effect of Immune Complexes on Plasminogen-Dependent and Independent Fibrinolysis Induced by MNL*

HSA in substrate	Plate incubated with anti-HSA	Percent fibrinolysis‡		Fibrinolytic units§	
		Without plasminogen	With plasminogen	Plasminogen independent	Plasminogen dependent
µg dried/plate					
0	—	1.9 ± 0.2	23.2 ± 0.6	1.0	10.7
0	+	3.8 ± 0.5	29.9 ± 0.9	1.7	11.7
10	—	6.0 ± 1.5	19.8 ± 2.5	2.6	5.9
10	+	30.1 ± 1.0	29.2 ± 1.2	11.9	0
50	—	11.7 ± 0.7	17.9 ± 0.2	5.1	2.7
50	+	31.3 ± 0.5	31.9 ± 1.0	13.2	0.3

* 1-ml vol of MNL (2 × 10^6/ml) were dispensed onto plates containing ^{125}I-fibrin with or without HSA in varying amounts. Some plates were incubated with anti-HSA before the addition of cells. Fibrinolysis was assayed for 3 h.

‡ The percent fibrinolysis is expressed as the mean ± 1 SD based upon results from three replicate plates.

§ Fibrinolytic units of activity were calculated by correlating plasminogen-dependent or independent fibrinolysis with monolayer DNA content, as described in Materials and Methods.

TABLE II

Comparison of Fibrinolytic Activities of MNL and Purified Lymphocytes*

Cell type	Immune complexes present	Percent fibrinolysis		Fibrinolytic units	
		Without plasminogen	With plasminogen	Plasminogen independent	Plasminogen dependent
MNL	—	2.7 ± 0.4	23.2 ± 0.8	5.9	44.6
Lymphocytes	—	0	0	0	0
MNL	+	23.0 ± 1.0	22.7 ± 1.0	40.4	0
Lymphocytes	+	1.5 ± 0.1	2.6 ± 0.8	2.1	1.2

* MNL (1 × 10^6/ml) and purified lymphocytes (4 × 10^6/ml) were added to plates coated with ^{125}I-fibrin with or without immune complexes.

TABLE III

Comparison of Fibrinolytic Activities of MNL and Purified Neutrophils*

Cell type	Immune complexes present	Percent fibrinolysis		Fibrinolytic units	
		Without plasminogen	With plasminogen	Plasminogen independent	Plasminogen dependent
MNL	—	4.1 ± 0.1	26.2 ± 1.3	5.7	30.7
PMN	—	8.5 ± 0.2	15.4 ± 2.8	26.6	20.6
MNL	+	28.3 ± 0.0	35.0 ± 0.8	30.1	7.1
PMN	+	67.4 ± 0.5	70.2 ± 1.6	107	4.4

* MNL (2 × 10^6/ml) and purified PMN (2 × 10^5/ml) were added to plates coated with ^{125}I-fibrin with or without adherent immune complexes.

however, would tend to underestimate the activity of the monocytes; lymphocytes constitute 35% of the monolayer cells and would add significantly to the adherent DNA content without contributing any fibrinolytic activity. The possible contribution of PMN to the fibrinolysis observed with MNL was further assessed after depletion of PMN in overnight culture.

TABLE IV

*Effect of Protease Inhibitors on Plasminogen-Dependent and Independent Fibrinolysis**

Inhibitor‡	Concentration	Plasminogen-dependent fibrinolysis		Plasminogen-independent fibrinolysis§	
		Percent inhibition	Percent inhibition corrected for DNA content	Percent inhibition	Percent inhibition corrected for DNA content
EACA (1)	1.0 mM	100	100	0	0
DFP (3)	0.1 mM	36	23	56	62
	1.0 mM	96	94	69	78
STI (3)	20 μg/ml	75	68	ND	ND
	50 μg/ml	100	87	ND	ND
	100 μg/ml	100	100	38	40
	600 μg/ml	ND	ND	48	44
α-1-Antitrypsin (1)	100 μg/ml	46	ND	40	ND
	300 μg/ml	ND	ND	44	ND
Chymostatin (3)	0.1 mM	70	66	19	31
Elastatinal (2)	0.1 mM	0	0	0	0
Pepstatin A (3)	0.01 mM	0	0	0	0
	0.1 mM	ND	ND	0	0
TLCK (2)	0.1 mM	15	0	3	17
MeO-Suc-Ala-Ala-Pro-ValCH₂Cl (3)	0.1 mM	6	0	45	54
	1.0 mM	ND	ND	45	48
Ac-Ala-Ala-Pro-ValCH₂Cl (1)	0.1 mM	0	0	26	25

