SECRETION OF PLASMINOGEN ACTIVATOR BY BONE MARROW-DERIVED MONONUCLEAR PHAGOCYTES AND ITS ENHANCEMENT BY COLONY-STIMULATING FACTOR

By HSIU-SAN LIN* and SAIMON GORDON

From Sir William Dunn School of Pathology, University of Oxford, Oxford, England

Various mononuclear phagocyte populations differ considerably in their capacity to produce and secrete plasminogen activators (PA)[1] in vitro. For example, resident peritoneal macrophages show very little PA activity, whereas, thioglycollate medium-induced peritoneal exudate macrophages secrete high levels of this enzyme (1). It has been shown that the majority of exudate macrophages are freshly recruited blood monocytes (2–4) and there is evidence that recent arrivals are the cells that secrete PA (5).

Large numbers of mononuclear phagocytes can be obtained when bone marrow cells are grown in liquid culture in the presence of a growth factor, called colony-stimulating factor (CSF) (6–9). They are presumably derived from the committed hemopoietic stem cells for both granulocytes and monocytes-macrophages (CFU-C) (10, 11). Recent studies have shown that these adherent mononuclear phagocytes can be rendered cytotoxic in vitro and they can also serve as indicator cells for migration inhibitory factor (8, 9).

In this study, we investigated PA secretion by mononuclear phagocytes derived from bone marrow cells in culture and also the factors which modulate the secretion of PA by these cells, particularly the role of CSF.

Materials and Methods

Animals. Swiss mice of both sexes, bred in this Department and weighing 25–30 g, were used in all studies.

Media. α-Minimal essential medium (α-MEM) (Flow Laboratories, Irvine, Scotland) without nucleosides was used for growing bone marrow cells and mouse L cells. Dulbecco's MEM was used during the incubation of cells for PA secretion.

Preparation of Gelatin-Coated Flasks. Gelatin solution (0.1% wt/vol in glass-distilled water) was prepared and sterilized by autoclave. After cooling to ≅50°C, 3 ml of this solution was pipetted into each flask (Falcon, 24 cm^2, Falcon Labware, Div. of Becton, Dickinson, & Co., Oxnard, Calif.). These flasks were left at 4°C with the side to be coated down. After 2 h, the excess gelatin solution was removed by suction. The flasks were then dried at 37°C for 2 d with their caps loosened. Gelatin-coated flasks were stored at room temperature.

* Reprint requests, Section of Cancer Biology, Division of Radiation Oncology, Mallinckrodt Institute of Radiology, Washington University, School of Medicine, St. Louis, Missouri.

[1] *Abbreviations used in this paper:* ATDS, acid-treated dog serum; BMDM, bone marrow-derived mononuclear phagocytes; CFC, colony-forming cells; CFU-C, the committed stem cells for both granulocytes and macrophages; Con A-SCM, medium conditioned by concanavalin A-stimulated spleen cells; CSF, colony-stimulating factor; FCS, fetal calf serum; LCM, L-cell-conditioned medium; MEM, minimal essential medium; PA, plasminogen activator; PBS, phosphate-buffered saline; RPM, resident peritoneal macrophages; TPM, thioglycollate medium-induced peritoneal macrophages.

J. Exp. Med. © The Rockefeller University Press · 0022-1007/79/08/0231/15 $1.00

Volume 150 August 1979 231–245

Bone Marrow Cell Culture. Cells from femoral bone marrow were obtained by flushing the femur with a 23-gauge needle attached to a 2-ml syringe containing α-MEM. To each gelatin-coated flask, 1×10^6 bone marrow cells in 5 ml of growth medium were added. The growth medium contained α-MEM supplemented with 10% fetal calf serum (FCS) (Flow Laboratories), 5% horse serum, 10% L-cell-conditioned medium (LCM) and 2 mM glutamine. It also contained 100 U/ml penicillin, 100 μg/ml streptomycin, and 100 μg/ml kanamycin. These cultures were flushed with 5% CO_2 in air before the caps were tightened. They were kept at 37°C and the cells were harvested at various times as described later. Adherent cells were detached by incubation for 5–10 min in lidocaine solution (15 mM in Dulbecco's MEM supplemented with 10% FCS) and washed twice with Dulbecco's MEM before use (12). Bone marrow cultures were also prepared in bags of teflon FEP fluorocarbon film (13) (type 100A, Du Pont, Richard Klinger Ltd., Sidcup, Kent) or in Petriperm dishes (Heraeus, TR International Chemicals Ltd., London) for comparison with gelatin-coated flasks.

Peritoneal Cells. Resident peritoneal cells were washed out with Ca^{2+} and Mg^{2+}-free phosphate-buffered saline (PBS) from normal unstimulated mice. Thioglycollate medium-induced exudate cells were obtained 4 d after an intraperitoneal injection of 1 ml Brewer's thioglycollate medium. These cells were plated, $3–5 \times 10^6$ cells per gelatin-coated flask, in 5 ml of growth medium. 24 h later, the nonadherent cells were removed by washing the flasks twice with α-MEM and the adherent cells harvested with lidocaine solution.

Blood Mononuclear Cells. Blood was obtained from mice by cardiac puncture. Acid citrate dextrose solution was used as anticoagulant and blood mononuclear cells were obtained by a Ficoll-Hypaque technique described by Boyum (14). Between 3 and 6% of the cells in this fraction could be identified morphologically as monocytes.

Pulmonary Alveolar Cells. After mice were killed, a sterile polyethylene tube was inserted through the trachea and the lungs lavaged with Ca^{2+}- and Mg^{2+}-free PBS (15). About 3×10^5 cells were obtained from a single mouse, of which >95% were alveolar macrophages.

Preparation of LCM. Mouse L cells (strain 929) were seeded in 75-cm^2 tissue culture flasks (Falcon Labware, Div. of Becton, Dickinson, & Co.) at 1×10^6 cells in 20 ml of α-MEM supplemented with 10% FCS. 7 d later, the medium was harvested and filtered through a 0.22-micron Millipore filter (Millipore Corp., Bedford, Mass.) to insure a cell-free conditioned medium. Conditioned medium was stored frozen until use. For serum-free LCM, L cells were grown for 4 d. The medium was then removed and the flask washed twice with α-MEM. 20 ml of α-MEM was added and the cultures were incubated at 37°C for 3 more d before the medium was harvested.

