MACROPHAGE PLASMINOGEN ACTIVATOR: INDUCTION 
BY PRODUCTS OF ACTIVATED LYMPHOID CELLS*

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The synthesis and secretion of plasminogen activator by mouse peritoneal 
macrophages are correlated with inflammation: macrophages obtained from 
animals after intraperitoneal injection of inflammatory agents produce the 
enzyme, whereas "resident" macrophages from untreated mice do not (1). Since 
enzyme formation can be both induced 1 and suppressed (2) in vitro, the synthesis 
of macrophage plasminogen activator can be used as a sensitive and specific 
assay for potential regulatory mediators of the inflammatory response.

That secretion products of stimulated lymphocytes can influence macrophage 
biology has been suggested for some time: inhibition of macrophage migration 
(3), macrophage "activation" (4), stimulation of macrophage collagenase produc-
tion (5), and many other effects have been attributed to such materials. How-
ever, purification and identification of these lymphocyte products has proven 
difficult, owing perhaps in part to the lack of convenient assays.

We show here that conditioned medium from concanavalin A (Con A) 2-
stimulated mouse spleen cells can both induce the synthesis of plasminogen 
activator by "resident" macrophages obtained from normal, untreated mice, and 
in addition stimulate enzyme production by macrophages "primed" by intraperi-
toneal injection of thioglycollate medium or endotoxin. Macrophage plasmino-
gen activator production is a sensitive, quantitative, and rapid assay for further 
study of the inducing factor secreted by lymphocytes.

Materials and Methods

Con A (3× crystallized) was obtained from Miles Laboratories Inc., Miles Research Products, 
Elkhart, Ind., and α-methyl-D-mannoside from Sigma Chemical Co., St. Louia, Mo. Con A 
[3H(G)], 50.8 Ci/mmol, was obtained from New England Nuclear, Boston, Mass. The sources of all 
other materials have been given elsewhere (2). All incubations were carried out at 37°C in a 5% 
CO2-95% air atmosphere.

Preparation of Conditioned Medium from Cultured Spleen Cells. Spleens were obtained from 
NCS (The Rockefeller University) mice (25-30 g) and teased apart with forceps in cold Dulbecco's
medium; tissue clumps were separated by repeated gravity sedimentation (three times for 3 min) and discarded; the suspension of single cells was washed in cold Dulbecco's medium, sedimented by centrifugation (225 g, 10 min, 4°C), counted in a hemocytometer in Turk's solution, and resuspended at a density of 6 × 10^6 nucleated cells/ml in Dulbecco's medium supplemented with 5% of fetal bovine serum that had been heat inactivated at 56°C for 30 min (HI FBS). Spleens yielded between 5 × 10^7 and 1 × 10^8 cells, and the cells obtained from 5 to 10 spleens were pooled for each experiment. The cells were cultured for 20 h in Falcon 100-mm tissue culture Petri dishes (no. 3003; Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), at a density of 6 × 10^7 cells per plate, in presence or absence of 4 μg/ml Con A. After resuspension by gentle pipetting, the cells from 10 plates were pooled, centrifuged (225 g, 10 min, 4°C), and washed twice by further resuspension and centrifugation in 125 ml of Dulbecco's medium; after another hemocytometer count in Turk's solution, the cells were again resuspended in Dulbecco's medium at a density of 6 × 10^6 cells/ml, replated in fresh culture dishes (Falcon no. 3003) at 6 × 10^7 cells per plate, and incubated at 37°C. Lymphocyte-conditioned medium (LCM) was collected 24 h later, centrifuged for 15 min at 500 g to remove the cells, and either used immediately or stored at -20°C for up to several weeks without loss of activity; a precipitate that formed occasionally upon thawing was removed by centrifugation (900 g, 15 min) and discarded before assay.