* MNL (2 × 10^6/ml) were plated onto ^{125}I-fibrin with or without incorporated immune complexes. Inhibition was calculated as the percent reduction in plasminogen-dependent or independent fibrinolysis or as the percent reduction in fibrinolysis expressed in fibrinolytic units (i.e., corrected for the DNA content of the cell monolayers). ND indicates that the measurement was not done.

‡ The number of experiments with each inhibitor is indicated in parentheses. When multiple experiments were performed, the data are expressed as the mean.

§ No correction was made for the amount of deiodination.

Fibrinolysis by MNL Freed of PMN. MNL were incubated at 37°C overnight in siliconized glass culture vessels, then harvested and centrifuged again on Ficoll-Hypaque gradients. These preparations contained no PMN among 200 cells counted. Fibrinolysis was compared with that of fresh MNL obtained from the same donor. With cell concentrations adjusted so that the MNL preparations contained equal numbers of peroxidase-positive cells, fibrinolysis of the two preparations on surface-bound complexes was identical. We concluded that monocytes were the major source of the enzyme activity observed in MNL and not lymphocytes or the small numbers of PMN present.

Characterization of the Enzyme Activities. The enzymes responsible for the observed fibrinolytic activities were further characterized by including protease inhibitors with known specificities in the culture medium before the plating of cells. The inclusion of inhibitors (with two exceptions) did not affect cell viability as determined by trypan blue dye exclusion or monolayer DNA content.

Plasminogen-dependent fibrinolysis was abolished by EACA, STI, and DFP (Table

Table V

Deiodination by MNL*

Immune complexes present	Plasminogen present	Enzyme inhibitors present	Percent apparent fibrinolysis‡	Amount of free iodide as a percentage of§	
				Soluble radioactivity	Total radioactivity
−	−	None	4.8 ± 1.2	27.1 ± 4.3	1.2 ± 0.3
−	+	None	9.7 ± 3.9	19.2 ± 3.1	1.8 ± 0.4
+	−	None	24.4 ± 7.4	43.9 ± 1.5	11.4 ± 3.5
+	+	None	28.5 ± 6.5	38.9 ± 6.3	11.4 ± 4.5
+	−	DFP (1 mM)	8.3 ± 1.3	48.9 ± 7.2	4.1 ± 1.3
+	−	MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl (0.1 mM)	18.2 ± 3.3	62.8 ± 5.5	11.5 ± 3.2
+	−	STI (600 μg/ml)	16.3 ± 2.8	62.9 ± 9.6	10.5 ± 3.4

* MNL from three donors were plated onto ^{125}I-fibrin with or without adherent immune complexes, and the supernates were analyzed for the presence of free iodide as described in Materials and Methods. The data are expressed as the mean ± 1 SD of the results obtained with three plates under each set of conditions.

‡ The apparent percent fibrinolysis was not corrected for deiodination.

§ The free iodide in the supernate is expressed as a percentage of the soluble radioactivity (counts per minute in the supernate) and of the total radioactivity (counts per minute in the supernate and remaining on the plate).

IV). Plasminogen-dependent fibrinolysis was also inhibited 70% by 0.1 mM chymostatin, an inhibitor of chymotrypsin-like enzymes (26). Cell-free fibrinolysis activated by urokinase was also inhibited by chymostatin in amounts comparable to that which inhibited cell-mediated plasminogen-dependent fibrinolysis. Plasmin was not directly inhibited by chymostatin. Two other inhibitors of chymotrypsin-like enzymes, the chloromethyl ketones TPCK and Z-Gly-Leu-PheCH$_2$Cl, were toxic to MNL, reducing monolayer viability by 50% and adherence by 90%. No inhibition of plasminogen-dependent fibrinolysis was observed in the presence of pepstatin A, an inhibitor of acid proteases (26), the elastase inhibitor elastatinal (26), or TLCK (Table IV).