Preparation of Conditioned Medium from Concanavalin A-Stimulated Spleen Cells. Spleens were removed from mice and cell suspensions prepared. The method described by Vassalli and Reich (16) was used to obtain conditioned medium from concanavalin A (Con A)-stimulated spleen cells. The cells suspended in Dulbecco's MEM supplemented with 5% heat-inactivated FCS were cultured at 37°C for 20 h in Falcon 100-mm tissue culture Petri dishes at a density of 6×10^6 cells/ml, 10 ml per dish, in the presence or absence of 4 μg/ml Con A. The cells were washed twice and resuspended in Dulbecco's MEM at a density of 6×10^6 cells/ml. They were replated in new Petri dishes at 6×10^7 cells per dish and incubated at 37°C in CO_2 incubator. Medium was collected 24 h later, centrifuged for 15 min at 500 *g* to remove cells and stored at −20°C until use. In comparison to LCM, these conditioned media contained very little colony stimulating activity (<1/100 by volume).

CSF. Highly purified CSF, obtained from Dr. D. M. Chen of Washington University, St. Louis, was prepared from serum-free LCM according to the method of Stanley and Heard (17).

Assay for Colony-Forming Cells. The number of mononuclear phagocyte colony-forming cells was determined by a previously described method (15, 18, 19). Cells were mixed with agar medium containing 10% fetal calf serum, 5% horse serum, 10% LCM, and 0.3% Bacto agar in α-MEM. 1 ml of this mixture was plated in 35-mm Falcon culture dishes. These dishes were incubated in a 37°C incubator continuously flushed with 5% CO_2 in air. Cells were also grown in 1 ml of liquid medium which contained the same ingredients as the agar medium except agar.

Assay of Fibrinolytic Activity. Fibrinolysis was assayed on ^{125}I-fibrin-coated Linbro plates (Linbro Chemical Co., Hamden, Conn.), prepared as described (20). To measure fibrinolysis by intact cells, adherent cells were detached from gelatin-coated flasks with lidocaine solution

and washed twice with Dulbecco's MEM without serum. Different cell concentrations, 2×10^4–2×10^5 in 0.5–1 ml of Dulbecco's MEM, were added to ^{125}I-fibrin-coated wells and incubated for 30–60 min at 37°C in the presence of 5% CO_2 to adhere. 25–50 μl of acid-treated dog serum (ATDS, pH 2 for 30 min at room temperature) were then added to each well as a source of plasminogen, final concentration 5% vol/vol. The cultures were incubated at 37°C in the presence of 5% CO_2. 100-μl samples of medium were withdrawn at 2.5 and 4 h after the addition of ATDS to assay for release of radioactivity in a Packard gamma counter (Packard Instrument Co., Inc., Downer's Grove, Ill.). Assays were performed in duplicate and appropriate controls included in all experiments. This procedure was modified, as described later, to measure the effect of various test solutions on fibrinolysis by cells.

For cell-free assays of fibrinolysis, serum-free conditioned media and cell lysates were prepared as described elsewhere (20). Bone marrow-derived mononuclear phagocytes (BMDM) were cultivated for 6 d and thioglycollate medium-induced peritoneal macrophages (TPM) and resident peritoneal macrophages (RPM) for 1 d, in α-MEM with 10% FCS and 10% LCM. The adherent cells were washed three times and fed 3 ml Dulbecco's MEM supplemented with 0.1% wt/vol lactalbumin hydrolysate for 2 d before collecting conditioned media and cell lysates. Samples (10–50 μl) were assayed on ^{125}I-fibrin in 400 μl 0.1 M Tris HCl, pH 8.1, containing 100 μg bovine serum albumin, in the presence or absence of 10 μg human plasminogen which had been purified by lysine sepharose chromatography (20). A unit of fibrinolytic activity was defined as the solubilization of 10% of the total radioactivity releasable by trypsin, per 4 h, at 37°C.

Agarose-Overlay Assay for the Detection of Plasminogen Activator Production. For the detection of PA secretion by individual colonies, we used a method adapted from that of Goldberg (21). The overlay mixture consisted of 0.5% agarose, 1.33% nonfat dry milk, Dulbecco's medium, and 5% ATDS. Flasks with discrete colonies were washed twice with warm PBS and the agarose overlay added. The overlay was allowed to harden at room temperature for 5 min and then incubated at 37°C for 4–12 h.

Protein Assay. Protein was assayed using the method of Lowry et al. (22).

Materials. Gelatin was obtained from Oxoid, Ltd., London; bactoagar from Difco Laboratories, Detroit, Mich.; agarose from BDH, Poole, Dorset; dog serum from Gibco-Biocult, Paisley, Scotland.

Results

Bone Marrow Cell Culture. The replication of bone marrow cells in growth medium began within 1 d, first as small clusters of nonadherent cells and yielding aggregates of 8–32 adherent cells by day 3. The continuous clonal growth of these cells, presumably derived from CFU-C, resulted in fusion of the colonies and formation of a confluent monolayer of adherent cells between days 6 and 8. After day 8, some adherent cells became detached unless the cultures were refed with complete growth medium. Both granulocyte and mononuclear phagocyte colonies were present during the early stage of culture. However, after day 5, >98% of adherent cells were mononuclear phagocytes. We routinely used adherent cells from 6- to 8-d-old cultures as the source BMDM. When LCM was omitted, there was no appreciable proliferation of bone marrow cell suspensions plated at 1×10^6 cells per flask.