Conditioned medium from other cultured lymphoid cell populations was prepared in the same way. These included spleen cells obtained after filtration through a column of nylon wool (Leukopak; Fenwal Laboratories, Morton Grove, Ill.), thymus cells, and lymph node cells shaken free of fragmented thymus and axillary and mesenteric nodes, respectively. The amount of Con A present in the conditioned medium from stimulated lymphocytes (sLCM) was determined by stimulating the spleen cell preparation with [3H]-Con A (0.5 μCi/ml, 4 μg/ml), and counting an aliquot of the sLCM in Aquasol-2 (New England Nuclear) at the time of collection.

Culture of Peritoneal Macrophages. Cells were obtained from NCS (The Rockefeller University) mice (25-30 g) by washing the peritoneal cavity with 4 ml cold phosphate-buffered saline (PBS); “normal” resident peritoneal macrophages were cultured from mice obtained from the specific pathogen-free colony immediately before sacrifice; “endotoxin” and “thioglycollate” macrophages were cultured from mice that had been injected 4 days previously with 30 μg endotoxin (Salmonella minnesota lipopolysaccharide mR 595(5418)) in 1 ml PBS or with 1 ml Brewer's thioglycollate medium, respectively.

Fibrinolysis by Normal Macrophages. Peritoneal cells (1 × 10^6), from uninjected mice were plated onto 125I-fibrin-coated wells of Linbro Disposo Trays (FB16-24TC; Linbro Chemical Co., New Haven, Conn.) prepared as previously described (2), in Dulbecco's medium containing 0-50% of LCM in the presence of 5% of HI FBS and 100 μg/ml soybean trypsin inhibitor (STI). After 20 h of incubation, 2.5 × 10^7 latex particles [1.01 μm diameter, prepared as described elsewhere (2)] were added to some of the cultures; 1 h later all of the cultures were washed twice with 0.024 M Tris (pH 7.4), 0.1 M NaCl, 0.05 M KCl, and 0.0037 M Na2HPO4 (TD), and placed in Dulbecco's medium supplemented with 8 μg/ml human plasminogen. Aliquots of the supernatant medium were removed 3 h and 6 h later, and assayed for solubilized radioactivity in a Packard Autogamma Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Fibrinolysis by Endotoxin Macrophages. Peritoneal cells (1 × 10^6) from endotoxin-injected mice were plated onto 125I-fibrin-coated wells of Linbro Disposo Trays in Dulbecco's medium supplemented with 5% of HI FBS and 100 μg/ml STI. After 20 h of incubation the cultures were washed twice with TD and placed in Dulbecco's medium containing 0-30% LCM, and supplemented with 5% of acid-treated HI FBS (1). Aliquots of medium were removed 6, 12, and 24 h later and assayed for solubilized radioactivity.

Detection of Plasminogen Activator Production by Single Cells. Peritoneal cells (1 × 10^4) were plated in Falcon 60-mm tissue culture Petri dishes (no. 3002), in Dulbecco's medium supplemented with 10% of HI FBS. The cultures were washed after 6 h, and placed in Dulbecco's medium containing 0-30% LCM in the presence of 10% of HI FBS. The plates were washed 20 h later with TD, overlaid with a casein-agar mixture, incubated for 9 h, fixed, and stained; a full description of the procedure has been given elsewhere. The overlay mixture was prepared either with or without human plasminogen (40 μg/ml), and did not contain any LCM.

Secretion of Plasminogen Activator by Thioglycollate Macrophages. Peritoneal cells (5 × 10^6) from thioglycollate medium-injected mice were plated onto wells of Linbro Disposo Trays, in 1 ml Dulbecco's medium supplemented with 5% of HI FBS. The cultures were washed 20 h later with
TD, and placed in Dulbecco's medium containing 0–100% of LCM. After a further incubation of 24 h the medium was collected and stored at –20°C until assayed for plasminogen activator as previously described (2); the cells were washed three times with PBS and dissolved in 1 N NaOH for determination of cell protein by the method of Lowry et al. (6). One unit of plasminogen activator was defined as the amount that stimulated the release of 10% of the initial radioactivity in 4 h (1).