Plasminogen-independent fibrinolytic activity was reduced 69% by DFP, 48% by STII, and 40% by alpha-1-antitrypsin (Table IV). The elastase inhibitors MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl and Ac-Ala-Ala-Pro-ValCH$_2$Cl inhibited activity by 45 and 26%, respectively, and chymostatin inhibited 19%. No inhibition of plasminogen-independent fibrinolysis was observed in the presence of EACA, pepstatin A, elastatinal, or TLCK. When MNL were incubated overnight before assay of plasminogen-independent fibrinolysis, an identical degree of inhibition was observed in the presence of MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl. There was no augmentation of inhibition when chymostatin and MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl were assayed together, nor was there significant recovery of plasminogen-dependent activity.

Deiodination by Monocytes.   As no protease inhibitor reduced plasminogen-independent fibrinolysis by >70%, we investigated the possibility that nonproteolytic enzyme activity was being detected in these assays.

Analysis by ascending paper chromatography of supernatant media from MNL in contact with surface-bound complexes revealed that 39–44% of the soluble radioactivity (representing 11.4% of the total radioactivity) migrated with the mobility of free iodide (Table V). When MNL were incubated on fibrin without adherent immune

complexes, <2% of the total radioactivity was solubilized as free iodide. The enzyme inhibitors STI and MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl did not affect the amount of free iodide solubilized when expressed as the percentage of total radioactivity although there were increases in the relative amounts of free iodide in the supernates. When cells on immune complexes were incubated in the presence of DFP, there was a 65% decrease in free iodide solubilized.

Discussion

When the surface to which human peripheral monocytes adhere is modified by the incorporation of immune complexes, the cells exhibit differences in the nature and pattern of neutral enzyme secretion from what has been generally noted with resident, elicited, or activated animal macrophages. Fibrinolysis by monocytes in contact with unmodified ^{125}I-fibrin is largely plasminogen dependent, proceeds linearly with time, and reflects the continuous secretion of plasminogen activator, as has been described by others (11). Such monocytes also exhibit a minor continuous degree of plasminogen-independent fibrinolysis. When monocytes are incubated on surface-bound immune complexes, however, a burst of plasminogen-independent fibrinolysis occurs. Under these conditions, plasminogen-dependent fibrinolysis is absent and does not emerge even when the plasminogen-independent activity diminishes to the background levels of unstimulated monocytes. Plasminogen-independent fibrinolysis is also elicited if large amounts of albumin, another surface-bound phagocytic stimulus, are dried onto the surface. This pattern of fibrinolysis is similar to that observed with PMN stimulated with phorbol myristate acetate (PMA) (27). A lower degree of plasminogen-independent fibrinolysis also was observed by Vassalli et al. (28) in mouse macrophages stimulated with PMA.

Critical in our studies is the identification of the monocyte as the source of the plasminogen-independent enzyme activity observed with MNL preparations. PMN constituted about 1% of the total cells and 5-7% of the peroxidase-positive cells in the MNL. The cells adherent to the fibrin substrate contained 2% or less PMN, but the possibility exists that PMN in the supernate interacted with the surface-bound complexes and contributed to the observed fibrinolysis. Purified PMN exhibited three- to fourfold greater levels of activity in comparison with the MNL preparations when fibrinolysis was correlated with cellular DNA contents. When MNL preparations were cultured overnight, the number of cells possessing stainable peroxidase granules was reduced by one-half and no PMN were identified. In cell preparations adjusted to contain equal numbers of peroxidase-positive cells, fresh MNL and MNL incubated overnight exhibited identical levels of plasminogen-independent fibrinolytic activity during 3-h culture on surface-bound complexes. In addition, purified lymphocytes showed no fibrinolytic activity when plated on the complexes. These results support the conclusion that the observed plasminogen-independent fibrinolysis was due to enzymes released from monocytes in the MNL preparations.