We did not notice any difference in the growth of bone marrow cells in gelatin-coated and standard uncoated tissue culture flasks. However, the use of gelatin-coated flasks facilitated the recovery of viable adherent cells from the flasks after treatment with lidocaine. We recovered 85–99% of adherent cells, of which 80–95% were viable by trypan blue dye exclusion. In contrast, only 70–80% of adherent cells could be recovered from uncoated flasks, of which 65–80% were viable. This was also true for peritoneal macrophages. Bone marrow cultures in Teflon-film bags or in Petriperm dishes yielded more loosely adherent mononuclear phagocytes which could be de-

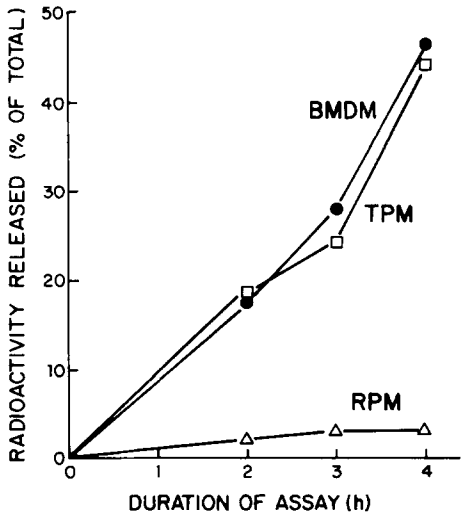

FIG. 1. Fibrinolysis by BMDM, TPM, and RPM. To each ^{125}I-fibrin-coated well, 5×10^4 BMDM or TPM or 1×10^6 RPM were added.

tached by vigorous pipetting without the use of lidocaine. Experiments done with such BMDM gave similar results to those with cells prepared routinely in gelatin-coated flasks.

Fibrinolytic Activity of BMDM. BMDM obtained from bone marrow after 7 d in culture were tested for fibrinolytic activity as described earlier and compared with RPM and TPM which had been cultured in gelatin-coated flasks in the same growth medium for one day. As shown in Fig. 1, the BMDM displayed a high level of fibrinolytic activity, similar to that of TPM, whereas fibrinolysis by resident macrophages was very low. Fibrinolysis by BMDM was proportional to cell number and, in eight separate experiments, was consistently at least 20-fold greater than that of resident peritoneal macrophages. The activity per cell was equal to or only slightly less than that of TPM. The data on resident and exudate peritoneal macrophages were in good accord with a previous report (1).

Conditioned medium and cell lysates prepared from similar cultures were next examined to define the fibrinolytic activity of BMDM. Table 1 shows that the high level of fibrinolysis in cultures of BMDM and TPM was strictly dependent on the presence of plasminogen and was found almost entirely in the conditioned medium fractions, confirming that BMDM secreted plasminogen activator as actively as TPM. The intracellular level of PA was somewhat lower in BMDM than in TPM, possibly as a result of the presence of inhibitors of fibrinolysis. Resident macrophages secreted only low levels of PA and inhibition of fibrinolysis could be detected in both intra- and extracellular fractions.

Relationship between Fibrinolytic Activity and Colony-forming Cells. TPM and RPM differ in proliferative activity as well as in production of plasminogen activator. TPM contain between 5 and 20% of cells that are able to proliferate extensively in the presence of CSF and form colonies in vitro (PE-CFC), whereas <0.1% of resident macrophages are colony-forming cells (18). Because fibrinolysis by BMDM was comparable to that of TPM, it was of interest to compare the colony-forming capacities of BMDM and TPM.

TABLE I

Fibrinolysis by Different Macrophages

Cells	Fibrinolytic activity				Cell protein per culture
	Conditioned medium		Cell lysate		
	Plg indep.*	Plg dep.‡	Plg indep.	Plg dep.	
	U/mg protein				*mg*
RPM	−6.5	+4.4	−3.9	−0.5	1.0
BMDM	0	+78	−5.3	+4.5	0.66
TPM	0	+84	0	+17	1.6

* Plg indep., plasminogen independent.
‡ Plg dep., plasminogen dependent.

TABLE II

CFC and Fibrinolytic Activity of Various Mononuclear Phagocyte Populations

Cells	Incidence of CFC per 10^3 cells		Fibrinolytic activity*
	Liquid	Agar	
BMDM	112 ± 24‡	101 ± 28	32
RPM	<1	<0.1	3
TPM	62 ± 18	77 ± 20	38
Blood mononuclear cells	46 ± 5	2 ± 1	1
Alveolar macrophages	114 ± 29	120 ± 31	21

* Radioactivity released (percentage of total) in 2.5 h. The wells contained either 1×10^5 BMDM, TPM, or alveolar macrophages, or 1×10^6 RPM or blood mononuclear cells.
‡ Mean ± SEM.

When 7-d BMDM cultures were cloned in liquid and agar, we observed a class of colony-forming cell with growth kinetics intermediate between those of the fast growing CFU-C and slow growing peritoneal exudate colony-forming cells. Replication began within 2–3 d and aggregates of >50 cells had formed after 7–10 d in liquid culture or somewhat more slowly in agar, with colonies containing 100–200 cells after 10 d. Equal numbers of colonies were formed in liquid and agar cultures and the incidence of colony-forming cells in BMDM from 7-d-old cultures was between 5 and 15%. Only macrophage-like cells were found in all these colonies. Although they had a shorter lag period and grew faster than PE-CFC, it is not clear at present whether this class of colony-forming cell is different from PE-CFC.

The relationship between enhanced fibrinolysis and proliferation was explored further with other populations of mononuclear phagocytes known to contain colony-forming cells (15, 19). 12% of alveolar washout cells formed colonies in liquid or agar culture and alveolar macrophages also expressed a high level of fibrinolytic activity, thus resembling 7-d BMDM and 1-d TPM in both respects (Table II). A mononuclear cell fraction obtained from blood contained ≅5% of cells which formed colonies in liquid culture, but no colonies were observed in agar, nor could fibrinolysis be detected in wells containing up to 2×10^6 total mononuclear cells. In other experiments, a monocyte-rich fraction was obtained by adherence of blood mononuclear cells to gelatin-coated flasks for 3 h and harvest with 15 mM lidocaine solution. These preparations contained ≅20% of colony-forming cells in liquid culture, but showed very little fibrinolytic activity at 2×10^5 cells/well. These experiments showed that

a high level of fibrinolysis by a population of mononuclear phagocytes was associated with the presence of cells able to form colonies in agar rather than in liquid culture.