Results

**Induction of Plasminogen Activator Production in Cultures of Normal Macrophages.** To test for a possible effect of lymphocyte products, macrophages obtained from untreated mice were first cultured for 20 h in the presence of LCM, and then assayed for plasminogen-dependent fibrinolytic activity. As seen in Fig. 1, sLCM greatly enhanced fibrinolysis by macrophages; the increased rate of fibrinolysis was a function of the concentration of sLCM and, after 6 h of assay, stimulation by as little as 0.3% of sLCM was detectable. In contrast, no enhancement of fibrinolysis was obtained in macrophage cultures exposed to conditioned medium from nonstimulated lymphocytes (NsLCM). All of the fibrinolytic activity induced by sLCM was plasminogen dependent and hence was attributable to macrophage plasminogen activators.3

Two extrinsic factors that affect macrophage plasminogen activator synthesis are corticosteroids and phagocytizable particulates. Low concentrations of anti-inflammatory steroids, which are known to inhibit enzyme production under other conditions (2) also blocked the effects of sLCM; addition of dexamethasone (10⁻⁷ M) reduced the macrophage response to sLCM (30%) by more than 85% when the drug was present both during the preincubation period and the period of fibrinolysis assay.

Exposure of macrophage cultures to phagocytizable particles may increase and/or prolong plasminogen activator secretion (7). When cultures of normal macrophages were exposed to latex particles immediately before assay, the observed fibrinolytic activity was slightly increased (Fig. 1, insert); a similar effect of latex was detected in cultures pretreated with sLCM, but latex did not alter the shape of the dose response curve to sLCM.

To test for the possibility that a minor subpopulation of normal macrophages was responding to sLCM, the proportion of plasminogen activator-producing cells in stimulated cultures was determined by means of a casein-agar overlay procedure that detects proteolysis by single cells. As shown in Fig. 2, the proportion of proteolytically active cells was a function of the concentration of LCM in the preincubation medium. A majority of macrophages responded to high concentrations of sLCM and all of the proteolytic activity was due to plasminogen activators, since no plaques were found when the casein-agar overlay was deprived of plasminogen. In this, as in other tests (vide supra) macrophages did not produce any detectable plasminogen activator in response to conditioned medium from lymphocyte cultures that had not been stimulated by mitogens.

Apart from inducing plasminogen activator production, high concentrations

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3 It should be pointed out that LCM itself was not present during the fibrinolysis assay, and therefore its continuous presence was not needed to maintain plasminogen activator production. LCM contained no detectable plasminogen activator.
FIG. 1. Fibrinolysis by normal macrophages plated on I25I-fibrin and preincubated in presence of LCM. Normal macrophages were preincubated either in the absence (○) or in the presence of various concentrations of sLCM (□) or NsLCM (△), washed, and tested for fibrinolytic activity. For additional details see the Materials and Methods. Insert: Fibrinolysis by normal macrophages plated on I25I-fibrin and preincubated in presence of LCM; effect of phagocytosis. Same experiment as in Fig. 1. At the end of the preincubation period the cells were exposed to latex particles (closed symbols) before being tested for their fibrinolytic activity; 3 h assay; (open symbols), no latex. (□—□, ■—■), sLCM; (△—△), NsLCM; (○, ○), no LCM.

FIG. 2. Proportion of normal macrophages secreting plasminogen activator after preincubation in presence of sLCM. All cells surrounded by a clear and concentric zone of lysis in the casein-agar overlay were scored as positive, irrespective of the size of the lysis zone.

(10-30%) of sLCM also stimulated morphological changes in normal macrophage cultures: sLCM-treated macrophages were square or round in outline, and they spread over a larger surface than the untreated, star-shaped controls. In con-
contrast, neither the plating efficiency of normal macrophages, nor their survival were affected by sLCM.