The reasons for the absence of plasminogen activator secretion from the cells cultured on immune complexes are not known. It is unlikely that the plasminogen activator was destroyed by other enzymes as no plasminogen-dependent fibrinolysis emerged even when the cells were cultured for an additional 18 h after a change in culture medium. During this time, plasminogen-independent activity was no higher than that of cells cultured on plain fibrin. Cytoplasmic inhibitors of plasminogen

activators have been described in rabbit endothelial cells (29) but no free inhibitors were detected in the supernates of cultured mouse peritoneal macrophages (3). The reduction in viability seen with monocytes cultured on immune complexes may signify cell damage sufficient to lead to the observed decrease in plasminogen activator secretion. The localization of plasminogen activators in macrophages and the mechanisms of increased secretion from elicited or activated cells are not known. If this enzyme is present primarily in the plasma membrane, as has been described in rabbit endothelial cells (29) and guinea pig mast cells (30), culturing of monocytes on surface-bound immune complexes may have led to an inhibition of mobility or turnover of membrane constituents. Mouse peritoneal macrophages cultured on surface-bound immune complexes exhibit a selective membrane immobilization manifest by a loss of Fc-triggered phagocytosis (13). In addition, mixed B lymphocytes and macrophages plated on immobilized immune complexes display an inhibition of mitogenesis (31) and a decrease in proliferation into antibody-forming cells (32).

The fibrinolytic enzymes released from the human monocytes in our studies were further characterized by mixing protease inhibitors with cells before culturing. A limitation of this technique, however, is that inhibition of enzyme activities might be incomplete in the microenvironment of cells plated directly onto the substrate. In addition, the possibility cannot be excluded that added inhibitors reduced enzyme secretion rather than enzyme activity or affected a step in enzyme activation. This technique has an advantage, however, in that it permits analysis of enzyme activities from small numbers of cells without the potential losses induced by handling conditioned medium or cell lysates.

Plasminogen-dependent fibrinolysis was abolished by DFP, EACA, and STI. DFP and EACA are known to inhibit plasmin as well as plasminogen activators, whereas STI inhibits only plasmin (33). Chymostatin inhibited plasminogen-dependent fibrinolysis by 70%. Fibrinolysis activated by urokinase in the absence of cells was also inhibited to a similar degree, indicating that the chymostatin did not simply reduce plasminogen activator secretion. Furthermore, chymostatin was found by ourselves and others to have no direct inhibitory effects on plasmin (26). Thus, at the concentrations used in our experiments, chymostatin was a selective inhibitor of both urokinase and monocyte-derived plasminogen activators. At these concentrations chymostatin also inhibits proteases other than chymotrypsin and cathepsin G (26, 34). Therefore, inhibition of plasminogen activators does not imply that these enzymes are chymotrypsin-like. Evidence from other studies indicates that cell-derived plasminogen activators have a trypsin-like substrate specificity (33). Plasminogen activators, however, are not inhibited by STI or other macromolecular trypsin inhibitors (33). Further study of the interaction of chymostatin and plasminogen activators might help to elucidate the active sites of these enzymes.

The plasminogen-independent fibrinolysis seen in our studies with monocytes incubated on surface-bound immune complexes was inhibited 69% by DFP and 48% by STI. The chloromethyl ketones specific for leukocyte elastase (35), MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl and Ac-Ala-Ala-Pro-ValCH$_2$Cl, inhibited 45 and 26%, respectively, of this activity. These results suggest that the predominant monocyte protease is similar to leukocyte elastase. Elastatinal was not inhibitory, but the preparation used in our studies had only 10% of the activity toward pancreatic elastase as has been reported for this compound (26). Furthermore, elastatinal is weakly inhibitory

toward leukocyte elastase in comparison to its effect on porcine pancreatic elastase (34). 20–30% inhibition of plasminogen-independent fibrinolysis by chymostatin was observed. At the concentrations used in our experiments, the chymostatin could be inhibiting a chymotrypsin-like enzyme such as cathepsin G (34) or partially blocking elastase. The absence of additive inhibitory effects with chymostatin and MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl suggests the latter possibility.