PA Production by Individual Colonies. To determine what proportion of colonies of bone marrow-derived macrophages contained cells secreting PA, 5×10^4 bone marrow cells were grown in liquid culture in uncoated tissue culture flasks for 7 d to allow formation of discrete colonies. The agarose-overlay method showed that all of these colonies produced plaques of caseinolysis, which depended on the presence of ATDS for their development. We also tested PA secretion by colonies of macrophages derived from 7-d-old BMDM, peritoneal exudate and alveolar cells after growth in liquid culture for 10–14 d. Every colony present in these cultures also yielded a plaque of caseinolytic activity. These studies indicated that each colony-forming cell could give rise to cells that secreted PA.

Fibrinolytic Activity of Bone Marrow Cells at Different Stages of Cultivation. Experiments were designed to study the fibrinolytic activity of both adherent and nonadherent cells present in the early stages of bone marrow cultures.

Groups of gelatin-coated flasks containing 3×10^6 bone marrow cells in 5 ml of growth medium were incubated at 37°C. After 2, 24, 48, and 72 h, the culture medium with nonadherent cells was removed and saved. The flasks were washed twice with 5 ml of α-MEM and the media pooled with the original medium to prepare a suspension of all nonadherent cells. The adherent cells were then detached from the flasks with lidocaine solution and washed. The number of nucleated cells, cellular morphology, the content of fast and slow growing colony-forming cells, and fibrinolytic activity, per cell, of both fractions were then determined. Fast growing colony-forming cells (CFC) were defined as cells able to form colonies of >50 cells in agar within 7 d and slow growing CFC as cells which formed colonies between day 7 and day 14.

Although the results of three such experiments varied in detail, the trend was similar and a representative experiment is shown in Table III. The fibrinolytic activity of both adherent and nonadherent cells was low during the first 24 h of culture and then increased progressively in the case of the adherent cell fraction only. The 16-fold increase in fibrinolytic activity of adherent cells seen in this experiment was associated with a 7-fold increase in the proportion of macrophage-like cells and a 14-fold increase of slow growing CFC. Fast growing CFC varied between 0.4 and 1% of the nonadherent fraction, but were virtually absent among adherent cells. It should be noted that nonadherent cells contained a higher fraction of polymorphonuclear cells, but that all fractions rich in polymorphonuclear lymphocytes had only low fibrinolytic activity. These studies showed that active fibrinolysis and slow growing CFC were associated with the appearance of adherent macrophages in bone marrow cultures.

The fibrinolytic and proliferative activities of adherent cells obtained after 4–12 d in culture was investigated in another series of experiments. Cultures containing 1×10^6 cells in 5 ml of growth medium were fed every 4 d with 0.5 ml of fresh growth medium to supply nutrients. The number of adherent cells increased exponentially during the first 10 d of culture and then decreased, probably a result of overcrowding and detachment of cells (Fig. 2A). Very few fast growing CFC (<0.1%) were present in adherent cell fractions after the 4th d. Once again, the fibrinolytic activity per cell of BMDM was more closely correlated with their content of slow growing CFC (Fig. 2b). The fibrinolytic activity per cell was relatively constant between 4 and 10 d in

TABLE III

Fibrinolytic Activity of Cells during the First 3 d of Bone Marrow Cell Culture

Group	Time in culture	Cell yield (× 10^5)	Fibrinolytic activity*	CFC‡/10^4		Morphology§ (percentage of total)			Lymphocytes
				Fast growing	Slow growing	Macrophages	Mature PMN	Immature cells‖	
	h								
Nonadherent cells	21	6.7	1.3	37	22	0.2	84	5.0	11
	24	1.7	2.9	33	25	1.0	93	3.2	2.6
	48	4.7	2.7	86	47	0.5	94	3.5	2.3
	72	8.3	2.9	104	55	2.7	89	7.1	1.3
Adherent cells	2	2.0	3.1	1	26	14	63	17	5.2
	24	0.3	3.8	6	21	—	—	—	—
	48	1.3	14	11	173	63	22	15	0
	72	1.5	20	4	350	93	4.0	2.9	0

* Radioactivity released (percentage of total). After 2.5 h, each well contained 5 × 10^4 cells.

‡ Fast growing CFC-formed colonies within 7 d and slow growing CFC-formed colonies between days 7 and 14.

§ Determined by Giemsa staining of cytocentrifuge preparations.

‖ Including all blast cells.

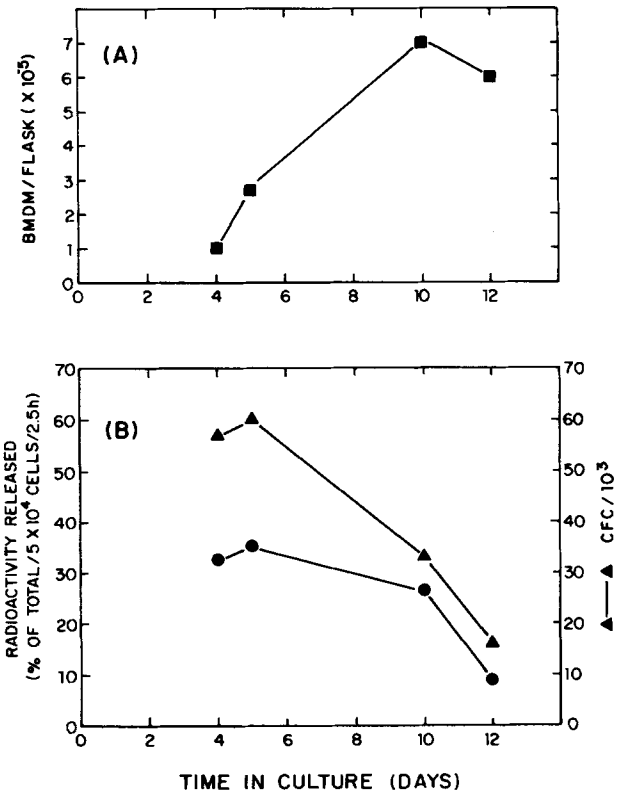

FIG. 2. Correlation between the age of bone marrow cell cultures and the incidence of macrophage CFC and the fibrinolytic activity of adherent cells.

TABLE IV
Effect of Con A on Fibrinolysis by BMDM

Con A	Fibrinolysis*
μg/ml	
0	24.5
0.4	29.8
2.0	33.7
10.0	39.5

* Radioactivity released (percentage of total) per 5×10^4 BMDM/2.5 h.

culture and then declined rapidly, in parallel with the proportion of slow growing CFC.