**Stimulation of Plasminogen Activator Secretion by Endotoxin- and by Thioglycollate Medium-Primed Macrophages.** Macrophages harvested from endotoxin-induced peritoneal exudates secrete little plasminogen activator, but they can be stimulated to much higher levels of enzyme production by exposure to phagocytizable particles (7), asbestos (8), Con A, or phorbol myristate acetate. As seen in Fig. 3, plasminogen-dependent fibrinolysis by these cells was rapidly and strongly enhanced upon exposure to sLCM; this effect persisted for at least 24 h, and its magnitude was a function of the concentration of sLCM. The proportion of plasminogen activator-secreting endotoxin macrophages, as detected by the casein-agar overlay technique, was also markedly increased after incubation with sLCM.

Macrophages cultured from thioglycollate-induced peritoneal exudates secrete high levels of plasminogen activator independent of any further stimulation in vitro (1); even so, enzyme production was increased as much as 10-fold in a dose-dependent manner when the cells were incubated in media enriched with sLCM. The results of experiments using different preparations of LCM and of thioglycollate macrophages are presented in Fig. 4. Although sLCM always caused a marked stimulation of plasminogen activator secretion, in contrast to the lack of effect of NsLCM, the extent of this stimulation was variable, reflecting perhaps in part a variation in the macrophage populations obtained after intraperitoneal injection of thioglycollate medium.

**The effect of sLCM is Not Due to Carry-Over of Con A.** Con A, when added directly to a purified population of cultured peritoneal macrophages, is a potent inducer and stimulant of plasminogen activator synthesis. The possibility that the effects reported here might be due to the presence of Con A in the sLCM therefore required investigation. The actual amount of Con A present in sLCM (100%) was determined by using $^3$H-Con A, and found to be 20 ng/ml, a concentration 20-50 times lower than that required to obtain stimulation of plasminogen activator synthesis. In addition, the effect of Con A and of sLCM
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FIG. 4. Plasminogen activator secretion by thioglycollate macrophages cultured in presence of LCM. The data are pooled from two separate experiments. The amount of plasminogen activator secreted per milligram cell protein in the control, untreated cultures was defined as 1, and represented for experiment (a) (closed symbols) 1,150 U/mg cell protein, and for experiment (b) (open symbols) 860 U/mg cell protein. (□), sLCM; (▲), NaLCM.

### TABLE I
The Effect of sLCM is Not Prevented by α-Methyl Mannoside

| Cells producing plasminogen activator | Plasminogen activator secreted |
|---------------------------------------|-------------------------------|
|                                       | Normal macrophages*             | Thioglycollate macrophages†    |
|                                       | α-Methyl mannoside               | α-Methyl mannoside             |
|                                       | %                              | U/mg cell protein              |
| Control                               | 3.5                            | 823                           |
| Con A (10 μg/ml)                      | 30.2                           | 8,865                         |
| sLCM (30%)                            | 57.8                           | 7,563                         |

* Peritoneal cells from normal mice were plated and treated as described for Fig. 2. α-Methyl mannoside (0.1 M) was present as indicated during the preincubation period, but was not included in the casein-agar overlay mixture.
† Peritoneal cells from thioglycollate medium-injected mice were plated and treated as described for Fig. 4. α-Methyl mannoside (0.1 M) was present as indicated during the period of collection of macrophage-conditioned medium; when added to cell-free-conditioned medium, it did not affect the assay for plasminogen activator.

on normal and on thioglycollate macrophages was compared (Table I): both agents stimulated macrophage plasminogen activator production, but whereas the effect of Con A was blocked by addition of α-methyl mannoside, the effect of sLCM was unaffected by this sugar. These results demonstrated that the effect of sLCM was not due to any detectable carry-over of Con A, but rather to a molecule or molecules generated in cultures of Con A-stimulated spleen cells.

Conditioned Medium from Other Lymphoid Cell Populations. To test whether the stimulating factor formed by spleen cells might be a product of lymphocytes, we have assayed for its presence in conditioned medium obtained both from cultured thymus and lymph node cells, and from the fraction of spleen
cells that was not retained by a column of nylon wool. All three cell types gave qualitatively the same results as the routine unpurified spleen cells: in each case plasminogen activator induction in cultures of normal macrophages, when tested with the casein-agar overlay technique as in Fig. 2, was observed only in presence of conditioned medium from Con A-stimulated lymphoid cultures. However, lacking any characterization of the active factor(s), we cannot assume that the results reflect the activity of a single inducing agent in all cases.