Up to 45% of the plasminogen-independent fibrinolysis produced by monocytes cultured on surface-bound immune complexes represented deiodination rather than proteolysis. Intracellular deiodination may be due to the activity of myeloperoxidase (36), which is found in the azurophilic granules of both monocytes and PMN (37, 38). Under certain conditions, myeloperoxidase may be active in the extracellular environment (36) although the site of deiodination in our experiments was not elucidated. The total amounts of deiodination were not reduced by STI or MeO-Suc-Ala-Ala-Pro-ValCH$_2$Cl, whereas the relative amounts of free iodide in the culture supernates were increased. These results indicate that the inhibitors were affecting plasminogen-independent fibrinolytic proteases and not the deiodinating enzymes. The reduction in total deiodination in the presence of DFP could reflect reduced secretion or a direct inhibition of the deiodinating enzymes, or could indicate a requirement for partial proteolysis to precede deiodination. A role for deiodinating enzymes in the induction of tissue injury has not been defined although the activity of such enzymes must be considered in the in vitro study of phagocytic cells and iodinated substrates. Under certain experimental conditions, deiodination could be mistaken for proteolysis.

An elastaselike enzyme with a pattern of response to inhibitors characteristic of leukocyte elastase has not previously been described in monocytes. The elastase found in conditioned medium from mouse peritoneal macrophages is not inhibited by STI and it is secreted in a continuous fashion (5). Monocytes stain weakly with naphthol AS-D chloroacetate (39), a histochemical substrate for leukocyte elastase and cathepsin G (40). Monocytes also stain, again weakly, with a fluoresceinated antiserum to leukocyte elastase (41). PMN show strong histochemical and immunological reactivity. Similarly, the level of elastaselike activity of PMN in our studies was threefold greater than that observed with monocytes. The azurophilic granules of the PMN contain peroxidase, elastase, and cathepsin G (37). It is probable that the deiodinating and elastaselike enzymes described in the human monocytes in our studies were derived from a similar intracellular structure. Azurophilic granules are lost as monocytes mature into macrophages, reflected in a disappearance of stainable peroxidase (38) and probably also of stainable esterase.

Monocyte- or macrophage-derived neutral proteases may play an important role in tissue destruction in human diseases where surface-bound immune complexes are present. Fibrin degradation products derived from proteases other than plasmin have been identified in rheumatoid synovium and synovial fluid (42). Although serum protease inhibitors may inactivate enzymes in body fluids, their effectiveness may be much reduced in the microenvironment of a cell directly adherent to the tissue injured (43). Increased numbers of immature monocytes are found in the blood of patients with rheumatoid arthritis or systemic lupus erythematosus (44). Newly recruited monocytes may interact with adherent immune complexes in the joints or glomeruli of these patients, stimulating the release of neutral enzymes capable of degrading collagen, elastin, and proteoglycans (40). More mature macrophages in the tissues

may have a different pattern of enzyme release. Further studies are necessary to characterize the response of human macrophages in various stages of activation or differentiation to surface-bound immune complexes.

Summary

The effect of surface-bound immune complexes on the secretion of neutral proteases by human peripheral monocytes was examined. Monocytes cultured on ^{125}I-fibrin secreted plasminogen activator in a continuous fashion. Monocytes incubated on ^{125}I-fibrin with surface-bound immune complexes displayed a burst of plasminogen-independent fibrinolytic activity, whereas no release of plasminogen activator was observed through 21 h. The plasminogen-independent fibrinolytic enzymes were derived from monocytes and not from lymphocytes or contaminating polymorpho-nuclear neutrophils. The effects of various protease inhibitors on the secretion of plasminogen-dependent and independent enzymes were determined. Chymostatin selectively inhibited the monocyte-derived plasminogen activators. Similar effects of chymostatin were observed on human urokinase in the absence of cells. The predominant protease producing plasminogen-independent fibrinolysis exhibited responses to inhibitors characteristic of leukocyte elastase. When monocytes were cultured on ^{125}I-fibrin with adherent immune complexes ≅40% of the solubilized radioactivity represented deiodination and not proteolysis. It was concluded that culture of human monocytes on surface-bound immune complexes stimulates the secretion of plasminogen-independent fibrinolytic proteases, primarily elastase, and of deiodinating enzymes. Under these conditions, plasminogen activator secretion is inhibited. Neutral proteases secreted from newly recruited monocytes may contribute to tissue injury in human diseases characterized by the presence of adherent immune complexes.

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