Effect of Various Substances on Fibrinolysis by BMDM. Con A has been shown to stimulate the production and secretion of plasminogen activator in cultured peritoneal macrophages (23). The effect of Con A on the fibrinolytic activity of BMDM was therefore investigated.

7-d BMDM were plated for 1 h on ^{125}I-fibrin at 5×10^4 cells per well. The adherent cells were washed twice with serum-free medium and 1 ml of Dulbecco's MEM containing 0.1% lactalbumin hydrolysate added with various concentrations of Con A ranging from 0.4 μg to 10 μg/ml. After 3 h of further incubation at 37°C, 50 μl of ATDS were added to each well.

As shown in Table IV, Con A enhanced the fibrinolytic activity of BMDM up to 160% depending on the dose.

The effect of conditioned medium from Con A-stimulated lymphoid cell cultures on fibrinolysis by BMDM was also studied. The experimental procedure was essentially the same as above except that cultures of BMDM on ^{125}I-fibrin were exposed to serum-free medium conditioned by normal spleen cells or by Con A-stimulated spleen cells for 24 h. The wells were then monitored for release of radioactivity before washing twice with prewarmed medium and adding 0.5 ml of Dulbecco's MEM containing 5% ATDS to each well for assay. As seen in Table V, conditioned medium from Con A-stimulated spleen cells stimulated fibrinolysis by BMDM up to 240%, depending on the concentration used. This enhancement was not a result of any remaining Con A itself, because the addition of α-methyl mannoside at the time of exposure to conditioned medium did not abolish the effect. Fibrinolysis was not increased in BMDM cultures exposed to conditioned medium from unstimulated spleen cells.

We also tested the effect of phagocytosis on fibrinolytic activity of BMDM. 7-d BMDM were plated in ^{125}I-fibrin-coated Linbro plates and exposed to 1.01-μm latex particles for 30 min at 37°C. At the end of incubation, almost all BMDM contained >10 latex particles per cell. These wells were washed twice to remove free latex particles and 0.5 ml of Dulbecco's MEM containing 5% ATDS was added to each well. There was no enhancement of fibrinolytic activity after uptake of latex by these cells.

Role of CSF in Regulation of Fibrinolysis by BMDM. Because LCM was used routinely to generate BMDM in culture, its influence on fibrinolysis was investigated. We first studied the effects of different concentrations of LCM, varying from 0.2 to 20% by volume, during the entire period of culture, on cell yield and fibrinolysis. The yield of

TABLE V

Effect of Con A-Stimulated Spleen Cell-Conditioned Medium on Fibrinolysis by BMDM

Treatment	Fibrinolysis*
Control	17.5
NSCM 20%	21.5
50%	18.5
Con A-SCM 20%	36.6
Con A-SCM α-MM‡	35.7
Con A-SCM 50%	41.1
Con A-SCM α-MM‡	40.7

* Radioactivity released (percentage of total)/5 × 10^4 per cells 2.5 h.
‡ α-Methyl mannoside 0.1 M.

TABLE VI

*Effect of Withdrawal and Addition of LCM on the Fibrinolytic Activity of BMDM**

Treatment	Cells/flask	Fibrinolysis‡/ 10^5 cells	Agar colony-forming cells/ 10^3§
+LCM	10 × 10^5	28.8	88 ± 6‖
−LCM	3 × 10^5	13.8	36 ± 7
−LCM → +LCM	5 × 10^5	50.8	86 ± 11

* 5-d-old bone marrow cell cultures were washed and refed with medium with or without LCM for 2 d, after which LCM was added to one set of cultures for a further day (−LCM → +LCM). All cells were harvested on day 8.
‡ Radioactivity released (percentage of total)/2.5 h.
§ Colonies counted on day 14.
‖ Mean ± SEM.

BMDM on day 7 was dependent on the concentration of LCM and reached a maximum at 10–20%, but fibrinolytic activity per cell showed very little difference among cultures.

We next designed a withdrawal and refeeding experiment to explore the role of LCM further.

BMDM which had been cultured for 5 d in the presence of complete growth medium with LCM were washed twice with α-MEM to remove nonadherent cells and LCM; they were then divided into three groups. To the first group, we added 4.5 ml of complete growth medium with LCM, whereas the second and third groups received 4.5 ml of growth medium without LCM. After a 2-d incubation at 37°C we added 0.5 ml of LCM to the third group and 0.5 ml of α-MEM supplemented with 10% FCS to the first and second groups. After further cultivation for 1 d, the cells were harvested, counted, and assayed for fibrinolytic activity and their content of CFC in agar.

As shown in Table VI, the withdrawal of LCM resulted in a lower yield of adherent cells and a twofold reduction in fibrinolytic activity per cell and in the incidence of CFC compared with cultures which had been maintained continuously in the presence of LCM. This part of the experiment showed that LCM influenced PA production as well as the growth of BMDM and again indicated a correlation between fibrinolytic activity of a cell population and its content of CFC in agar. The cultures which had been refed LCM 2 d after withdrawal showed some increase in cell yield and a striking

TABLE VII
Enhancement of PA Secretion of BMDM by CSF

Cells	Source of CSF	Dilution of CSF	Colony-stimulating activity (colonies*/5 × 10^4 BM cells)	Fibrinolytic activity‡
BMDM (1 × 10^5/cells)	None		0	7.1
	Serum-free LCM§	1	172	28.7
		¼	135	15.7
		1/16	68	7.7
	Highly purified CSF‖	1	156	25.0
		¼	142	17.8
		1/16	58	9.6
None	Serum-free LCM	1	172	0.4
	Highly purified CSF	1	156	0.5

* Colonies were counted after 7 d.
‡ Radioactivity released (percentage of total)/2.5 h.
§ Specific activity: 5.4 × 10^5 colonies/mg of protein.
‖ Specific activity: 6.8 × 10^7 colonies/mg of protein.

fourfold increase in fibrinolytic activity per cell, higher than the cells which had been on LCM continuously. The proportion of agar colony-forming cells in the third and first groups were, however, similar. One possible explanation for this apparent dissociation between fibrinolytic activity and the incidence of CFC was that the freshly added LCM might have acted as an inducer of PA (24).