Discussion

Macrophages that have entered the peritoneal cavity in response to injected inflammatory agents produce relatively large amounts of plasminogen activator (2). There are persuasive grounds for concluding that the secretion of this enzyme is a characteristic property of migratory cells and enables them to transect the structural barriers encountered during migration (9-11). In this view, plasminogen activator production would be one component in the cellular response to stimuli that provoke migration, and it might be expected that humoral factors capable of recruiting macrophages to sites of inflammation could be detected by the ability to induce enzyme synthesis. Our data show that cultured spleen cells exposed to the mitogenic lectin Con A yield products that (a) induce plasminogen activator synthesis by normal macrophages which do not spontaneously form this enzyme, and (b) stimulate enzyme synthesis over and above the appreciable amounts already produced by endotoxin and thioglycollate macrophages. A number of considerations suggest that this material may be of some physiological significance: (a) The stimulatory effect of sLCM on plasminogen activator synthesis is detectable at high dilution, and the quantitative dose-response pattern of macrophages resembles that commonly observed in the responses of other cellular systems to hormones. (b) At the level of individual cells macrophage response to sLCM is not a rare event: essentially all macrophages are induced to synthesize plasminogen activator at the highest concentrations of sLCM. (c) Plasminogen activator production in this and other tissues, including ovarian cells (12), skin cells (H. Green and E. Reich, unpublished observations), and fibroblasts (13), can be induced by physiological levels of hormones or by correspondingly low concentrations of hormone-like substances such as phorbol esters; and enzyme production by macrophages (2) as well as by certain cell lines (14) is suppressed by physiological concentrations of anti-inflammatory steroids. Hence inducibility of macrophage plasminogen activator by a product of spleen cells is not surprising, and the active material could conceivably represent a humoral antagonist to the effect of glucocorticoids.

Several details of the present work require brief comment. The spleen cells used for production of LCM are complex mixtures, and we have not, in fact, definitively identified lymphocytes as the source of LCM. Nevertheless, it seems safe tentatively to assume that the active factor is a lymphocyte product, since the replating procedure used in these experiments eliminates macrophages and other adherent cells, and washing incidentally also removes any macrophage plasminogen activator that might have accumulated during the preincubation period. Furthermore, the production of a similar inducing factor(s) by cultured thymic, lymph node, and partially purified nonadherent spleen cells suggests
the lymphoid source of the active factor(s), although we cannot as yet assume
that they are identical.

We do not know whether or to what extent the active spleen cell factor in our
experiments may be related to other activities of lymphocyte origin such as those
responsible for modifying macrophage migration (migration inhibition factor
(MIF)) (3), adherence to plastic (4), and electron-dense surface coats (15), one or
more of which might in fact reflect the action either of plasminogen activator, or
plasmin, or both. It may be significant that the MIF-like activity present in
conditioned medium produced by cultures of transformed mouse cells has been
attributed to plasminogen activator (16). The definition of the relationships, if
any, between these factors awaits their purification and characterization; since
macrophage plasminogen activator synthesis is a useful assay for an inducing
factor, the purification of the putative molecule is the subject of current efforts.

Summary

Macrophages obtained from the peritoneal cavity of untreated mice do not
ordinarily synthesize plasminogen activator. However, induction of enzyme
synthesis and secretion occurs when such macrophages are cultured in presence
of conditioned medium from Con A-stimulated spleen cells. Plasminogen activa-
tor production by macrophages from endotoxin or thioglycollate medium-in-
jected mice, which spontaneously secrete substantial amounts of the enzyme, is
also markedly increased in presence of such conditioned medium.

These results suggest that macrophage plasminogen activator production may
be regulated in part by lymphocytes. They provide further evidence to link
macrophage plasminogen activator with cell migration and inflammation, and
also support the view that in macrophages, as in certain other cell types, synthesis
and secretion of this enzyme are under hormonal control.

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