To investigate whether LCM contained an inducer of PA, BMDM were treated with LCM, directly on ^{125}I-fibrin, for short periods before assay.

7-d cultures of BMDM which had been starved of LCM for 2 d, were plated, 1 × 10^5 per well, in 0.5 ml Dulbecco's MEM supplemented with 0.1% lactalbumin hydrolysate. After incubation for 30 min at 37°C to allow cells to adhere, various amounts of test materials or fresh medium, all serum free, were added. The cultures were incubated for 4 more h and 30 μl of ATDS then added to each well, already containing 0.6 ml, so that the final concentration of ATDS was 5% vol/vol. Samples of culture medium were removed after 2.5 and 4 h and assayed for solubilized radioactivity.

As shown in Table VII, the addition of serum-free LCM stimulated fibrinolysis by BMDM fourfold. Control experiments showed that the LCM itself did not contain PA activity and that colony-stimulating activity was readily detectable in the LCM. The increased fibrinolysis in LCM-treated cultures was not a result of cell proliferation because the number of macrophages in treated and untreated wells did not differ over the short period of treatment or assay. Increased fibrinolysis after treatment with LCM has been observed in >10 separate experiments using different preparations of LCM, although the extent of enhancement has varied between 150–400% depending, in part, on the basal level of fibrinolysis by the BMDM used in each experiment. Stimulation of fibrinolysis by LCM was dose dependent, could be seen with LCM prepared in the presence or absence of serum and was measurable within 2 h of exposure. It made no difference whether or not BMDM were starved for LCM before harvest.

TABLE VIII

Differential Enhancement by LCM of Fibrinolysis by Various Mononuclear Phagocytes*

Cells	Fibrinolysis‡	
	Control	+LCM
RPM (1×10^6 cells)	0.5	1.0
TPM (2×10^5 cells)	14.2	22.4
BMDM (2×10^5 cells)	15.2	37.0

* BMDM (day 7), TPM (1 d), and RPM (1 d) were harvested with lidocaine solution, washed, and plated on ^{125}I-fibrin in Dulbecco's MEM with 2% FBS. LCM or control medium with serum was added (10% vol/vol) for 4 h at 37°C. The cells were then washed twice and fibrinolysis assayed in 5% ATDS.

‡ Radioactivity released (percentage of total)/2.5 h.

These experiments indicated that the LCM contained an inducer for fibrinolysis by BMDM. Because crude serum-free LCM contained materials other than CSF, we used highly purified CSF from LCM to test whether CSF itself was responsible for the enhancement. As shown in Table VII, CSF preparations also stimulated fibrinolysis up to fourfold, in a dose-dependent manner, in parallel with their colony stimulating activity. The CSF preparations contained no intrinsic PA activity. This result has been confirmed with three independent preparations.

These experiments established that CSF could account for the enhancement of fibrinolysis by LCM. To examine the role of the BMDM target we compared the effects of LCM on macrophages obtained from the peritoneal cavity and from bone marrow. Table VIII shows an experiment in which TPM and BMDM displayed comparable high levels of fibrinolysis in the absence of LCM. Addition of LCM further stimulated fibrinolysis 2.5-fold in BMDM and 1.5-fold in the TPM. The resident macrophages showed no significant fibrinolysis with or without LCM. We concluded that LCM also enhanced fibrinolysis in targets other than BMDM, but that macrophages derived from bone marrow in culture responded most readily to its inducing activity.

Discussion

These results have shown that mononuclear phagocytes derived from bone marrow precursor cells in culture are as active as thioglycollate medium-elicited peritoneal macrophages in producing and secreting plasminogen activator. BMDM resemble TPM in that production of PA is unaffected by phagocytosis of latex particles (25), but can be enhanced by exposing them to the products of stimulated lymphoid cells and to Con A (16, 23, 26). The PA activity of BMDM harvested at different times and after cultivation under different conditions correlates well with the incidence of relatively slow growing colony-forming cells (CFC). However, it is not clear at present whether these CFC themselves produce PA and whether the same cells display enhanced fibrinolytic and proliferative activity.

A close relationship between high PA production and the presence of >5% CFC is also observed with TPM and alveolar macrophages, whereas RPM show little fibrinolysis and contain very few CFC. Blood monocytes, which do not secrete PA, form colonies in liquid culture, but not in agar (19). These findings indicate that

proliferation in agar may be more closely related to production of PA by a population of mononuclear phagocytes. Our results resemble previous reports linking PA production to the formation of colonies in agar by virus transformed fibroblasts. Primary cultures of normal embryo fibroblasts which proliferate in liquid culture and not in agar may secrete little PA, but acquire the ability to grow in agar and produce high levels of PA after transformation (27, 28).

One explanation for the high fibrinolytic activity of 5–10 d BMDM is that the LCM required for growth also contains a substance which enhances PA. It is likely that CSF stimulates both proliferation and PA in this system because these two activities cofractionate during extensive purification. Further studies should establish whether both activities are indeed mediated by the same molecule.

The physiologic role of CSF is not known at present, but it is required not only for the proliferation of CFU-C and other colony-forming cells related to the mononuclear phagocyte system, but also for the survival and maturation of these cells in vitro (18, 29, 30). The enhancement of fibrinolysis in BMDM by LCM and CSF was rapid and still detectable after 1 d of continuous exposure. Withdrawal experiments showed that the effect on proliferation and fibrinolysis was reversible. CSF, therefore, provides a unique mechanism to regulate the number as well as the specific fibrinolytic activity of mononuclear phagocytes. Different sources of mononuclear phagocytes vary in their response to CSF, but the number of target cells involved in each cell population and the mechanism of action of CSF remain to be determined.

The presence of CSF-like substances has been documented in inflammatory exudates (31–33). Fibroblasts in peritoneal exudate cultures produce CSF (34) and mononuclear phagocytes themselves may release CSF when appropriately stimulated (35–37). Production of CSF by other cells provides another pathway for inducing PA in macrophages which accumulate at a site of inflammation, analogous to the production of a lymphokine inducer of PA by T lymphocytes after immune stimulation and challenge (26). We have been unable to detect colony-stimulating activity in supernates of lymphoid cells containing high levels of PA inducing activity after Con A or BCG stimulation (H.-S. Lin and S. Gordon, unpublished observations), but further studies are needed to determine whether CSF and lymphokine are distinct inducers of PA.

The present studies add to our knowledge of the sequence of appearance of marker products as macrophages proliferate and differentiate in culture. High levels of PA production were found in cultures rich in adherent macrophages which also produce lysozyme and express receptors for Fc and C3, as described by others (38–40). Improved single cell methods will be needed to detect and identify cells which may produce PA during earlier stages of differentiation. The decline of PA observed in older bone marrow cultures may represent macrophage maturation, but could also be due to unfavorable conditions after prolonged cultivation. The generation of high levels of plasmin by BMDM could itself influence the differentiation process observed in culture (41, 42).

Our studies on PA regulation in BMDM underline the importance of the developmental history and the role of newly divided cells in macrophage activation (43–45). In addition to secreting PA, BMDM produce and release other neutral proteinases in culture (46) and can be stimulated by lymphokines to display enhanced cytocidal activity and inhibition of migration (8, 9). BMDM can therefore be regarded as

partially activated cells which are highly responsive to CSF and lymphokines compared with resident macrophages. The production of PA by BMDM provides a sensitive method to study the role of CSF and its interaction with cellular targets in macrophage activation.

Summary

We have studied the production of plasminogen activator (PA) by mononuclear phagocytes derived from mouse bone marrow precursor cells (CFU-C) in culture. Bone marrow-derived macrophages (BMDM) obtained after 6–8-d cultivation in a liquid medium containing L-cell-conditioned medium (LCM), a source of colony stimulating factor (CSF), showed a high level of fibrinolytic activity comparable to that of thioglycollate medium-induced peritoneal macrophages (TPM) and at least 20-fold higher than that of resident peritoneal macrophages (RPM). Fibrinolysis was a result of active secretion of PA into the culture medium and plaques of caseinolysis could be detected by an overlay assay over all macrophage colonies formed after cloning of bone marrow cells in culture.

When the fibrinolytic activity of BMDM harvested at different times was investigated, it was found that the level of PA activity of a given BMDM population correlated well with the incidence of cells (5–15%) able to proliferate and form colonies in agar after 7–14 d, somewhat more slowly than CFU-C. This correlation between the level of PA secretion and the incidence of agar colony-forming cells was also found with other mononuclear phagocyte populations. Active fibrinolysis and slow growing colony-forming cells were observed at the same time as adherent macrophages appeared, 2–3 d after the start of bone marrow culture, they persisted for 10 d before declining.

Some of the factors which influenced PA production by BMDM were examined. Fibrinolysis could be enhanced two- to fourfold by exposing the cells for 4 h to concanavalin A (Con A), to medium conditioned by Con A-stimulated spleen cells and to LCM, but not by phagocytosis of latex particles. The substance in LCM that stimulated PA production appeared to be identical to CSF. Mononuclear phagocyte targets differed in their response to LCM, which stimulated fibrinolysis readily in BMDM, to a lesser extent in TPM and not at all in RPM.

We conclude that CSF stimulates both proliferation and fibrinolytic activity in BMDM and that the level of macrophage activation, as defined by PA production, can be further enhanced by lymphokines. Induction of PA in BMDM provides a rapid and sensitive assay for measuring the activity of CSF and defining its role in macrophage activation.

We thank Julie Cordell and Pat Clarke for excellent technical help.

Received for publication 5 March 1979.

References

1. Unkeless, J., S. Gordon, and E. Reich. 1974. Secretion of plasminogen activator by stimulated macrophages. *J. Exp. Med.* **139**:834.
2. Volkman, A., and J. L. Gowans. 1965. The origin of macrophages from bone marrow in the rat. *Br. J. Exp. Pathol.* **46**:62.
3. Spector, W. G., M. N.-I. Walters, and D. A. Willoughby. 1965. The origin of the

mononuclear cells in inflammatory exudates induced by fibrinogen. *J. Pathol.* **90:**181.

4. van Furth, R., and Z. A. Cohn. 1968. The origin and kinetics of mononuclear phagocytes. *J. Exp. Med.* **128:**415.
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. Sumner, M. A., T. R. Bradley, G. S. Hodgson, M. J. Cline, P. A. Fry, and L. Sutherland. 1972. The growth of bone marrow cells in liquid culture. *Br. J. Haematol.* **23:**221.
7. Goud, T. J. L. M., C. Schotte, and R. van Furth. 1975. Identification and characterization of the monoblast in mononuclear phagocyte colonies grown in vitro. *J. Exp. Med.* **142:**1180.
8. Meerpohl, H. G., M.-L. Lohmann-Matthes, and H. Fischer. 1976. Studies on the activation of mouse bone marrow-derived macrophages by the macrophage cytotoxicity factor (MCF). *Eur. J. Immunol.* **6:**213.
9. Lohmann-Matthes, M.-L., and H. G. Meerpohl. 1977. Mouse bone marrow-cultured macrophage as indicator cells for mouse and human migration inhibitory factor (MIF). *Eur. J. Immunol.* **8:**585.
10. Pluznik, D. H., and L. Sachs. 1965. The cloning of normal "mast" cells in tissue culture. *J. Cell. Comp. Physiol.* **66:**319.
11. Bradley, T. R., and D. Metcalf. 1966. The growth of mouse bone marrow cells *in vitro. Aust. J. Exp. Biol. Med. Sci.* **44:**287.
12. Rabinovitch, M., and M. J. De Stefano. 1976. Cell shape changes induced by cationic anesthetics. *J. Exp. Med.* **143:**290.
13. van der Meer, J. W. M., D. Bulterman, T. L. van Zwet, I. Elzenga-Claasen, and R. van Furth. 1978. Culture of mononuclear phagocytes on a teflon surface to prevent adherence. *J. Exp. Med.* **147:**271.
14. Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* **21**(Suppl. 97):1.
15. Lin, H.-S., C. Kuhn, and T.-T. Kuo. 1975. Clonal growth of hamster free alveolar cells in soft agar. *J. Exp. Med.* **142:**877.
16. Vassalli, J.-D., and E. Reich. 1977. Macrophage plasminogen activator: induction by products of activated lymphoid cells. *J. Exp. Med.* **145:**429.
17. Stanley, E. R., and P. M. Heard. 1977. Factors regulating macrophage production and growth. *J. Biol. Chem.* **252:**4305.
18. Lin, H.-S., and C. C. Stewart. 1974. Peritoneal exudate cells. I. Growth requirement of cells capable of forming colonies in soft agar. *J. Cell. Physiol.* **83:**369.
19. Lin, H.-S. 1977. Colony formation *in vitro* by mouse blood monocytes. *Blood.* **49:**593.
20. Gordon, S., Z. Werb, and Z. Cohn. 1976. Methods for detection of macrophage secretory enzymes. *In In Vitro* Methods in Cell Mediated and Tumor Immunity. B. Bloom and J. David, editors. Academic Press, Inc., New York. 341.
21. Goldberg, A. R. 1974. Increased protease levels in transformed cells: A casein overlay assay for the detection of plasminogen activator production. *Cell.* **2:**95.
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. L. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **241:**265.
23. Vassalli, J.-D., J. Hamilton, and E. Reich. 1977. Macrophage plasminogen activator: Induction by concanavalin A and phorbol myristate acetate. *Cell.* **11:**695.
24. Newman, W., S. Gordon, U. Hämmerling, A. Senik, and B. R. Bloom. 1978. Production of migration inhibition factor (MIF) and an inducer of plasminogen activator (IPA) by subsets of T cells in MLC. *J. Immunol.* **120:**927.
25. Gordon, S., J. Unkeless, and Z. A. Cohn. 1974. Induction of macrophage plasminogen activator by endotoxin stimulation and phagocytosis. Evidence for a two stage process. *J. Exp. Med.* **140:**995.
26. Gordon, S., and Z. A. Cohn. 1978. Bacille Calmette-Guérin infection in the mouse.

Regulation of macrophage plasminogen activator by T lymphocytes and specific antigen. *J. Exp. Med.* **147**:1175.

27. Ossowski, L., J. P. Quigley, G. M. Kellerman, and E. Reich. 1973. Fibrinolysis associated with oncogenic transformation. *J. Exp. Med.* **138**:1056.
28. Pollack, R., R. Risser, S. Conlon, and D. Rifkin. 1974. Plasminogen activator production accompanies loss of anchorage regulation in transformation of primary rat embryo cells by Simian Virus 40. *Proc. Natl. Acad. Sci. U. S. A.* **71**:4792.
29. Metcalf, D. 1970. Studies on colony formation *in vitro* by mouse bone marrow cells. II. Action of colony stimulating factor. *J. Cell. Physiol.* **76**:89.
30. Sachs, L. 1978. Control of normal cell differentiation and the phenotypic reversion of malignancy in myeloid leukemia. *Nature (Lond.).* **274**:535.
31. Wynne, K. M., W. G. Spector, and D. A. Willoughby. 1975. Macrophage proliferation in vitro induced by exudates. *Nature (Lond.).* **253**:636.
32. Aldolphe, M., J. Fontagne, M. Pelletier, and J. P. Giroud. 1975. Induction of DNA synthesis in rat macrophages *in vitro* by inflammatory exudate. *Nature (Lond.).* **253**:637.
33. Cifone, M., P. Mocarelli, and V. Defendi. 1975. *In vivo* production of macrophage growth factor. *Exp. Ceil Res.* **96**:96.
34. Virolainen, M., and V. Defendi. 1967. Dependence of macrophage growth *in vitro* upon interaction with other cell types. *Wistar Inst. Symp. Monogr.* **7**:67.
35. Eaves, A. C., and W. R. Bruce. 1974. *In vitro* production of colony-stimulating activity. I. Exposure of mouse peritoneal cells to endotoxin. *Cell Tissue Kinet.* **7**:19.
36. Golde, D. W., and M. J. Cline. 1972. Identification of the colony-stimulating cell in human peripheral blood. *J. Clin. Invest.* **51**:2981.
37. Ralph, P., H. E. Broxmeyer, M. A. S. Moore, and I. Nakoinz. 1978. Induction of myeloid colony-stimulating activity in murine monocyte tumor cell lines by macrophage activators and in a T-cell line by concanavalin A. *Cancer Res.* **38**:1414.
38. Cline, M. J., and M. A. Sumner. 1972. Bone marrow macrophage precursors. I. Some functional characteristics of the early cells of the mouse macrophage series. *Blood.* **40**:62.
39. Rabellino, E. M., G. D. Ross, H. T. K. Trang, N. Williams, and D. Metcalf. 1978. Membrane receptors of mouse leukocytes. II. Sequential expression of membrane receptors and phagocytic capacity during leukocyte differentiation. *J. Exp. Med.* **147**:434.
40. Lotem, J., and L. Sachs. 1977. Control of normal differentiation of myeloid leukemic cells XII. Isolation of normal myeloid colony-forming cells from bone marrow and the sequence of differentiation to mature granulocytes in normal and D^+ myeloid leukemic cells. *J. Cell Physiol.* **92**:97.
41. Broxmeyer, H. E., and M. A. S. Moore. 1978. Communication between white cells and the abnormalities of this in leukemia. *Biochim. Biophys. Acta.* **516**:129.
42. Dexter, T. M., T. D. Allen, and L. G. Lajtha. 1977. Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. *J. Cell Physiol.* **91**:335.
43. North, R. J. 1978. The concept of the activated macrophage. *J. Immunol.* **121**:806.
44. Karnovsky, M. L., and J. K. Lazdins. 1978. Biochemical criteria for activated macrophages. *J. Immunol.* **121**:809.
45. Cohn, Z. A. 1978. The activation of mononuclear phagocytes; fact, fancy and future. *J. Immunol.* **121**:813.
46. Hauser, P., and G. Vaes. 1978. Degradation of cartilage proteoglycans by a neutral proteinase secreted by rabbit bone marrow macrophages in culture. *Biochem. J.* **172**:275.